Drug Discovery and Evaluation

Safety and Pharmacokinetic Assays

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(Co-Editors)

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Safety and Pharmacokinetic Assays

With 131 Figures and 125 Tables

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Preface

Drug discovery and evaluation has been a sequential process for a long period of time. It started with the selection of the most active compound from a series of newly synthesized compounds with the help of special pharmacological assays. Safety aspects were considered by testing the selected compound in high doses in tests directed to indications other than the intended indication of the new compound. These tests were followed by pharmacokinetic studies, which were mainly aimed at confirmation of a suitable half-life time and of oral activity. Safety relied on acute and subacute toxicity studies, which gave information more on organ structure than on organ function. Toxicological and pharmacokinetic studies were adapted to the progress of studies in clinical pharmacology and clinical trails. This strategy has been changed during the last 15 years for several reasons:

Some negative effects on organ function, e.g., ventricular tachy-arrhythmia were detected too late. On the other hand, negative findings in chronic toxicity studies in animals turned out to be irrelevant for human beings.

New scientific approaches, e.g. combinatorial chemistry, high-throughput screening, in silico models, pharmaco-genomics and pharmaco-proteomics offered new possibilities.

The success rate in the pharmaceuticals industry and the introduction of new chemical entities to the market per year dropped dramatically, whereas the development time for new compounds increased, sometimes exceeding the patent protection.

This forced a change of strategy:

- Parallel instead of sequential involvement of the various disciplines.
- The term "Safety Pharmacology" was coined.
- An International Conference on Harmonization (ICH) Safety Pharmacology working group was founded.
- Easily accessible and most informative tests must be selected.

Exposure of drug to the body by pharmacokinetic studies on absorption, distribution, metabolism and excretion must be investigated at an early stage of development and can contribute to the selection of a compound for development.

Toxicology experienced major improvements by the introduction of new methods, e.g., in silico methods, toxicogenomics and toxicoproteomics.

These aspects stimulated our decision to publish this volume as a counterpart to "Drug Discovery and Evaluation. Pharmacological Assays" (Second Edition Springer Verlag 2002). The current book contains three sections. Dr. Franz Jakob Hock shares with me the responsibility for the section "Safety Pharmacology"; Dr. Jochen Maas took over the responsibility for the section "Safety Pharmacokinetics" and Prof. Dr. Dieter Mayer for the section "Safety Toxicology".

As with the book on pharmacological assays, this book is intended to aid both scientists and students. The reader can find methods for selecting candidates for drug development at early stages, such as screening methods based on the stage of development, or methods up to advanced stages of development and which are considered necessary for international approval from the scientific and regulatory point of view.

H. Gerhard Vogel
Juni 2006

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Contents

Section I Safety Pharmacology

Section I
Safety Pharmacology

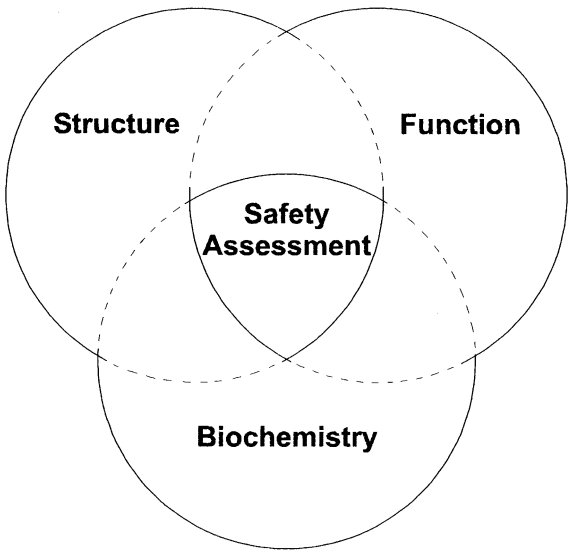
Chapter I.A
Introduction to Safety Pharmacology

Franz J. Hock

The adverse effects of new drug entities in animals and humans can be manifested by changes in the structural, biochemical or physiological status of the organism. In the preclinical safety assessment process, toxicological procedures have traditionally focused on the structural and biochemical consequences of drug actions. As a result, there has been a reliance on histopathological evaluation of organs and clinical pathology measures in the toxicological assessment of new drug candidates. Physiological or functional observations of drug action were mostly conducted outside of the safety assessment processes. The last decade has seen a growing awareness of the importance of physiological measures of drug toxicity. This awareness has been prompted by clinical issues of life-threatening effects e.g. cardiovascular functions. These issues have prompted regulatory action and involvement with respect to "Safety Pharmacology".

Safety pharmacology refers to the assessment of adverse effects of drugs on functional and physiological systems including central and peripheral nervous system, cardiovascular system, respiratory system, renal and gastrointestinal functions. The evaluation of functional or physiological toxicities plays a key role in the safety assessment process. It should be viewed as complementary to traditional assessments of toxicity based upon morphological or biochemical lesions. Safety pharmacology is not a new topic, but one with renewed interest in the pharmaceutical industry and the regulatory bodies governing the drug approval process.

Safety pharmacology can be thought of as one of the key areas to be integrated with structural and biochemical evaluations in the complete safety assessment program (see scheme below).



The authors of this section have assembled information to assist scientists in industry in i) understanding the recent regulatory status and expectations for safety pharmacology testing, and ii) the conduct of safety pharmacology studies in broad. As evidenced by their extensive experience, the authors share a deep commitment to the conduct of safety pharmacology studies and are leaders in this field of drug safety assessment. The emphasis of this section is to provide practical information and approaches to the assessment of central and peripheral nervous system, cardiovascular system, respiratory system, renal and gastrointestinal functions, and of other systems and functions involved in safety issues.

The following sections will detail approaches and options to assessing physiological functions as part of the overall safety assessment of new drug entities.

Finally we would like to thank the authors for their contributions to this section.

Chapter I.B
Status of Safety Pharmacology and Present Guidelines

Franz J. Hock

"The adverse drug reactions which the standard toxicological test procedures do not aspire to recognize most of the functional side-effects. Clinical experience indicates, however, that these are much more frequent than the toxic reactions due to morphological and biochemical lesions . . ." (Gerhard Zbinden 1979)

I.B.1 Origins of Safety Pharmacology

Serious injury and/or death of volunteers and patients participating in early clinical trials are rare and thus very disturbing when they occur (Marshall 2001a; 2001b; Miller 2000). The organ systems and functions most frequently responsible in these events are the central nervous (seizure), cardiovascular (hypotension, hypertension, and arrhythmia), respiratory (asthma/bronchoconstriction), and renal (glomerular filtration) systems, and the result is almost always a critical care emergency (Kinter et al. 1997). The origins of safety pharmacology are grounded upon observations that organ functions (like organ structures) can be toxicological targets in humans exposed to novel therapeutic agents and that drug effects on organ functions (unlike organ structures) are not readily detected by standard toxicological testing (see Mortin et al. 1997; Williams 1990; Zbinden 1984).

Already in the late 60s, even more in the 70s, major pharmaceutical companies, alerted by the thalidomide disaster, intensified the preclinical testing of drug candidates. For example, in the pharmacological laboratories of Hoechst AG, Germany, each compound proposed for development was thoroughly investigated under the term "General Pharmacology". In the specialized laboratories, e.g., for cardiovascular and respiratory function, nephrology, blood coagulation, psychopharmacology, neuropharmacology, analgesic and antiinflammatory research, gastroenterology, diabetology, atherosclerosis, and endocrinology, the potential new drug was studied according to internal rules for which the head of the department of pharmacology was responsible. For the performance of the tests, the heads of the special laboratories were responsible. Tests rarely used were performed in a laboratory for general pharmacology.

These rules emphasized:

- Application in the routes proposed for clinical use (e.g. oral, intravenous).
- Use of doses considerably higher than those found to be active in pharmacological tests for the proposed indication. If possible, doses at least 10 times higher were used.
- Use of the animal species considered the most sensitive in the special indication, though several species had to be studied.
- Multiple dose application, if effects could not be detected after single application, e.g. in atherosclerosis research or in endocrinology.
- In-depth analysis when unexpected results were found.

Unfortunately, these rules were never published, partially because pharmacologists wanted to perform their studies without supervision by GLP. However, the results were published if the investigational drug reached the market (Omosu et al. 1988). Since only 5 % of compounds proposed for development overcome all the hurdles, the majority of data were also not published.

As Kinter et al. (1994) reported, in many pharmacological laboratories prior to 1990, organ function testing was often conducted as an ancillary function of discovery research. The selection of specific studies for a candidate drug was based on concerns raised from its primary (those pharmacodynamic effects related to

Table 1 Comparison of different regulatory guidelines (before 1995).

DEUTSCHLAND (Germany)	EU	USA	JAPAN
Allgemeine Verwaltungsvorschrift zur Anwendung der Arzneimittelprüfrichtlinien (Bundesrat Drucksache 580/89; 3. Abschnitt, C 1; Okt. 1989) Pharmakodynamik • Wirkungsspektrum eines Stoffes ermitteln • Prospektive erwünschte Wirkungen • Wirkungen auf vitale Funktionen (allgemeine Pharmakodynamik) • Dosis-Wirkungs-Beziehung für erwünschte und unerwünschte Wirkungen sollen ins Verhältnis gesetzt werden	Notice to Applicants (Part III F; Jan. 1989) Pharmacodynamics Studies conducted to establish the pharmacodynamis effects and the mode of action should be evaluated in the following order: • studies demonstrating desired therapeutic effects (special pharmacodynamics) • studies demonstrating effects in addition to desired effects (general pharmacodynamics) • studies to detect drug interaction	Guidelines for the Format and Content of the Nonclinical Pharmacology/Toxicology Section of an Application (FDA; Feb. 1987) Part II. D. 1. Pharmacology studies should be presented in the following order, with pharmacodynamic ED_{50} in dose-ranging studies preceding mechanism of action studies • effects related to the therapeutic indication (primary and secondary activities) • effects related to possible adverse reactions • interactions with other drugs	Guidelines for General Pharmacology Studies (MHW 1995) • effects on general activity and behavior • effects on the central nervous system • effects on the autonomic nervous system and smooth muscle • effects on the respiratory and cardiovascular systems • effects on the digestive system • effects on water and electrolyte metabolism • other important pharmacological effects • and special additional tests

Table 2 Excerpts from international regulatory documents.

" . . . studies that otherwise define the pharmacological properties of the drug or are pertinent to possible adverse effects." (United States of America, 21 CFR (Section 314:50, paragraph 2)
" . . . a general pharmacological characterization of the substance, with special reference to collateral effects." (European Economic Community, Directive 91/507/EEC)
"A general pharmacological profile of the substance is required, with specific reference to collateral effects . . . Methods of screening will vary . . . but the aim should be to establish a pattern of pharmacological activity with in major physiological systems using a variety of experimental models." (United Kingdom, Medicines Act 1968, Guidance notes on applications for product licences, MAL 2, p A3F-1)
"Secondary actions – studies related to secondary pharmacological actions of the new drug which may be relevant to expected use or to adverse effects of the new drug." "Other studies – pharmacological activities of the drug that may be pertinent to safety and which may or may not be relevant to proposed clinical trials . . . " (Canada, RA5 Exhibit 2, Guidelines for preparing and filing drug submissions, p 21)
"Studies should reveal potentially useful and harmful properties of the drug in a quantitative manner which will permit an assessment of the therapeutic risk . . . Investigations of the general pharmacological profile should be carried out." (Australia, Guidelines for preparation and presentation of applications for investigational drugs and drug products under clinical trial exemption scheme, pp 12, 15)
"New drugs should be studied in a biological screening program so as to define any action over and above that which is desirable for the therapeutic use of the product." (Nordic countries)
"The objective of general pharmacological studies is to examine extensively the kind and potency of actions, predict potential adverse effects likely to manifest in clinical practice..." (Japanese Guidelines for Safety Pharmacology Studies 1995)

a drug's targeted indication; special pharmacology) or secondary (those pharmacodynamic effects unrelated to a drug's targeted indication; general pharmacology) pharmacology, or known effects associated with the drug's pharmacological, therapeutic, or chemical class. This ad hoc approach to safety evaluation led to non-systematic decisions regarding study designs and organ systems studied. Often the study designs employed were those available for the assessment of efficacy, not safety endpoints (e.g., blood pressure determinations in anesthetized felines). In addition, study designs employed dose levels that exceeded the projected clinical efficacy levels by small multiples, if any. Systemic exposures associated with those dose levels were seldom documented; indeed, investigators were sufficiently aware of this criticism that early organ function testing was often conducted using intravenous administration, regardless of the intended

clinical route of administration (Kinter et al. 1997). These early organ function assessments were normally disjointed and disconnected from the results of the toxicology program. Attempts to add organ function endpoints to toxicology protocols were frustrated by the fact that data were collected without regard to the physiological status of the subjects and/or pharmacokinetic parameters (Lufy & Bode 2002; Morgan et al. 1994).

Prior to 1990, regulatory guidances on organ function testing were limited. There were some "guidelines" but each country or area had their own regulatory ones (Table 1). These guidelines were very unspecific except the Japanese one (see below). The international regulatory documents differ as well (Table 2). The U.S. and European regulations provided only general references to evaluations of drug effects on organ system functions (Gad 2004; Kinter et al. 1994; Lumley 1994). Organ function assessments included with investigational new drug applications (INDs) and registrations (NDAs) were inconsistent and often viewed as unimportant (Green 1995; Proakis 1994). However, in Japan, the Ministry of Health and Welfare (now referred to as the Ministry of Health, Labour, and Welfare) had promulgated comprehensive guidances for organ function testing as early as 1975 (see Table 3). These guidelines described which organ systems would be evaluated (including central and peripheral nervous systems, cardiovascular, respiratory, gastrointestinal, and renal) as a first tier evaluation (Category A studies) and made specific recommendations regarding study designs (including description of models, criteria for dose selection, and which endpoints would be included in the investigation). The guidelines also described a second tier of studies (Category B) to be conducted based on the significant findings in the Category A investigations. Because the Japanese guidelines were the most comprehensive of their time, they became the de facto foundation for organ function safety testing throughout the pharmaceutical industry (Kinter & Valentin 2002). The organ function studies included in Categories A and B were intertwined with studies whose aim was to catalogue additional pharmacological functions and activities (secondary or general pharmacology) in addition to the primary pharmacological function/activity. Kinter et al. (1994) first distinguished two subgroups of objectives embedded in the Japanese studies as safety and pharmacological profiling. This concept was enlarged upon by the International Conference on Harmonization (ICH) safety pharmacology expert working group to define three categories of pharmacological characterizations: primary and secondary pharmacodynamic, and safety pharmacology (see ICH S7A, Table 3).

During the same period, European, U.S., and Japanese regulatory agencies prepared positions on general pharmacology/safety pharmacology in the form of guidance and concept papers (Bass & Williams 2003; Kurata et al. 1997, Table 3). Draft documents appeared from Japan, Europe, and United States by 1998. Later that year, the Ministry of Health and Welfare and the Japanese Pharmaceutical Manufacturer's Association proposed to the ICH Steering Committee the adoption of an initiative on safety pharmacology. This proposal was accepted and given the designation of Topic S7.

The origin of the term safety pharmacology is obscure. It first appeared in drafts of the ICH guidelines "Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals", Topic M3, and "Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals", Topic S6 (see Table 3). ICH S6 stated that "The aim of the safety pharmacology studies should be to reveal functional effects on major physiological systems (e.g., cardiovascular, respiratory, renal, and central nervous system)". The ICH Topic S7 Expert Working Group began their work in the first quarter of 1999, and a harmonized safety pharmacology guideline was finalized and adopted by the regional regulatory authorities over 2000–2001 (ICH S7A). The guideline describes the objectives and principles of safety pharmacology, differentiates tiers of investigations ("safety pharmacology core battery", "follow up", and "supplemental" studies), establishes the timing of these investigations in relationship to the clinical development program, and embraces GLP procedures (when applicable).

A significant issue that was extensively debated by the ICH Topic S7 Expert Working Group was how to evaluate the potential of new drugs to produce a rare but potentially life threatening ventricular tachy-arrhythmia (torsade de pointes) in susceptible individuals (Ackerman 1998; Anderson et al. 2002; De Ponti et al. 2001; Haverkamp et al. 2000). The incidence of torsade de pointes with drugs that are targeted at non-cardiac indications can be very low, for example, 1 in 120 000, and hence the imperative to find non-clinical surrogates to identify those drugs with the potential to elicit this serious cardiac arrhythmia (Malik & Camm 2001; Moss 1999; Thomas 1994; Viskin 1999; Webster et al. 2002). The surrogates of cardiac ventricular repolarization prolongation have included in vitro assessment of drug effects on repolarizing cardiac ion currents (e.g., sodium current, I_{Na}, calcium

Table 3 Current international guidelines and draft documents on Safety Pharmacology.

Year	Document
1975	Notes on Application for Approval to Manufacture (Import) New Drugs, issued in 1975 (MHW-Japan)
1995	Japanese Guidelines for Non-Clinical Studies of Drugs Manual 1995. Yakuji Nippo Tokyo, 1995
1998	Guideline for Safety Pharmacology Study (draft 3.17, 1998; Japan; personal communication, Dr. K. Fujimori)
	Committee for Proprietary Medicinal Products (EU). Note for Guidance on Safety Pharmacology Studies in Medicinal Product development (Draft 1998) FDA DRAFT concept paper on safety pharmacology
	Committee for Proprietary Medicinal Products (EU). Points to Consider: The assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products
1997	ICH S6: Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals, July 1997
	ICH M3: Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals, July 1997
	Guidance for Industry. S7A Safety Pharmacology Studies for Human Pharmaceuticals. US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, ICH. July 2001
	Therapeutic Products Directorate Guidance Document (Canada). Assessment of the QT prolongation potential of nonantiarrhythmic drugs (2001)
2002	The clinical evaluation of QT/QTc interval prologation and proarrhythmic potential for nonanti-arrhythmic drugs. FDA DRAFT preliminary concept paper. Nov. 15, 2002
2003	ICH Guideline on Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals (S7B). Sept, 2, 2003 The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Nonanti-arrhythmic Drugs. ICH E14 Step 1 Draft 2 (July 17, 2003)
	FDA (draft) Guidance for Industry: Non-clinical Studies for Development of Pharmaceutical Excipients. February, 2003
	FDA (draft) Guidance for Industry: Non-clinical Studies for Development of Medical Imaging Agents. February, 2003
	CPMP Position Paper on Von-clinical Safety Studies to Support Clinical Trials with a Single Microdose. July 2003
	FDA (draft) Guidance for Industry: Non-clinical Safety Evaluation of Pediatric Drug Products. February, 2003
2004	S7B Revised: The Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals. Step 3, June 2004
	E14: Principles for Clinical Evaluation of New Antihypertensive Drugs. Step 3, June 2004
	Draft EMEA: Guideline on the Non-Clinical Investigation of the Dependence Potential of Medicinal Products (EMEA/CHMP/SWP/94227/2004)
2005	S7B (Step 5; May 2005): EU: Adapted by CHMP May 2005, issued as CHMP/ICH/423/02. Date for coming into operation: November 2005 MHLW: To be notified FDA: Published in the Federal Register, Vol 70, N^{o} 202, pages 61134–61135; October 20, 2005
	EMEA Drafts: Guideline on the Nonclinical Development of Fixed Combinations of Medicinal Product (CHMP/EMEA/CHMP/SWP/258498/2005) Guideline for the Need for Nonclinical Testing in Juvenile Animals on Human Pharmaceuticals for Pediatric Indications (EMEA/CHMP/SWP/169215/2005) FDA Draft: Safety Testing of Drug Metabolites (Guidance for Industry; June 2005)

current, I_{Ca}, rapid, delayed potassium rectifying current, I_{Kr}, slow, delayed potassium rectifying current, I_{Ks}, and inward rectifying potassium current, I_{K1}) and cardiac cell action potential waveforms (Hammond et al. 2001; Redfern et al. 2003), and in vivo electrocardiography assessments of QT interval prolongation (with heart rate correction, QTc), monophasic action potentials, and effective refractory periods (Batey & Doe 2003; Hammond et al. 2001; Spence et al. 1998). The controversial issue is the accuracy of these models to identify problematic drugs and how these data may be assimilated into an assessment of human risk (Kinter & Valentin 2002). Recognizing that resolution would not be easily forthcoming, the ICH S7 Expert Working Group proposed to the ICH Steering Committee that a new initiative be accepted to generate guidelines on the assessment of drugs for effects on cardiac ventricular repolarization. This proposal

was accepted in November 2000 and was designated ICH Topic S7B, "Guideline on Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals". The guideline on safety pharmacology finalized at the same ICH meeting was redesigned Topic S7A, "Safety Pharmacology Studies for Human Pharmaceuticals".

Shortly after the adoption of the ICH Topic S7B, the U.S. Food and Drug Administration and the Pharmaceutical Research and Manufacturers of America proposed to the ICH Steering Committee the adoption of a parallel initiative to prepare guidelines on clinical testing of new therapeutics for their potential to prolong ventricular repolarization. This proposal was accepted as ICH Topic E14, entitled "The Clinical Evaluation of QT/QTc Interval Prolongation and Pro-Arrhythmic Potential for Non-Antiarrhythmic Drugs". In November 2003, the ICH Steering Committee directed the ICH Topic E14 and S7B expert working groups to align their respective guidelines, in particular the role that non-clinical findings will serve in the design of the clinical study to assess a drug's effect on ventricular repolarization (QT interval). Both guidelines were recommended for adoption at Step 5 of the ICH process in May 2005 by the ICH Steering Committee. The Committee for Medicinal Product for Human Use (CHMP) of the EMEA has adopted both guidelines in May 2005 (Shah 2005a,b) and issued as CHMP/ICH/423/02 (S7B) and CHMP/ICH/2/04 (E14). The date for coming into operation of the S7B was November 2005 and in the Federal Register (FDA) it was published in Vol 70, N^{o} 202, pages 61133–61134; October 20, 2005. The date for coming into operation of the E14 was November 2005 and it was published in the Federal Register, Vol 70, N^{o} 202, pages 61134–61135; October 20, 2005.

I.B.2 Practice of Safety Pharmacology (ICH S7A)

Safety Pharmacology is "those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relationship to exposure in the therapeutic range and above" (ICH S7A, Table 3). Three primary objectives are encompassed in these investigations.

(1) To provide a perspective of the potential pharmacodynamic risk posed to humans by exposure to a new therapeutic agent. This is accomplished through the pharmacodynamic characterization of the new drug on central (peripheral) nervous (Haggerty 1991; Mattsson et al. 1996; Moser 1991; Porsolt et al. 2002; Ross et al. 1998), cardiovascular (Bunting & Siegl 1994; Lacroix & Provost 2000; Kinter & Johnson 2003), and respiratory (Murphy 1994, 2002; Sarlo & Clark 1995) systems (safety pharmacology core battery studies), and other major organ systems (supplemental studies; e.g., gastrointestinal Baldrick et al. 1998; Kinter 2003; Mojaverian 1996 and renal, Chiu 1994; Kinter 2003) as appropriate based on concern for human safety.

(2) To investigate the underlying mechanism(s) of observed effects to refine and improve upon the integrated assessment of the risk posed by the drug when adverse findings have been noted in non clinical or clinical investigations. These may be follow-up studies of the safety pharmacology core battery or the study of other major organ systems (supplemental studies) based on a potential clinical concern (Gad 2004; Kinter & Dixon 1995; Williams & Bass 2003).

(3) To determine the temporal relationship between the pharmacodynamic responses noted with the test substance and the peak blood levels of parent drug and any major metabolites. This information will be used to identify the peak drug levels at the low-observed-effect level (minimal dose level tested that produces an effect; LOEL) and no-observed-effect level (maximum dose level tested without an effect; NOEL), the relationship between parent drug and/or major metabolite and the pharmacodynamic response, and whether the pharmacodynamic changes noted with the test material may be related to animal-specific metabolites. These data are critical to defining a margin of safety between the NOEL and the projection of plasma levels needed to achieve clinical efficacy. They also serve to define the human risk posed by exposure to the new drug (e.g., little risk if the response can be attributed to an animal-specific metabolite) and the possible timing of onset and recovery from any observed effects (Williams & Bass 2003).

The safety pharmacology core battery and any supplemental studies deemed to be necessary to assure human safety are to be conducted in advance of initial clinical trials ("first in human" studies) so that a new drug can progress safely into the clinical phases with an appropriate level of monitoring. In situations where the adverse effects are judged to be potentially serious, or when unexpected pharmacodynamic effects occur in humans, the next tier of testing, investigational safety pharmacology studies (e.g., follow up or supplemental studies), may be appropriate (ICH S7A; see Table 3).

The ICH S7A guideline has brought uniformity to the evaluations of new drugs for effects on organ

functions, mandating with few exceptions, that all drug candidates will be evaluated in the safety pharmacology core battery studies and in follow up and supplemental as appropriate to assure human safety. The cardiovascular, respiratory, and central (peripheral) nervous system functions were selected for the safety pharmacology core battery based on the concern that an acute failure of these systems would pose an immediate hazard to human life. The examination of additional organ systems (e.g., gastrointestinal, renal, etc.) may also be appropriate based on a cause for concern for human safety. The experimental models, endpoints, and study designs chosen should be relevant to the prediction of the potential human response. Preference is given to studying animals in the conscious versus the anesthetized states and in the unstressed/unrestrained versus stressed/restrained conditions, to the extent possible within Animal Welfare guidelines. The clinical route is the preferred route, unless otherwise justified, for example, intravenous rather than oral route to achieve higher blood levels of the parent drug, where oral bioavailability in the test species may be low. Data are collected for a period that has the potential to define the onset, duration, and recovery from possible pharmacodynamic effects. This data collection period would be initially based on the pharmacokinetic (or toxicokinetic) properties of a drug in the selected species and, at a minimum, encompass the time at which the maximum plasma concentrations of the parent drug and any major metabolites are achieved. The demonstration of reversibility/recovery from pharmacodynamic effects may be accomplished by waiting five or more half-lives before terminating the data collection. In the event that human-specific metabolites are detected in the early clinical phases, consideration would be given to non-clinical pharmacodynamic studies that would be appropriate to assure continued human safety.

I.B.3 Institutional Strategies

The impact of the implementation of the ICH S7A guideline on the organization, philosophy and practices in safety pharmacology in the pharmaceutical industry shows some controversies and challenges.

In a recent survey, Valentin and Bass (2004) showed the impact in various pharmaceutical companies. The types of safety pharmacology studies conducted are different. During the pre-candidate drug selection phase primarily in vitro and in vivo vascular studies were conducted. The focus in these studies was on cardiac repolarization. CNS studies were of lesser extent. In the post-candidate phase, the "core battery" of S7A as well as "follow-up" and "supplementary" studies were performed. In late phases, during clinical development, studies followed on a case by case basis.

Should early Safety Pharmacology studies replicated in order to claim GLP compliance? This question was answered differently.

YES for those who think: it increases the statistical power, shows higher exposure of the test article, slightly different focus, GLP, "box-ticking" reasons.

NO for those who mentioned: studies sometimes acceptable by regulatory agencies, animal welfare (3Rs), limited resources, cost/time vs. benefit, limited resources, avoid generating conflicting results.

The design and the execution of safety pharmacology studies are focused upon the safety of human volunteers and patients in clinical trials. ICH S7A and may be soon S7B strive for effective integration of safety pharmacology results with those of the non-clinical (toxicology) and clinical safety databases.

Overall the ICH S7A guideline is successfully implemented in the pharmaceutical world. The "core battery" is in general performed prior to "First in Man". The guideline increases the visibility of safety pharmacology within companies and increases focus by regulatory agencies.

I.B.4 Future of Safety Pharmacology

The future of safety pharmacology will depend, in part, upon the scientific and technological advances and regulatory challenges that envelop pharmaceutical development (Cavero & Crumb 2005; Porsolt et al. 2005). With advances in molecular biology and biotechnology, which allow for the identification of new clinical targets, newer pharmaceutical agents are being identified that act at these novel molecular sites in an attempt to ameliorate the disease condition. Inherent in the novelty of new targets is the risk of unwanted effects that may or may not be detected with current techniques. The scientific challenge facing safety pharmacology is to keep pace, to adapt, and to incorporate new technologies in the evaluation of new drugs in non-clinical models and identifying the effects that pose a risk to human volunteers and patients.

Recent examples include safety pharmacology's embracement of modern electrophysiological techniques to evaluate the effects of new drugs on the ionic components of the cardiac action potential (Redfern et al. 2003), and telemetry techniques to permit the

chronic monitoring of physiological functions in unstressed animals (Kinter & Johnson 1999; Kramer & Kinter 2003; Kramer et al. 1998). Efforts continue to construct databases relating the similarities and differences between animal and human responses to pharmaceutical agents (Igarashi et al. 1995; Olsen et al. 2000). As an example, non-clinical safety studies, including safety pharmacology studies, are typically conducted in normal, healthy, young adult or adult animals. However, these tests may not appropriately detect specific responses in humans at other ages (e.g., neonates, adolescents, and geriatrics) or those with underlying chronic diseases (e.g., heart failure, renal failure, and type II diabetes), conditions which may alter the pharmacodynamic response to a drug. In some cases, animal models that overexpress or are deficient in the unique targets, or are otherwise manipulated to model the human pathophysiological conditions, may provide additional focus and sensitivity to detect and interpret the potential unwanted effects of new drugs in terms of human risk (Hondeghem et al. 2001).

The future of safety pharmacology is also intertwined with international regulatory guidelines such as ICH Topics S7A, S7B, and E14. The discipline is considered integral to the evolving regulatory strategies for safely accelerating the introduction of these drugs into the clinical phases [e.g., EMEA (CPMP), "Position Paper on Non-clinical Safety Studies to Support Clinical Trials with a Single Microdose" and the U.S. Food and Drug Administration, Screening Investigational New Drug Application]. Additionally, safety pharmacology is also considered important to newly emerging regulatory guidelines from U.S. Food and Drug Administration, such as the "Safety Evaluation of Pediatric Drug Products and Nonclinical Studies for Development of Pharmaceutical Excipients" and further guidelines (see Table 3).

The introduction of pharmaceutics into the environment is gaining the attention of both regulators and pharmaceutical industry (Calamari 2003; Huggett et al. 2003; Kopin et al. 2002). While this is not currently the subject of any international environmental guideline, the use of organ function endpoints may become an important component in bridging safety data collected in mammalian vertebrates (including humans) to aquatic species for purposes of the identification of relevant target species and organ functions and the design of specific environmental toxicology studies.

The future of safety pharmacology will be contained within the vision of its current and future leaders, the issues and concerns that they face, and the solutions to the important problems that they generate.

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Online Links

These are the text-versions published in November 2005, for modifications and newer versions see the recent web-pages.

S7A Safety Pharmacology Studies for Human Pharmaceuticals:
http://www.ich.org/LOB/media/MEDIA504.pdf

S7B The Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) By Human Pharmaceuticals (Step 5, May 2005):
http://www.ich.org/LOB/media/MEDIA2192.pdf

E14 The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs (Step 5, May 2005):
http://www.ich.org/LOB/media/MEDIA503.pdf

S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals:
http://www.ich.org/LOB/media/MEDIA503.pdf

Points to consider: The Assessment of the Potential QT Interval Prolongation by Non-Cardiovascular Medicinal Products: http://www.emea.eu.int/pdfs/human/swp/098696en.pdf

Chapter I.C
Central Nervous System (CNS) Safety Pharmacology Studies

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I.C.1 General Considerations

Definition of Safety Pharmacology

Safety pharmacology studies are "those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic dose-range and above" (Anon 2000). There has, unfortunately, been considerable confusion about the term safety pharmacology, and many other kinds of study have also been included. This is no doubt explained by the multitude of terms which have been used to describe the area: general pharmacology, ancillary pharmacology, secondary pharmacology, high-dose pharmacology and regulatory pharmacology (Porsolt 1997).

Scope of CNS Safety Pharmacology

What kinds of studies come within the scope of CNS safety pharmacology? This topic was extensively developed in the Japanese Guidelines (Anon 1995), which divided recommended CNS safety pharmacology studies into two categories, A and B. Category A included so-called core battery studies such as general behavioral observation, measures of spontaneous motor activity, general anesthetic effects and potential synergism/antagonism with general anesthetics, effects on convulsions (proconvulsant activity and synergy with convulsive agents), analgesia and body temperature. Category B included effects of the test substance on the electroencephalogram (EEG), the spinal reflex, conditioned avoidance response and locomotor coordination. The governing notion of Category A was that the studies mentioned were obligatory, whereas those mentioned in Category B were to be carried out when necessary. More recently, the European Agency for the Evaluation of Medicinal Products has proposed a new set of guidelines ICH S7A (Anon 2000), that came into effect in June 2001, and have since been adopted in the USA and Japan. The European guidelines are much less specific than the Japanese guidelines, and include as core battery CNS studies "motor activity, behavioral changes, coordination, sensory/motor reflex responses and body temperature" with the remark that "the central nervous system should be assessed appropriately". Follow-up studies should include "behavioral pharmacology, learning and memory, ligand-specific binding, neurochemistry, visual, auditory and/or electrophysiology examinations, etc". In general, core battery studies should be carried out prior to first administration in humans, whereas the follow-up studies should be carried out prior to product approval. A final recommendation is that core battery studies should be carried out in accordance with GLP, whereas follow-up studies,

because of their unique characteristics, require only assurances of "data quality and integrity".

Comparison of the Japanese Guidelines and ICH S7A suggests a clear intent by the European and USA authorities to free safety pharmacology from the constraints of a cookbook approach. On the other hand, their vagueness does not provide any clear idea of what could or should be done. In particular, only passing mention is made of drug abuse/dependence assessment, despite this evident safety concern for a large variety of pharmacological agents.

In vivo versus in vitro

CNS safety pharmacology makes use mainly of in vivo methods in conscious animals. The primary reason is that CNS function is best evaluated in an intact and freely moving animal. In contrast to cardiovascular safety pharmacology, where important aspects of function can be evaluated in anesthetized animals or even isolated organs or cells, behavior is the global result of multiple mechanisms, many unknown, and by definition occurs only in the conscious animal or person. Possible exceptions are drug effects on CNS electrophysiology, where drug effects on cerebral cells or structures can be investigated under anesthesia. On the other hand, CNS electrophysiology can also be investigated using EEG techniques in conscious animals with higher predictability for real life situations.

Core Battery CNS Safety Pharmacology Studies

ICH S7A distinguishes between core battery studies and supplementary or follow-up studies. Core battery CNS procedures are typically simple tests, using traditional techniques, which can be carried out rapidly in a routine fashion. They are the first techniques to be employed in safety assessment, and are frequently applied at the very beginning of the discovery process as a screen to eliminate substances with a potential for CNS risk. Because of their use early in the safety evaluation process, such studies are conducted almost exclusively in the rodent. ICH S7A recommends that core battery studies should include measures of drug-induced observable signs, measures of spontaneous locomotion and motor coordination. Three other kinds of measure, originally recommended by the Japanese Guidelines Category A but dropped from the ICH S7A core battery, are the convulsive threshold, interaction with hypnotics and the pain threshold. In contrast to ICH S7A, it seems to us that such measures could usefully be included in a core battery of CNS safety pharmacology procedures. Decreases in the convulsive threshold are by no means negligible in the assessment of CNS safety. Several substances, including antipsychotics like clozapine, do not induce frank convulsions at any dose but clearly decrease the convulsive threshold. Even anticonvulsive activity, which in itself is not a risk factor, could be a useful predictor of cognition impairing effects. Several anticonvulsants, such as benzodiazepines and NMDA antagonists, are known to impair cognition in addition to their anticonvulsant activity. Thus the presence of anticonvulsant activity could represent a useful first screen for potential cognition impairing effects (Porsolt et al. 2002). In a similar fashion, sleep-inducing or sleep-attenuating activity could be unmasked by a barbiturate interaction procedure. Benzodiazepines, for example, do not by themselves induce sleep but their sleep enhancing activity can be readily detected in interaction with barbiturates. The same is true for psychostimulants which may or may not induce signs of excitation in a primary observation procedure, but clearly block barbiturate-induced sleep. Finally, a drug-induced increase in pain sensitivity would certainly constitute a CNS risk factor which could be assessed fairly simply using a nociception procedure. Whereas analgesic ctivity is not in itself a risk factor, it has even been suggested (Franklin 1998) that the presence of analgesic activity constitutes a predictor of abuse liability. Compared with current cardiovascular and respiratory procedures, inclusion of such tests in a core battery would not represent a major expense, but would considerably improve the assessment of CNS risk. ICH S7A guidelines recommend that core battery studies be conducted in compliance with GLP. Protocols for such tests are included below (Section 2).

Supplementary CNS Safety Pharmacology Studies

Supplementary or follow-up studies are more wide-ranging and cover cognitive function (learning, memory and attention), brain function (EEG) and dependence/abuse potential. Because of their complexity, there exist no standard protocols and there is no requirement that such studies be carried out in compliance with GLP. A more stringent requirement is that such procedures be carried out according to internationally accepted scientific standards of excellence. Protocols which we have found useful for evaluating such effects are also included below (Section 3).

Choice of Animal Species

Most of the core battery studies can be performed in the mouse or the rat. The protocols described below

provide the rat version and have been standardized in our own laboratory using male Wistar rats weighing between 150 and 250 g depending on the tests. Apart from general observation procedures, where the behavioral repertoire in the rat is richer than that in the mouse, there is no theoretical reason for preferring the rat. On the other hand, the rat is the species of choice for many other areas of drug development (chronic treatment studies, toxicology, biochemistry, pharmacokinetics). It is therefore preferable to use the rat for CNS core battery studies to ensure a maximum of coherence with other available data. Although the mouse is clearly more economical both in terms of cost and the quantity of test substance required for carrying out the experiments, such considerations are less relevant later in the drug development process (just before Phase I), where larger quantities of test substance are available and the costs of such experiments are minor in comparison with other development costs.

For supplementary studies, the rat remains the species of choice. A possible exception is the use of primates for assessment of abuse potential. The reason is not that drug effects differ between the species, but more because primates are closer to man in terms of active doses and pharmacokinetics, thereby increasing predictability in areas of more complex CNS function.

Route of Administration

Test substances can be administered by different routes of administration. ICH S7A guidelines suggest that safety pharmacology studies should be performed using the same route of administration as that intended in man. Because in most cases drugs are administered by the oral (p.o.) route, the p.o. route is used most frequently for CNS core battery studies. On the other hand, there is no guarantee that rats have similar absorption and metabolism to man via the oral route. Indeed some drugs, for example the neuroleptics haloperidol or sulpiride, are poorly absorbed in the rat after oral administration but are given orally in man. The risk of poor absorption in the rat might indeed provide a justification for using a more effective route, intraperitoneal (i.p.) or sub-cutaneous (s.c.) for safety pharmacology studies, where the aim is to establish potential risk. Thus, showing an absence of adverse effect by a pharmacologically sensitive route, should in principle provide the best estimate of safety.

Statistical Analyses

The aim of safety pharmacology is the detection of risk. It is therefore essential not to miss elements which could compromise the safety of a potential therapeutic agent. In other words, the number of false negatives should be kept to a minimum. False positives (erroneous detections of possible risk), although troublesome, are less serious and can usually be corrected by supplementary testing.

Translated into statistics, this implies that for safety pharmacology the risk of Type 2 errors (false negatives) should be decreased as much as possible, even if there is an increase in the risk of Type 1 errors (false positives). In other words, the statistical tests employed in safety pharmacology should err in the direction of oversensitivity rather than the reverse. A test substance found not to have significant safety risks based on preclinical studies, even after the use of oversensitive statistics, is more likely to be truly devoid of risk. As a consequence, the statistical analyses proposed for the CNS safety procedures described below (mainly two-by-two comparisons with control using Student's t tests) have been selected for maximal sensitivity to possible effects per dose at the acknowledged risk of making more Type 1 errors.

Ethical and Animal Welfare Issues

As with all procedures involving living animals, important considerations in the choice of method are the ethical issues surrounding it. Most regulatory bodies have made pronouncements on this subject, and the reader should consult these documents for more detailed information. However, the guiding principles are to use as few animals as necessary and to avoid suffering as much as possible.

In the field of safety pharmacology, where the aim is to assess the risk of unwanted effects, the possibility of causing suffering is potentially higher than in other areas of pharmacology. This is particularly true for CNS safety pharmacology studies which use intact and conscious animals. The experimenter must therefore maintain awareness of these issues, not only in planning and devising the protocols but also during the experiments, where procedures for stopping the experiment in the event of well-defined events (e.g. pain or death) should be in place. Conditions should be fixed in advance such that these events are absent or exceptional, and allow decisions to be made to stop the experiment if they occur.

Safety pharmacology generally uses larger group sizes than efficacy pharmacology, and it is important that the results are sufficiently reliable for regulatory decisions to be made. However, in some circumstances, methods exist to reduce animal use while at the same time maintaining scientific validity. One example is

the supplanting of the traditional LD50 acute toxicity test, which uses a large number of animals to obtain very limited data. Similar data, with fewer animals and considerably more information, can be obtained using the Irwin procedure.

The test procedures described below have been selected and conceived to comply with the ethical requirements outlined above.

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I.C.2 Core Battery CNS Studies

The present Section describes basic protocols satisfying ICH S7A recommendations for core battery CNS studies. Included are protocols for measuring general behavioral signs induced by test substances (Irwin Test), effects on spontaneous locomotion (Activity Meter Test), effects on neuromuscular coordination (Rotarod Test), effects on the convulsive threshold (Electroconvulsive Shock (ECS) Threshold and PTZ Seizure Tests), interaction with hypnotics (Barbital Interaction Test) and effects on the pain threshold (Hot Plate Test).

I.C.2.1 Irwin Test

PURPOSE AND RATIONALE

The primary aim of the Irwin Test, first described in the mouse by Irwin (1968) but easily adapted to the rat, is to evaluate the qualitative effects of the test substance on behavior and physiological function, from the first doses that have observable effects up to doses which induce clear behavioral toxicity or even death. The Irwin Test also permits a reasonable estimate of the test substance's duration of action on the different parameters observed.

Because most measures involve subjective assessment of different aspects of the animal's behavior, the test must be performed in a highly standardized manner by well trained observers to ensure reproducible findings on different occasions, or at different observation times on the same day. Indeed, because of systematic changes in the behavior and reactions of test animals over a test day, drug-treated animals are always evaluated with reference to a simultaneously treated group of vehicle controls under non-blind conditions to gauge whether an observed effect is truly a consequence of treatment with the test substance.

PROCEDURE

Rats are administered the test substance and are observed simultaneously with a control group given vehicle. Although the test treatment can be blinded, the observer is always aware of which group received vehicle. Two treated groups are compared simultaneously with a single control group. The first 2 doses investigated are generally the highest and lowest planned doses. The order of testing the other doses depends on the effects observed.

Behavioral modifications, physiological and neurotoxicity symptoms, rectal temperature and pupil diameter are recorded according to a standardized observation grid derived from that of Irwin. The grid contains the following items: death, convulsions, tremor, Straub tail, sedation, excitation, jumping, abnormal gait (rolling, tiptoe), motor incoordination, altered muscle tone, loss of grasping, akinesia, catalepsy, loss of traction, loss of balance, fore-paw treading, writhing, piloerection, stereotypies (sniffing, chewing, head movements), head-twitches, scratching, altered respiration, aggression, altered fear, altered reactivity to touch, ptosis, exophthalmia, loss of righting reflex, loss of corneal reflex, analgesia, defecation/diarrhea, salivation, lacrimation, rectal temperature (hypothermia/hyperthermia) and pupil diameter (myosis/mydriasis). Further details of the evaluation of the signs/symptoms are provided in Table 1.

Observations are performed 15, 30, 60, 120 and 180 minutes after administration of the test substance and also 24 and 48 hours later. The symptoms marked (*) are observed continuously from 0 to 15 minutes after administration.

4 rats are studied per group.

The test substance is usually evaluated at 6 doses, administered p.o. immediately before the test. For safety pharmacology studies, the lowest dose is close

Table 1

SYMPTOMS	OBSERVATIONS
Death*	Presence Indicate the time of death if < 15 min.
Convulsions*	Presence Indicate the time of appearance if < 15 min.
Tremor*	Presence Whole or part body trembling
Straub tail*	Presence Rigid tail held upright and tending to curve over the back of the animal
Sedation	3 intensities: +: After removing the cage cover and manipulation, the animal moves more slowly than controls ++: Under the same conditions, the animal moves very slowly +++: Under the same conditions, the animal does not move at all
Excitation	3 intensities: +: After removing the cage cover and manipulation, the animal is slightly more active than controls ++: Under the same conditions, the animal moves rapidly (frequently stopping) +++: Under the same conditions, the animal moves very rapidly (occasionally stopping)
Jumping*	Presence
Abnormal gait*	Presence e.g. Rolling gait. Motor coordination preserved, occasional staggering Locomotion on tiptoe
Motor incoordination*	Presence Motor coordination impaired. Disorganized locomotion (e.g. crossing of paws etc.)
Altered muscle tone	Increase (↑) or decrease (↓) Hardness or softness of the abdomen when pressed laterally between forefinger and thumb is compared with controls
Loss of grasping	Presence When placed on a horizontal wire grid, the animal does not grasp grid when pulled backward by the tail
Akinesia	Presence When placed on a wire grid, the animal does not move when the grid is progressively moved towards a vertical position
Catalepsy	Presence When placed in Buddha position (upright posture on its hind paws), the animal does not move
Loss of traction	Presence When placed on a horizontal bar by the fore-paws, the animal fails to bring its hind-paws up onto a second bar placed below the first one (3 trials maximum)
Loss of balance*	Presence Motor coordination impaired. The animal falls on its side when it moves
Fore-paw treading*	Presence Repeated stamping of fore-paws without displacement
Writhing*	Presence Coordinated contraction of abdominal muscles usually resulting in hollow flanks and stretching of hind-limbs
Piloerection*	Presence Fur standing on end
Stereotypy*	Presence Abnormal repeated movements: Sniffing Chewing Head movements
Head-twitches*	Presence Rapid saccadic side-to-side axial movements of the head

SYMPTOMS	OBSERVATIONS
Scratching*	Scratching with fore- or hind-paws anywhere on the body.
Altered respiration*	Increase (↑) or decrease (↓) Respiration rate is compared with controls
Aggression*	Presence Biting attempts when approached towards head with a ballpoint pen. Biting attempts toward the other animals or toward the experimenter
Altered fear	Increase (↑) or decrease (↓) Animal's reaction (flinch, jump...) when fingers are snapped above cage is compared with controls
Altered reactivity to touch	Increase (↑) or decrease (↓) Animal's flight reaction to downward finger pressure on the hindquarters is compared with controls
Ptosis	Presence Eyelids partially or completely closed
Exophthalmia	Presence Protrusion of the eyeballs
Loss of righting reflex	Presence When placed on its back, the animal does not right itself
Loss of corneal reflex	Presence When the corneal surface is lightly touched with the tip of a pen, the animal does not close eye
Analgesia	Presence When pinched at base of tail with forceps, the animal does not react (turning towards forceps, vocalization)
Defecation/Diarrhea	Presence
Salivation	Presence Dampness visible around mouth
Lacrimation	Presence Dampness visible around eyes
Hypothermia	Presence 3 levels based on mean temperature measured in treated and control animals +: decrease > 1° ++: decrease > 2° +++: decrease > 3°
Hyperthermia	3 levels based on mean temperature measured in treated and control animals +: increase > 1° ++: increase > 2° +++: increase > 3°
Myosis	3 levels based on mean pupil diameter measured in treated and control animals (1 unit =1/30 mm) +: decrease > 10 units ++: decrease > 20 units +++: decrease > 30 units
Mydriasis	3 levels based on mean pupil diameter measured in treated and control animals (1 unit =1/30 mm) +: increase > 10 units ++: increase > 20 units +++: increase > 30 units

to the therapeutic dose as estimated in tests predictive of the indication, and the highest dose can be 100 or 300 times this dose if the substance's toxicity or physical characteristics permit administration of such a dose.

EVALUATION

Being mainly a qualitative assessment procedure no formal statistical analysis is conducted. Most symptoms are evaluated by their presence or absence. Some (sedation, excitation) are rated on a 3-point scale.

Table 2 Effects of diazepam in the Irwin Test

1 (mg/kg p.o.)	2 (mg/kg p.o.)	4 (mg/kg p.o.)	8 (mg/kg p.o.)	16 (mg/kg p.o.)	64 (mg/kg p.o.)
No change	Hypothermia + at 30'	Sedation + ↓ Traction Hypothermia (++ → 30') (+ at 60' and 120')	Sedation +↓ Traction Hypothermia (++ → 60') (+ at 120')	Sedation ++ ↓ Traction ↓ Reactivity to touch ↓ Muscle tone Abnormal gait Hypothermia ++ (+++ at 30') (+ at 120')	Sedation ++ ↓ Traction ↓ Reactivity to touch ↓ Muscle tone Abnormal gait Hypothermia (+++ → 120) (++ at 180') (+ at 24 h)

Note the dose-dependent increase in sedation and decreases in reactivity to touch, traction and muscle tone accompanied by abnormal gait.
N = 4 for each treatment group.
+ = slight; ++ = moderate; +++ = marked. (‘): minutes
Observations were performed at 15, 30, 60, 120, 180 minutes after administration.
The symptoms which did not necessitate handling were also observed up to 15 minutes immediately following administration.
Hyperthermia and mydriasis were evaluated by comparison of the mean scores obtained in treated and control animals.

Others (respiration, fear, reactivity to touch) are scored in 2 directions. Finally, certain parameters (rectal temperature, pupil diameter) are measured quantitatively.

CRITICAL ASSESSMENT OF THE METHOD

The Irwin Test serves at both ends of the drug discovery/development spectrum.

In the exploratory phase of drug development, it is frequently the first test employed in vivo. During this phase the purpose of the test is to provide a rapid detection of the test substance's toxicity, active dose-range and principal effects on behavior and physiological function. A first estimate of safety is provided by the difference between the first doses inducing observable effects and those inducing frank behavioral toxicity or lethality. Even when the test substance is intended for a non-psychotropic indication, the Irwin Test permits the choice of doses for subsequent tests. Furthermore, the Irwin Test can frequently permit identification of the kind of activity exerted by the test substance in the psychotropic domain. As is illustrated in Tables 2 and 3, clearly distinct and identifiable profiles are observed with drugs such as the anxiolytic diazepam and the analgesic morphine.

At later stages of drug development (before Phase I), when the test substance has been more fully characterized, the Irwin Test provides a clear over-all index of the test substance's margin of safety. Furthermore, although the test is mainly behavioral, it can give global indications of drug effects on vital functions such as respiration or intestinal motility. On the other hand, the test provides little information about effects which are not visible from direct observation, including the whole range of cardiovascular, pulmonary and gastrointestinal parameters.

MODIFICATION OF THE METHOD

The Irwin Test, although originally described in the mouse, was rapidly applied to the rat (Esteve et al. 1988). Indeed, the principle of systematic observation of drug-induced symptoms using standardized observation criteria can be applied to a wide range of animal species, and even constitutes the basis of symptom check-lists in man. Every laboratory has its own manner of doing the Irwin Test. That described above is what we have been practising for over 20 years. ICH S7A simply requires that animals be observed on a systematic basis over a wide dose-range. Alongside variants of the Irwin procedure, ICH S7A mentions another systematic observation procedure, the Functional Observation Battery (FOB) more recently described by Mattson et al. (1996) as being functionnally equivalent. This test, derived from classical toxicology, is more specifically used for testing neurotoxicity.

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Mattson JL, Spencer PJ, Albee RR (1996) A performance standard for clinical and Functional Observational Battery examination of rats. J Am Coll Toxicol 15:239–250

Table 3 Effects of morphine in the Irwin Test

1 (mg/kg i.p.)	2 (mg/kg i.p.)	4 (mg/kg i.p.)	8 (mg/kg i.p.)	16 (mg/kg i.p.)	32 (mg/kg i.p.)
No change	Abnormal gait (rolling) 30' ↑ Reactivity to touch 60'	Excitation + at 30' and 120' Abnormal gait (rolling) 15' → 30' ↑ Reactivity to touch 60' Exophthalmia15'	Straub 30' Sedation + at 15' Excitation + at 30' and 120' Abnormal gait (tip-toe) 15' and 30' ↑ Reactivity to touch 60' Analgesia 30' Hyperthermia+ 60' → 120'	Straub 30' → 120' Sedation + 30' → 60' Excitation+ 120' → 180' Abnormal gait (tip-toe) → 120' ↑ Fear 30' ↑ Reactivity to touch 15' → 30' and 120' → 180'	↑ Muscle tone 60' → 120' Exophthalmia 30' → 120' Analgesia 30', 60' and 120' Hyperthermia+ at 120' Lethality(3/4) at 60'

Note the dose-dependent occurrence of Straub tail, analgesia, sedation followed by excitation, accompanied by tip-toe gait.
N = 4 for each treatment group.
+ = slight. (‘): minutes.
Observations were performed at 15, 30, 60, 120, 180 minutes after administration.
The symptoms which did not necessitate handling were also observed up to 15 minutes immediately following administration.
Hyperthermia and mydriasis were evaluated by comparison of the mean scores obtained in treated and control animals.

I.C.2.2 Activity Meter Test

PURPOSE AND RATIONALE

Locomotor activity can be quantified in rodents by a variety of means. The method described below uses interruptions of photoelectric beams. The difference between Irwin estimations of drug effects on spontaneous activity and activity meter tests is mainly a question of quantification. Activity meter tests are generally carried out using automated apparatus with a larger number of animals, and are therefore less labor intensive but permit more precise statistical analyses. The quantitative data obtained enable the generation of dose-response curves and more precise estimations of the minimal effective dose (MED) or the dose which increases or decreases locomotion by 50 % (ED50).

PROCEDURE

The activity meter we use (Imetronic, Bordeaux, France) consists of 12 covered Plexiglas cages (40 × 25 × 25 cm) each equipped with two photocell assemblies placed at the ends of the cage which is contained within a darkened enclosure. The number of beam-crossings by each animal (one per cage) from one photocell to the other is recorded by computer over 10 minute periods for 30 minutes. The scores are cumulated over the 30 minute period. In addition, another set of beams placed vertically higher permit quantification of rearing behavior.

Ten rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 4 doses, administered p.o. 60 minutes before the test, and compared with a vehicle control group.

Caffeine (32 mg/kg p.o.) and chlorpromazine (16 mg/kg p.o.), administered under the same experimental conditions, are used as reference substances.

The basic experiment therefore includes 7 groups.

EVALUATION

The basic measure taken is the global effect of the test substance over the whole measurement period. More detailed information can be obtained by analyzing the drug effects over sequential time slots, for example every 10 minutes. For example, some drugs (diazepam, nicotine) attenuate high activity levels (first 10 minutes) but increase low levels (last 10 minutes). Further analyses can compare the effects of the test substance on rearing with its effects on locomotion within the enclosure. Finally, comparison of single photobeam interruptions with paired interruptions of the beams at each end of the enclosure can permit distinctions between drug effects on small and large movements.

The data are usually analyzed using Student’s t tests.

CRITICAL ASSESSMENT OF THE METHOD

Locomotion tests estimate whether a test substance possesses psychostimulant or sedative activity, but

the data obtained must be interpreted with caution. Locomotion can be decreased because the animals are sedated, but also because the animals have drug-induced motor impairment or are otherwise debilitated by the test substance. Even substances with marked psychostimulant properties can decrease activity meter scores because the animals are rotating rapidly in a small space or are showing other stereotyped behaviors. It is therefore unwise to interpret activity meter scores in isolation from direct observation (Irwin Test above) or measures of neuromuscular coordination (Rotarod Test below).

Another complication with activity meter tests is the phenomenon of habituation. All animals placed in an unknown environment will tend to explore it more at the beginning of the exposure with a decline in exploration with time. This can be clearly seen in most activity meters despite the apparent simplicity of the test environment. Thus, when locomotion is followed over sequential time slots, an apparent increase or decrease in drug effect over time cannot simply be interpreted as a change in its pharmacological activity. The substance may be interacting with the process of habituation. It is for this reason that activity meters are not the ideal means of measuring the duration of drug action.

Because the activity meter evaluates spontaneous behavior, the behavioral baseline is intrinsically variable and subject to many kinds of influence, including lighting, apparatus cleanliness, ambient temperature, noise level, and even time of day. As a consequence, particular care has to be taken to ensure constant experimental conditions to obtain reproducible results. Furthermore, when comparing different drug treatments, it is important to take advantage of the fact that several animals can be evaluated simultaneously, by distributing the different treatments in a balanced fashion over the test period, and even in the positions within the experimental apparatus or of the observation chambers in the experimental room.

MODIFICATION OF THE METHOD

Locomotor activity can be quantified in rodents by a variety of means, interruptions of photoelectric beams, activity wheels, changes in electromagnetic fields, Doppler effects, video-image analysis, telemetry (Reiter and McPhail 1979). As has been suggested above, it is not very important how locomotion is measured because the main outcome measure is whether a test substance increases or decreases spontaneous locomotion.

More important is the time at which a test substance is administered in relation to placing the animal in the activity meter. Because of potential interactions of the test substance with the process of habituation, we think it is important to start testing when the drug effect has had time to reach its maximum, i.e. an appropriate interval after drug administration. If the animal is treated and immediately placed in the activity meter, the kind of drug effect observed may critically depend on the interaction between the onset of drug action and the habituation process.

Another procedural variant, aimed to minimize the role of habituation, is to prehabituate the animal to the environment before administering the test substance. In this way it is hoped to assess drug action on "baseline" behavior in already habituated animals. This approach appears to be a reasonable one but considerably increases the time and costs for conducting the test. Furthermore, there are cases where drug effects are dramatically attenuated in pre-habituated animals (Porsolt et al. 1970). In particular, this approach is much less sensitive to drug-induced decreases in motor activity.

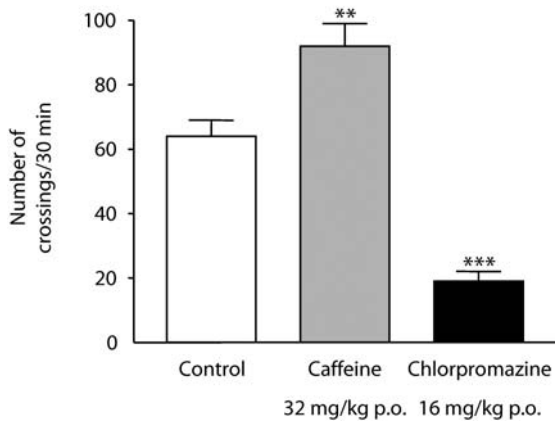

Fig. 1. Activity meter test. Effects of caffeine and chlorpromazine (p.o.) on locomotion in the rat.

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I.C.2.3 Rotarod Test

PURPOSE AND RATIONALE

Locomotor coordination is most commonly assessed using a rotarod (Dunham and Miya 1957). The rotarod consists of a circular rod turning at a constant or increasing speed. Animals placed on the rod will naturally try to remain on the rod rather than fall onto a platform some 30 cm below. This test therefore provides an estimate of the animal's level of neuromuscular coordination. Drugs which are known to perturb neuromuscular coordination (e.g. benzodiazepines) clearly reduce the time the animals stay on the rod.

This test lends itself readily to automation with several animals being tested simultaneously on the same rod, each animal being separated by vertical barriers.

PROCEDURE

Habituation

Rats are placed on a rod (diameter: 7 cm) rotating at a speed of 12 revolutions per minute for a 2 minute period (first habituation session). The rotarod apparatus we use consists of 4 positions separated by a screen, permitting the automatic measurement of 4 animals simultaneously (Ugo Basile, Model 7750). If the rats fall off during this period they are replaced on the rod. At the end of the first habituation session, rats are placed on the rod under the same conditions as used during the test session (i.e. for a maximum of 3 minutes) but are not replaced on the rotarod if they fall off (second habituation session).

Test

The test session is performed at least 2 hours after the second habituation session. Rats are placed on the rotarod for a maximum period of 3 minutes. The number of animals which fall off before the end of this period is counted and the latency to fall off is recorded (maximum: 3 minutes).

Ten rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 4 doses, administered p.o. 60 minutes before the test, and compared with a vehicle control group.

Diazepam (8 mg/kg p.o.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes 6 groups.

EVALUATION

Two basic measures are taken, the number of animals per group which fall off the rod within the 3 minute test and the drop-off times. The quantal data (number of animals falling) are analyzed using Fisher's Exact probability Test. The quantitative data (drop-off times) are analyzed using Student's t-tests.

CRITICAL ASSESSMENT OF THE METHOD

The rotarod test is one of the oldest used in the behavioral assessment of drug action. It provides a simple first estimation of whether a test substance has any effect on neuromuscular coordination and has been found sensitive to a variety of agents which are known to disturb it, for example the benzodiazepines.

When preceded by the habituation procedure (described above), the test is very reliable and reproducible. Generally speaking, the doses impairing rotarod performance are higher than those affecting locomotor activity, except for drugs having particular effects on motor coordination (notably the benzodiazepines). Furthermore, in contrast to the Activity Meter Test, the Rotarod Test is not particularly sensitive to environmental factors such as lighting, ambient temperature or noise. This is no doubt due to the fact that the test imposes a behavior on the animal (staying on the rod), where the consequences of falling are mildly aversive.

On the other hand, the Rotarod Test can be considered only as an initial screen for neuromuscular impairment. More complex tests, for example analyses of gait, are required to further understand the motor aspects, whereas electrophysiological procedures, for example the electromyogram (EMG), are required for better understanding the neuromuscular aspects.

In contrast to most of the other core battery CNS tests, the rotarod is mainly unidirectional, detecting principally the capacity of substances to decrease neuromuscular coordination. This is not a serious limitation in that the risk factor evaluated is whether the test substance causes impairment. When used in conjunction with locomotor activity tests, the rotarod test provides a useful quantification of the margin of safety between doses of test substances which alter spontaneous activity and those which disturb motor function.

MODIFICATIONS OF THE METHOD

The only major modification of the method is the use of an accelerating rotarod (Capacio et al. 1992). With this procedure, the test starts with the rotarod turning at

a very slow speed (about 5 turns per minute), with the speed progressively increased over a 3–5 minute test. The maximum speed is about 40 turns per minute. The measure taken is the latency to fall off the rod, which therefore represents the maximum speed tolerated by the animal.

This procedure possesses the advantage that little or no prior habituation is required and that all latency measures can be employed for measuring drug effects, in principle allowing the use of more powerful parametric statistical tests. On the other hand, it is not clear whether the data obtained using this version of the procedure yields markedly different results from those obtained with the more simple version described above.

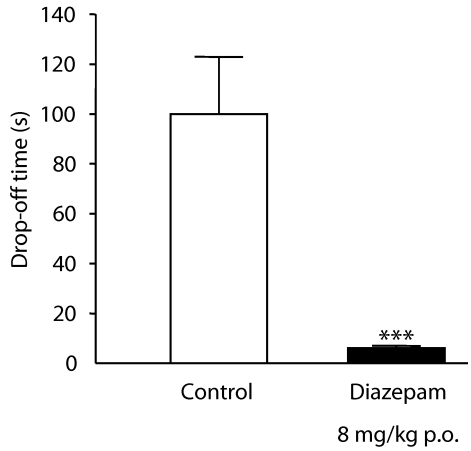

Fig. 2. Rotarod test. Effects of diazepam (p.o.) on rotarod performance in the rat.

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I.C.2.4 Convulsive Threshold Tests

PURPOSE AND RATIONALE

Tests for the convulsive threshold estimate whether a test substance induces changes in the probability of convulsions occurring either spontaneously or, more importantly, in association with other treatments. Although overt convulsions can usually be detected using the Irwin procedure, proconvulsant activity can occur in the absence of overt convulsions and therefore needs to be evaluated in specific tests. The existence of anticonvulsive activity is less dramatic as a risk factor. Nonetheless, as suggested in the General Introduction above, such activity might be an indicator of potential cognitive disturbance. There is therefore a need, in CNS safety pharmacology, to include procedures which assess changes in the convulsive threshold in both directions.

Described below are two procedures whereby convulsions are induced either electrically (ECS Threshold Test), following Swinyard et al (1952), or chemically (PTZ Seizure Test), following Krall (1978). We recommend using two methods for inducing convulsions because of considerable past experience showing that congruent findings are not always obtained between the two methods. In terms of predictability, it is important to obtain coherent findings between different means of inducing convulsions before concluding as to the presence or absence of risk.

PROCEDURE

ECS Threshold Test

Rats are administered ECS (rectangular current, 1.5 s, 200 Hz) via earclip electrodes connected to a constant current shock generator (Ugo Basile: Type 7801).

Treatment groups of 15 rats are exposed to ECS as follows:

Animal nr. 1 is exposed to 30 mA of ECS. If animal nr. 1 does not convulse (tonic convulsions) within 5 seconds maximum, animal nr. 2 is exposed to 40 mA, with increases of 10 mA until the first tonic convulsion is observed. Once the first tonic convulsion is observed, the intensity of ECS is decreased by 5 mA for the next animal and then the intensity is decreased or increased by 5 mA from animal to animal depending on whether the animal convulses or not. The minimum intensity given is 5 mA and the maximum intensity given is 95 mA.

The results are represented as the mean intensity administered and as percent change from control. The number of deaths is also recorded approximately 30 minutes after the animal has been tested for convulsions.

The test is performed blind.

A positive percent change indicates an anticonvulsant effect. A negative percent change indicates a proconvulsant effect.

The test substance is usually evaluated at 4 doses, administered p.o. 60 minutes before ECS, and compared with a vehicle control group.

Ro 15-4513 (16 mg/kg p.o.) and diazepam (16 mg/kg p.o.), administered under the same experimental conditions, are used as reference substances.

The basic experiment therefore includes 7 groups.

PTZ Seizure Test

Rats, placed in individual macrolon cages (25 × 19 × 13 cm), are injected with PTZ (100 mg/kg s.c.). The occurrence and latency of clonic convulsions, tonic convulsions and deaths are noted over a 60 minute period.

The results for the number of convulsions and deaths are also represented as percent change from control.

Ten rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 4 doses, administered p.o. 60 minutes before PTZ, and compared with a vehicle control group.

Ro 15-4513 (16 mg/kg p.o.) and diazepam (16 mg/kg p.o.), administered under the same experimental conditions, are used as reference substances.

The basic experiment therefore includes 7 groups.

EVALUATION

ECS, if sufficiently intense, induces immediate full clonic-tonic convulsions but only occasionally death. In contrast, with PTZ, clonic convulsions can be observed without necessarily being followed by tonic convulsions. On the other hand, when tonic convulsions occur, they are almost invariably followed by death.

With the ECS threshold procedure, the primary measure is the mean electroshock intensity administered per group of 15 animals. If less current is required to induce a clonic-tonic convulsion after a particular treatment, this is reflected by a decrease in the mean intensity administered (proconvulsant effect). Conversely, if more current is required to induce a clonic-tonic convulsion, this is reflected by an increase in the mean intensity (anticonvulsant effect). In other words, the mean intensity administered is a direct representation of the electroconvulsive threshold.

With the PTZ procedure, pro- or anti-convulsant activity is indicated both by the frequency of the convulsant events (clonic convulsions, tonic convulsions, deaths) and by their latency of occurrence. Latency is the measure which most clearly identifies pro-convulsant activity, whereas frequency is the measure most useful for detecting anticonvulsant activity. A statistical problem arises when the test substance completely blocks convulsions in some of the animals. In such cases, we recommend that parametric statistics be applied to the latency data as long as a minimum of 4 animals show convulsions, to avoid score bias by inclusion of floor and ceiling effects.

In both procedures, quantal data (number of occurrences) are analyzed using Fisher's Exact Probability Test and quantitative data (mean shock intensity, latencies) are analyzed using Student's t tests.

CRITICAL ASSESSMENT OF THE METHOD

It is generally accepted that there is a good correlation between potentiation or antagonism of experimentally induced convulsions in animals and effects observed in man (Kupferberg 2001). Most substances with antiepileptic properties in man antagonize experimental convulsions, whereas many substances which either induce convulsions or lower the convulsive threshold in man (certain CNS stimulants, some neuroleptics) show similar effects in animals. There are nonetheless differences in the efficacy of certain substances in antagonizing experimental convulsions. For example, sodium valproate appears to be more widely effective in different experimental models than benzodiazepines (Löscher 2002).

The existence of such differences is an argument for employing at least two different methods in a core battery for evaluating drug effects on the convulsive threshold. If signs of risk are apparent, further models can be employed either to establish the mechanism or the anatomical locus in the brain. To establish mechanisms, use can be made of different convulsive agents (strychnine, bicuculline, picrotoxin, NMDA). As far as anatomical locus is concerned, the kindling model can be used with recordings taken from different brain regions (amygdala, temporal lobe, hippocampus, prefrontal cortex) to establish the development and regional distribution of the convulsive phenomena (Ebert et al. 1977). The simple EEG trace recording from the cortex is also useful for identifying the existence of convulsive brain phenomena at doses inducing no overt behavioral effects.

MODIFICATIONS OF THE METHOD

The ECS method described above involves titration of the shock level between successive test animals to determine the mean intensity required to induce convulsions after different treatments. This procedure represents a variant of the more habitual procedure where a set level of ECS is selected to induce convulsions in 100 % of the animals (maximal electroshock). Another variant would be to select a low electroshock

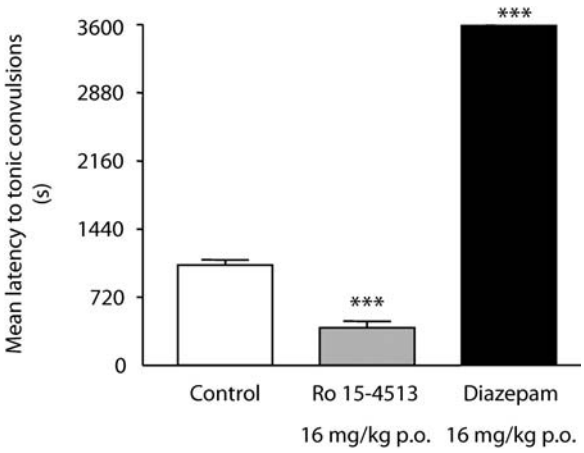

Fig. 3. Convulsive threshold in the Rat: PTZ Test. Effects of Ro 15-4513 and diazepam (p.o.) on the latency to tonic convulsions induced by PTZ in the rat.

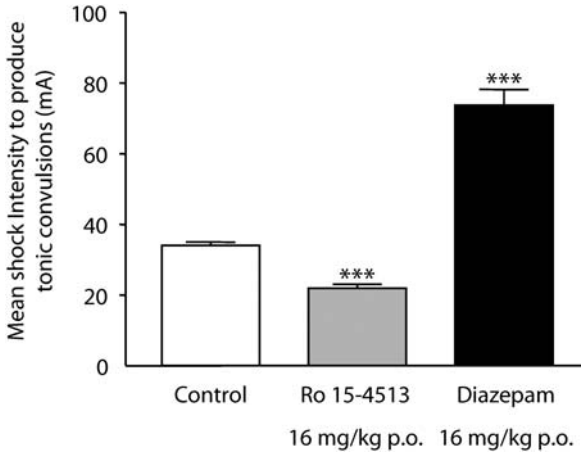

Fig. 4. Convulsive threshold in the Rat: ECS Test. Effects of Ro 15-4513 and diazepam (p.o.) on the mean shock intensity required to induce tonic convulsions in the rat.

level (subconvulsant) to detect proconvulsant activity. The present method possesses the advantage of permitting within a single test the evaluation of pro- and anti-convulsant activity. A further advantage of the present method is that it generates quantitative data (mean intensity) as opposed to quantal data (frequencies), permitting more powerful statistical analyses.

With the PTZ procedure described above, using 120 mg/kg PTZ, convulsions are observed in 100 % of the animals. Variants exist whereby lower doses of PTZ, for example 60 mg/kg, are administered with the aim of rendering the test more sensitive to proconvulsant effects. The problem with this approach is that convulsions are not observed in 100 % of the animals, thereby weakening the possibilities for a sensitive statistical analysis. When 100 % of the control animals convulse after PTZ, the latency measures become the indices of choice for demonstrating pro- or anti-convulsant activity.

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I.C.2.5 Barbital Interaction Test

PURPOSE AND RATIONALE

Procedures evaluating the interaction between the test substance and hypnotics were included in the Japanese Guidelines Category A, but were dropped from the ICH S7A core battery. We suggest that simple tests of this kind can usefully be included in a CNS core battery. The additional expense is minimal, and such procedures provide useful information not provided by the other tests. For example, although several substances with psychostimulant activity (caffeine, modafinil) induce frank signs of excitation at higher doses, this kind of activity can be detected at lower doses when tested by interaction with a barbiturate. Similarly, sleep-enhancing effects can be readily detected in barbiturate interaction procedures by substances, for example benzodiazepines and neuroleptics, which do not induce sleep even at high doses when administered alone. Thus interaction tests with barbiturates can serve to unmask stimulant or sedative activity not otherwise readily apparent. Furthermore, there is a high correlation between the effects observed in such procedures and those observed in more complex tests and in man.

A further consideration is which barbiturate to employ. We recommend the use of barbital because this substance, in contrast to many other barbiturates, undergoes no metabolism (Remmer 1972; Simon et al. 1992). For this reason, results obtained can be more readily interpreted in terms of a pharmacological interaction without confounding by pharmacokinetic or metabolic factors.

PROCEDURE

Rats, placed in individual macrolon cages (25 × 19 × 13 cm), are injected with barbital sodium (150 mg/kg i.p.). The latency to sleep and the duration of sleep (maximum: 6 hours after barbital injection) are then recorded. Sleep is indicated by loss of the righting reflex. To avoid disturbing the experiment, no righting reflex test is performed before 50 minutes has elapsed after barbital injection (i.e. minimum sleep latency = 50 minutes).

Ten rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 4 doses, administered p.o. 60 minutes before barbital, and compared with a vehicle control group.

Caffeine (16 mg/kg p.o.) and diazepam (8 mg/kg p.o.), administered under the same experimental conditions, are used as reference substances.

The basic experiment therefore includes 7 groups.

EVALUATION

Barbital at 150 mg/kg i.p. induces sleep in 100 % of the animals. The two principal parameters measured are the latency to sleep and the duration of sleep which are analyzed for statistical significance using Student's t tests. In addition, the number of animals sleeping is counted and differences from control are then analyzed using Fisher's Exact Probability Test.

A statistical problem arises when the test substance completely blocks sleep in some of the animals. In such cases, we recommend that parametric statistics be applied to the latency and duration data as long as a minimum of 4 animals show sleep, to avoid score bias by inclusion of floor and ceiling effects.

CRITICAL ASSESSMENT OF THE METHOD

For the reasons given in the Purpose/Rationale section above, barbiturate interaction tests yield useful information not readily provided by the other procedures in the core battery. Furthermore, the data obtained are highly correlated with results that could be expected in man. To our knowledge, no sleep enhancing agent used in man has been found inactive in a barbiturate interaction procedure. Conversely, no clinically known enhancer of wakefulness, for example caffeine, amphetamine and modafinil, has failed to reduce or abolish barbiturate-induced sleep. Potential to cause drowsiness, as is observed with many antihistamines, is also clearly picked up in barbiturate interaction procedures as are the potential sedative effects of a variety of neuroleptics. The procedure therefore possesses high predictive validity.

On the other hand, this procedure does not provide information about drug effects on the components of sleep, for example paradoxical versus slow-wave sleep, or on the cyclical changes which can occur during natural sleep over longer periods (24 hours).

Although procedurally simple, barbiturate interaction procedures are extremely subject to small environmental changes (lighting, ambient noise and temperature, time of day) and therefore have to be performed under highly controlled conditions to yield reproducible results.

MODIFICATIONS OF THE METHOD

Being one of the long-time standards in basic psychopharmacology, there are few major variants to the procedure apart from the kind of barbiturate employed. Several barbiturates undergo clear hepatic metabolism, for example pentobarbital and phenobarbital, thereby confounding interpretations because of pharmacokinetic and metabolic factors. Indeed, one modification of the method has been specifically employed to estimate enzyme induction in the liver as indicated by more rapid barbiturate metabolism. Animals given a pre-exposure to a test substance are then exposed to a standard dose of phenobarbital (80 mg/kg i.p.) 24 hours later and assessed for sleep duration. The presence or absence of a decrease in sleep duration is taken as an index of the hepatic enzyme induction produced by the test substance (Kushikata et al. 2003).

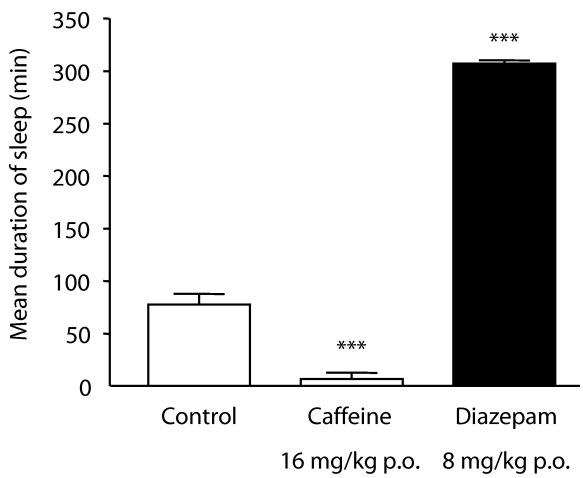

Fig. 5. Sleep/wakefulness in the Rat: Barbital Interaction Test. Effects of caffeine and diazepam (p.o.) on the duration of sleep induced by barbital in the rat.

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I.C.2.6 Hot Plate Test

PURPOSE AND RATIONALE

The Hot Plate Test (Eddy & Leimbach 1953) is a simple behavioral screen for estimating the effects of test substances on the threshold for pain sensitivity. It is based on the principle that rodents, placed onto a hot surface, will demonstrate the aversive effects of the stimulation first by licking their paws and subsequently by clear attempts to escape the situation (jumping). Substances changing the nociceptive threshold will either increase the latency to licking/jumping (analgesic effect) or decrease it (hyperalgesic effect).

PROCEDURE

Rats are placed onto a hot metal plate maintained at 52 °C surrounded by a Plexiglas cylinder (Height: 26 cm; Diameter: 19 cm). The hot plate apparatus we use is provided by Apelex, 92220 Bagneux, France (Model DS37). The latency to the first foot-lick is measured (maximum: 30 seconds).

Ten rats are studied per group. The test is performed blind.

The test substance is generally evaluated at 4 doses, administered p.o. 60 minutes before the test, and compared with a vehicle control group.

Morphine (128 mg/kg p.o.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes 6 groups.

EVALUATION

The principal parameter assessed in the Hot Plate Test is the latency to the first paw-lick response.

Data are analyzed for statistical significance using Student's t tests.

CRITICAL ASSESSMENT OF THE METHOD

The Hot Plate Test is one of the most frequently employed screens for evaluating the nociceptive threshold. It is reliable and reproducible, and remains robust in the face of moderate environmental variations. Most known analgesic substances show analgesia in the method which can therefore be considered as possessing a reasonable predictive validity. On the other hand moderate analgesics, in particular those with a primarily anti-inflammatory profile (aspirin, paracetamol, ibuprofen), show less marked effects than major analgesics such as the opioids.

Of the two parameters measured (foot-licking, jumping), foot-licking appears more sensitive to the analgesic properties of test substances, whereas decreases in jumping often can often reflect their locomotor effects. Other screening tests, for example chemically-induced writhing (phenylbenzoquinone, acetic acid), although revealing analgesic activity at lower doses, are sensitive to a wider range of substances and therefore yield more false positives.

The search for hyperalgesic effects is more delicate. Standard drugs which cause hyperalgesia are rare and more difficult to demonstrate, frequently requiring particular conditions of administration required, for example topical administration of capsaicin (Yoshimura et al. 2000).

The hot plate procedure possesses an advantage over other methods of thermal stimulation, for example the tail flick procedure (d'Amour and Smith 1941), in that it can be applied repeatedly in the same animals over a short period of time (2–3 hours) without causing tissue injury, particularly if the maximum observation duration is 30 seconds. This could be particularly useful for estimating time-course effects. The hot plate procedure also constitutes a more global estimate of nociceptive reactivity because it represents a complex willed behavior rather than a simple reflex like the tail flick.

MODIFICATIONS OF THE METHOD

Different hot plate temperatures have been used, varying from 48 °C to 58 °C. Lower temperatures are in principle more useful for detecting hyperalgesic activity. Although consistent with ethical requirements for decreasing pain, lower temperatures produce more variable responding and therefore require more animals to obtain statistically reliable data. A better way of satisfying the ethical requirement is to reduce the total possible duration of the test session. We have found that 30 seconds is largely sufficient to obtain measurable changes in the foot-licking latency in both directions.

Higher temperatures, although producing less variable data, reduce the range of analgesics which can be detected. The analgesic activity of most opioids can al-

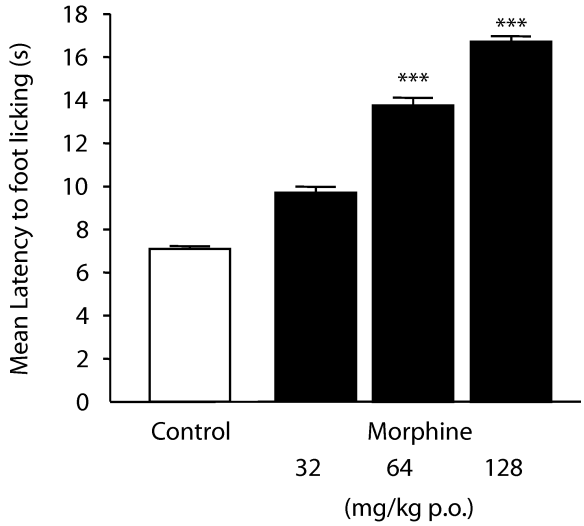

Fig. 6. Pain sensitivity in the Rat: Hot Plate Test. Effects of morphine po. in the Hot Plate Test in the rat.

most always be detected, but not the milder analgesic activity of agents such as salicylates.

In addition to foot-licking latency, another parameter frequently measured is the latency to jumping attempts to escape from the apparatus. Jumping is less selective as analgesic parameter because it can be influenced by substances without known analgesic activity, but which cause either locomotor stimulation or sedation, for example neuroleptics. In our own procedure, with a 30 second cut-off, jumping is not reliably observed during the observation period under control conditions, and is therefore not a useful parameter.

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I.C.3 Supplementary CNS Studies

Supplementary CNS safety studies are more complex procedures, investigating test substance effects on cognitive processes, electrophysiological brain activity or their potential to cause drug-dependence/abuse. The following Section is therefore divided into three sub-sections dealing with these different areas. As for the core battery studies described above, an essential aspect of CNS supplementary studies is that they are all performed in vivo, in the intact freely moving animal.

I.C.3.1 Cognitive Processes

Tests of higher cognitive function find their place during the later phases of CNS safety assessment because they are more time-consuming to perform and cannot therefore be performed on a routine screening basis. Included under the term cognitive function are learning, memory and attention. It is important that drugs be devoid of impairing effects on these functions, whatever their indication. It is thus essential that CNS safety pharmacology provide procedures for evaluating these effects in animal studies. There is no standardized set of procedures in this area. The following section provides an indication of the kinds of protocols that can be applied. The list is by no means exhaustive.

I.C.3.1.1 Passive Avoidance Test

PURPOSE AND RATIONALE

One of the simplest procedures for looking for adverse effects on learning/memory is the so-called one-trial passive avoidance task (Bammer 1982). A mouse or a rat receives an aversive stimulation in a recognizable environment and on a later occasion shows it has remembered by not going there (passive avoidance). Amnesia-inducing drugs (benzodiazepines, anticholinergics, NMDA antagonists), administered before the first exposure, attenuate the animal's memory for the aversive stimulus as shown by a decreased avoidance of the environment in which it was previously received.

PROCEDURE

The passive avoidance apparatus we use consists of two compartments, one (30 × 30 × 30 cm) brightly lit and the other (20 × 20 × 12.5) dark, connected by a small opening (8 × 8 cm) which can be closed by a guillotine door.

For the first trial (T1), rats are placed individually into the lighted compartment. After 30 seconds, the door to the dark compartment is opened. When the rat has entered the dark compartment, the door is closed and the rat immediately receives a 0.8 mA shock (Coulbourn Shock Generator) for 2 seconds. The animal is removed immediately after the shock and is replaced in its home cage.

48 hours later the animal is placed again in the lighted compartment with the door closed for the second trial (T2). The door is opened after 30 seconds and

the animal's latency to cross to the dark compartment is recorded (cut-off time = 180 seconds).

Amnesia-inducing drugs cause a significant decrease in the step-through latency at T2.

15 rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 3 doses, administered p.o. 60 minutes before T1, and compared with a vehicle control group.

Diazepam (8 mg/kg p.o.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes 5 groups.

EVALUATION

The principal measure taken is the animal's latency to cross to the dark compartment at T2. This score provides an estimate of the animal's retention of the shock received at T1. The latencies measured at T2 have a 180 second cut-off. The scores in the control group are therefore abnormally distributed because of the presence of numerous ceiling scores. It is therefore essential to apply non-parametric statistics, for example the Mann-Whitney U-test, to analyze the data.

A second measure taken is the animal's latency to cross to the dark compartment during T1. This measure is used to estimate the intrinsic effects of the test substance on exploration/locomotion. These scores are more normally distributed than at T2 but, for reasons of homogeneity, should also be evaluated using the Mann-Whitney U-test.

A clear drug effect on T2 latencies in the absence of an effect on T1 latencies can be more clearly interpreted as an effect on memory.

CRITICAL ASSESSMENT OF THE METHOD

Passive avoidance procedures provide the most rapid and apparently simple index of a drug's impairing effects on memory. They are therefore suitable as a first screen for potential cognition impairing activity. The fact that the learning occurs within a single trial distinguishes passive avoidance from the other procedures described below. On the other hand, passive avoidance procedures suffer problems of interpretation in terms of the memory/learning processes involved. Is a decrease in step-through latency at T2 due to the fact that the animal did not learn the association between the dark compartment and the shock, or because it could not retain this learning over the inter-test interval? A decrease in T2 step-through latency could also reflect an impairment of attention at T1 or even a decrease in pain sensitivity induced by the test substance during the learning trial.

A further problem with passive avoidance procedures is that, despite their apparent simplicity, the results obtained are notoriously variable on separate occasions and between different laboratories (Bammer 1982). Although part of the explanation for the interlaboratory variability lies with the multiplicity of passive avoidance procedures (see below), even using the same procedure from day to day provides varying results. To counteract the variability it is therefore necessary to include more animals per group.

Passive avoidance procedures are essentially procedures for detecting impairing effects of test substances. It is more difficult to demonstrate cognition enhancement because of ceiling effects. In terms of safety pharmacology, this is not a major drawback as impairment represents the major cognitive risk.

MODIFICATIONS OF THE METHOD

The passive avoidance procedure exists in multiple versions, but basically consists of two paradigms, step-down and step-through. The procedure described above is a step-through procedure (Glick & Zimmerberg 1972), involving two compartments, light and dark, whereas the step-down procedure (Kameyama et al. 1986) involves placing the animal on a platform just above an electrifiable grid floor. Both oppose the animal's natural tendencies (aversion to bright light or remaining in a restricted space) with avoidance of an electric shock.

With the step-through procedure, the dimensions of the apparatus can vary, in particular the relative sizes of the two compartments. In general, it is the light compartment which is larger. The size of the light compartment can radically change the latencies with which the animal crosses to the dark compartment, and thereby influence the amount the animal learns about this new environment before receiving electric shock. The manner of delivering shock can also vary. With some procedures the animal receives shock for a brief but fixed period. With other procedures, the animal receives shock until it has returned to the light compartment. The same is also true for the step-down procedure. Indeed, the existence of so many variants no doubt explains much of the variability of data between different laboratories (Sarter et al. 1992). As yet, no single "best" method can be recommended.

Most passive avoidance procedures used in CNS safety pharmacology employ shock levels of sufficient intensity to induce a high level of avoidance, thereby providing a behavioral baseline suitable for assessing

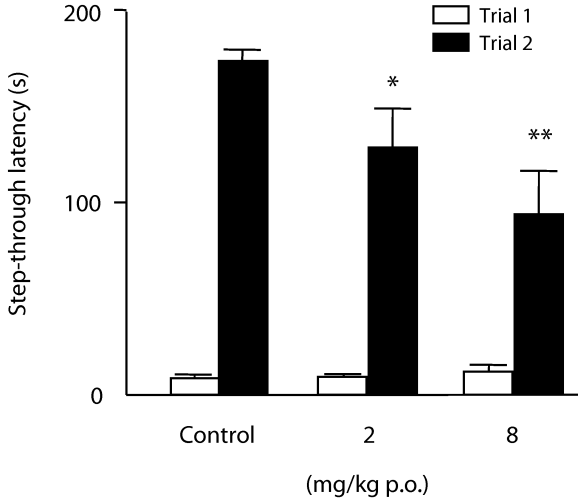

Fig. 7. Effects of diazepam on passive avoidance behavior in the rat. Note the absence of effects of diazepam on the step-through latency at Trial 1 and the presence of a dose-dependent decrease in step-through latency at Trial 2 in the absence of diazepam.

cognitive impairment. Other variants use lower levels of shock to induce less than maximal avoidance during a learning trial either to permit demonstrations of improved performance or the use of multiple trials for establishing learning curves. Multiple trial procedures permit the progress of learning to be monitored and the effects of test substances on the learning process to be assessed, thereby providing more readily interpretable data. On the other hand, multiple trials are time-consuming, decreasing the major advantage of one-trial procedures. Using lower shock levels in a one-trial procedure has the disadvantage of decreasing the interpretability because there is no independent means of establishing whether learning was present or whether the shock level was sufficient to sustain a passive avoidance response.

Another variant is the manner of administering the test substance. One of the interpretational problems with passive avoidance is the occurrence of other drug effects, for example on pain sensitivity or attention, which can confound the interpretation in terms of memory. Some of these effects can be controlled by administering the test substance immediately after T1, with the intention of acting on so-called memory "consolidation" (Slotnick & Jarvik 1966). Post-trial administration has other problems however. Administration of the test substance may have positive reinforcing or aversive effects which can influence the scores at T2. Another problem is their onset of action. A test substance with slow onset could miss the "consolidation" period altogether and thereby appear to be devoid of amnesic potential when this is not the case. The best general screening procedure therefore remains that where the test substance is administered before the learning trial, despite some difficulties in data interpretation.

Passive avoidance procedures generally use inter-trial intervals of 24–48 hours, and thereby measure longer term memory. It is possible to shorten the inter-trial interval to, for example, 1 hour to assess drug effects on shorter term memory (Bartus & Dean 1985). On the other hand, passive avoidance procedures are not frequently used for comparisons of short- and long-term memory, probably because other procedures (see below) offer better possibilities of interpretation.

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I.C.3.1.2 Morris Maze Test

PURPOSE AND RATIONALE

Another fairly simple procedure, which allows a greater degree of interpretation, is the Morris water maze (Morris 1981). In this test a mouse or a rat is placed into a circular tank containing water, and has to find an escape platform in a fixed place just beneath the surface. The escape platform is not visible to the animal because the water has been rendered opaque. After swimming around for a certain time, the animal will eventually come across the hidden platform and climb onto it to escape from the water. When placed again in the water on subsequent occasions, the animal will generally find the platform with increasing rapidity, indicating that it has learned the position of the platform. Although the maze is featureless, it is

important that sufficient distinctive cues are present in the experimental room to allow the animal to orient itself.

PROCEDURE

The rat Morris maze we use consists of a circular water tank (150 cm in diameter) filled with water and maintained at 27 °C with an escape platform (15 cm in diameter) 18 cm from the perimeter always in the same position 2 cm beneath the surface of the water. The water is made opaque by addition of milk powder rendering the platform invisible.

Rats are given a single training session on one day. A training session consists of 4 consecutive trials in the Morris maze separated by 60 seconds. For each trial the animal is placed in the maze at one of two starting points equidistant from the escape platform and allowed to find the escape platform. The animal is left on the escape platform for 60 seconds before starting a new trial. If the animal does not find the platform within 120 seconds the experimenter removes it from the water and places it on the platform for 60 seconds before beginning the next trial. During the 4 trials the animals start the maze twice from each starting point in a randomly determined order per animal.

Twelve rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 3 doses, administered p.o. 60 minutes before the session, and compared with a vehicle control group.

Diazepam (8 mg/kg p.o.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes 5 groups.

EVALUATION

The principal measure taken is the escape latency at each trial. Decreases in the escape latency from trial to trial indicate learning. A drug-induced flattening of the learning curve therefore indicates impairment of learning. These effects can be analyzed statistically by comparing performance between the treated groups and vehicle control at each learning trial using Student's t tests. ANOVA with repeated measures (trials) provides a more sophisticated and sensitive assessment by including within the same analysis all the scores obtained per animal.

Another measure of interest is the animal's escape latency at the first trial. The absence of any treatment effect at the first trial, the animal's first experience of the test situation, can suggest that the test substance is devoid of intrinsic effects on swimming behavior which could confound interpretation of the subsequent data.

One other measure frequently taken, particularly when the animal's behavior is scored using computerized analysis of the video records, is the animal's swimming path during the trials. By this means it is possible to quantify drug-induced changes in swimming patterns, for example wall clinging (thigmotaxis), and to calculate the animal's swimming speed, thereby providing another assessment of the intrinsic effects of the test substance on swimming performance.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to the one-trial passive avoidance procedure, the Morris maze permits the progress of learning to be evaluated within the test. Furthermore, subsequent learning can be compared with initial swimming performance. Both factors allow a clearer interpretation of drug effects. The Morris maze depends on the animal's use of extra-maze visual cues. The behavior can therefore be more readily interpreted in terms of the animal's capacity to learn to orient itself in space (spatial learning) and the effects thereon of the test substance.

Forcing the animal to swim is a powerful motivator and thereby induces a stable behavioral baseline, once the animal has experienced the escape platform. On the other hand, the situation represents a major stress, particularly if the animal has difficulty finding the escape platform on the first trial. When this happens, drug effects on emotional factors, for example "behavioral despair" (Porsolt et al. 1979) could confound interpretations in terms of learning/memory. The random nature of the first discovery of the platform is therefore a factor which increases variability, whereas subsequent learning varies little from one occasion to the next and is more clearly interpretable.

The capacity to conduct multiple variants of the procedure (see further details below) endows the Morris maze with a wide range of possibilities for interpreting drug-induced change in cognitive function. This together, with its functional simplicity, no doubt explains the popularity of the procedure.

MODIFICATIONS OF THE METHOD

The two major variants of the procedure are the grouping of the trials and the inclusion or not of probe trials.

In our own basic procedure, 4 trials are given on a single day with a 60 second timeout between trials. It is possible to repeat the same procedure over several consecutive days in the same test animals. Although

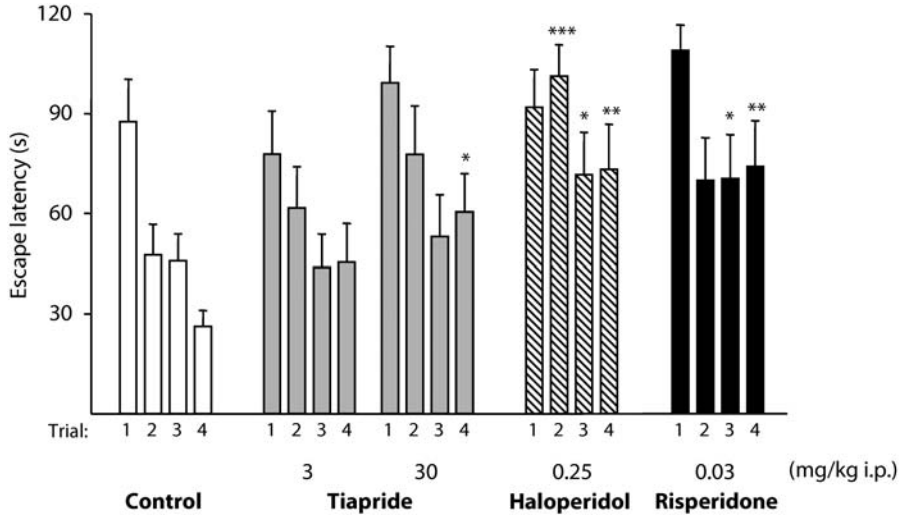

Fig. 8. Effects of 3 neuroleptics (tiapride, haloperidol and risperidone) on escape latencies during the first session of the Morris maze task in the rat. Note the clear decrease in escape latencies in the vehicle control group (short-term memory) and the absence of effect of the 3 substances on performance at the first trial (absence of intrinsic effects on swimming performance). Tiapride has modest but dose-dependent effects on the decrease in escape latency from trial to trial, whereas haloperidol and risperidone clearly attenuate this decrease (perturbing effect on short-term memory).

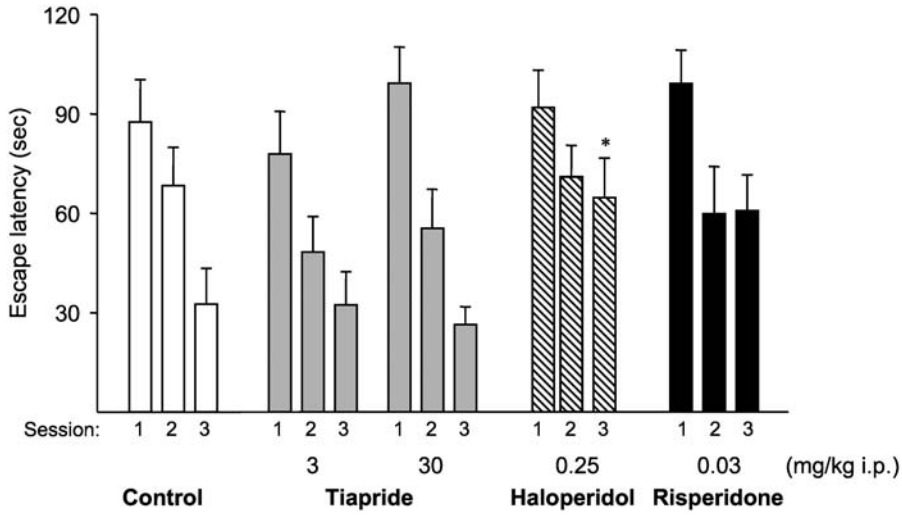

Fig. 9. Effects of 3 neuroleptics (tiapride, haloperidol and risperidone) on escape latencies during the first trial of the same Morris maze task on 3 consecutive days. Note the clear decrease in escape latencies in the vehicle control group from day to day (long-term memory). Tiapride has no effect on the decrease in the first trial escape latency from day to day (absence of effect on long-term memory), whereas haloperidol clearly attenuates this decrease with a similar tendency for risperidone (perturbing effect on long-term memory).

more time-consuming, repeated sessions on different days enable drug effects on short-term memory and long-term memory to be distinguished. Short-term memory is best assessed by examining performance on repeated trials on the same day. The decreases in escape latency from trial to trial are a direct reflection of the animal's retention from the immediately preceding trial. In contrast, long-term memory can best be assessed by examining day-to-day performance. Indeed the purest measure of long-term memory is the change in escape latency on the first trial of each day. This measure reflects the animal's retention from the previous day without confounding from the new learning occurring on subsequent trials on the same day. Another variant of the procedure is simply to give a single trial on each day. In this way the influence of within-day learning is totally excluded.

We recommend giving multiple trial sessions on consecutive days and analyzing them as described above. Examples are given below with

3 known antipsychotics (tiapride, haloperidol and risperidone) where the effects of the 3 substances on short- and long-term memory can be clearly differentiated.

Another modification is to include probe trials, where no escape platform is present (Czech et al. 2000). The retention measure is the time spent in the maze quadrant associated with the escape platform. An animal which remembers the location of the platform will spend more time in that quadrant of the maze. An essential requirement is that the probe trial remains short, say 1 minute, to avoid the animal learning that the platform is no longer present. If new learning can be thus avoided, probe trials provide the most sensitive index of the animal's retention of the position of the platform, unconfounded by random elements (the animal finding the platform by chance).

Further modifications are the size of the swimming tank and the position of the escape platform. Both parameters differ widely between different laboratories. In general, larger tanks increase the difficulty for the animal to find the escape platform with a consequently flatter learning curve. Indeed age- or drug-related changes in Morris maze performance depend critically on such factors (van der Staay 2000).

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I.C.3.1.3
Radial Maze Test

PURPOSE AND RATIONALE

Another task in which short-term memory can be differentiated from long-term memory is the radial maze (Olton 1986). This apparatus consists of a central platform with arms radiating from it like the spokes of a wheel. A hungry rat or a mouse is placed in the centre of the maze and can find food at the end of each arm. An ideal performance during a trial would be for the animal to visit each arm once to collect food. This task requires therefore that, during a trial, the animal remembers the arms already visited (short-term or "working" memory).

Long-term or "reference" memory can also be assessed by having a restricted number of arms baited, but always the same. The animal must visit only the baited arms during a test session.

The protocol described below assesses only "working memory" in that all the arms are baited.

PROCEDURE

The radial maze we use consists of a central platform (30 cm in diameter) with 8 arms (68 × 10 cm) surrounded by walls (Height = 10 cm). A food receptacle is located at the end of each arm. The receptacle is in the form of a small hole in the floor rendering the food invisible from the entrance to the arm. The apparatus is constructed of black Plexiglas and is elevated 80 cm above the floor.

Training

During the week preceding the beginning of the experiment the animals are placed on a restricted food regime (15 g standard diet per day) with free access to water.

In the following week, the animals are submitted to 4 maze habituation sessions. During the first session the animals are placed in the maze in groups of 5 and are left to explore freely for 10 minutes. The maze is baited with several food pellets (45 mg) dispersed around the maze. For the remaining 3 sessions, the animals are placed singly in the centre of the maze which is baited with a single pellet at the end of each arm. They are withdrawn from the maze after having consumed the food in all 8 arms or after 5 minutes have elapsed. In addition to the food pellets consumed in the maze, the animals receive 15 g food in their home cages after the last animal in the afternoon has been tested.

The number of errors (reentering the arms already visited) and the time taken to complete the maze are scored. The scores obtained on the 4th day are used to allocate the animals to matched groups which are then randomly assigned to a treatment condition.

Drug Testing

Subsequent to maze habituation, all animals are submitted to 3 test sessions on 3 consecutive days. During a test session each animal is placed individually in the centre of the maze and allowed to make 8 choices after which it is removed from the apparatus. If the animal does not complete 8 choices within 5 minutes, it is removed and its scores discounted for that day. If the animal is in the course of making its 8th choice

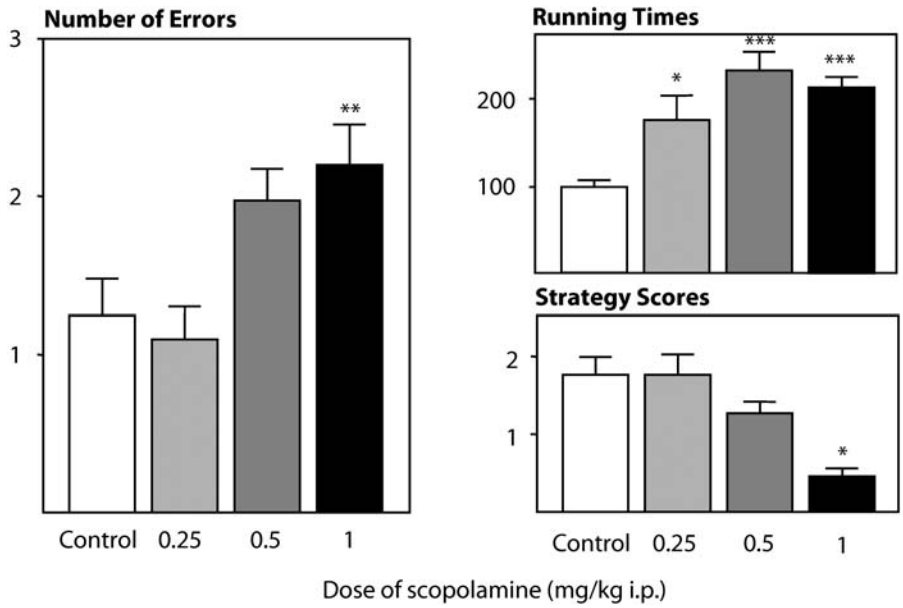

Fig. 10. The effects of scopolamine on the 3 parameters measured (errors, running times, response strategy) during the radial maze task in the rat (mean of 3 sessions). Note the dose-dependent increase in the number of errors and running times, with a statistically significant decrease in response strategy occurring at the highest dose.

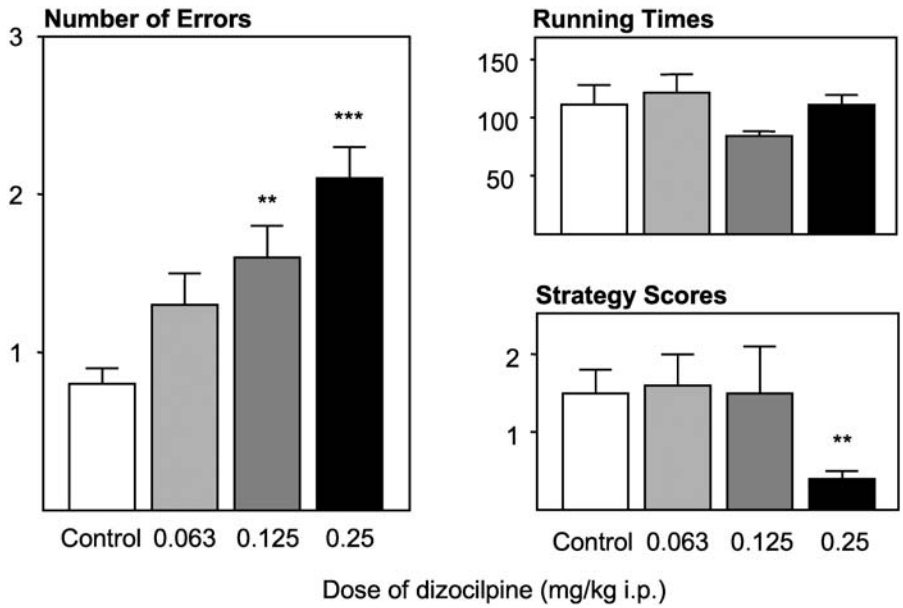

Fig. 11. The effects of dizocilpine (MK-801) on the 3 parameters measured (errors, running times, response strategy) during the radial maze task in the rat (mean of 3 sessions). Note the dose-dependent increase in the number of errors without any effect on running times. A decrease in response strategy occurs only at the highest dose tested.

when the 5 minutes comes to an end, it is left in the experiment if it takes less than 1 minute to make its last choice.

The animals are maintained on their restricted food regime throughout the experiment.

Twelve rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 3 doses, administered i.p. 30 minutes before each test session, and compared with a vehicle control group.

MK-801 (0.125 mg/kg i.p.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes 5 groups.

EVALUATION

Three parameters are scored during each test session:

Errors: whenever the animal enters an arm it has already visited during the same session.

Running time: the time taken to complete a session.

Response choice strategy: a numerical score based on the number of times the animal, on leaving an arm, visits the next or next-but-one arm in the same direction.

A global score is calculated per parameter consisting of the mean score over the 3 sessions.

These effects can be analyzed statistically by comparing performance between the treated groups and vehicle control at each test session, or pooled over the 3 sessions, using Student's t tests. ANOVA with repeated measures (sessions) provides a more sensitive assessment by including within the same analysis all the scores obtained per animal.

CRITICAL ASSESSMENT OF THE METHOD

The radial maze requires considerably more training, and is therefore more time-consuming than either passive avoidance or the Morris maze. On the other hand, with the three parameters, it offers data which are more clearly interpretable in terms of "working" memory, motor performance and response choice strategy (Cassel & Kelche 1989). Memory and strategy are very different aspects of cognition. Indeed, if an animal has been given sufficient training in the radial maze, it develops a clear strategy and has no need for memory to attain error-free performance.

The running time score provides a measure of the intrinsic effects of the test substance on motor performance, and thereby permits an estimate of the selectivity of a drug effect on "working memory". A test substance which markedly prolongs running time may also cause an increase in errors simply because the animal has to retain the required information (which arms were visited) over a longer period time.

Another aspect of radial maze performance is the use of positive motivation (food reward), in contrast to the aversive motivations maintaining the passive avoidance and Morris maze procedures. Data suggesting drug-induced cognitive impairment are more convincing if the impairments span the different motivational systems maintaining the behaviors.

MODIFICATIONS OF THE METHOD

The principal variant to the radial maze procedure described above is the inclusion of a "reference" memory component, by baiting only some of the arms (Beatty & Bierley 1985). In addition to remembering which arms were visited during a particular session ("working" memory), the animal has to remember which arms contain food reward ("reference" memory). With an 8-arm radial maze, exclusion of certain arms, diminishes the possible scores which can be obtained with the baited arms, and thereby reduces the sensitivity of the test. One way of counteracting this is the use of a larger maze, for example 12 arms (Magni et al. 1979), but such mazes require considerably more habituation training.

Another approach to "reference" memory is to use the radial maze task as a repeated acquisition task with different arms being baited during each test session (Levin et al. 1998). In this way the task can be modified to evaluate exclusively "reference" memory. As with repeated passive avoidance sessions, this use of the radial maze is very time-consuming, and we do not recommend it for safety pharmacology purposes.

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I.C.3.1.4 Social Recognition Test

PURPOSE AND RATIONALE

Social recognition refers to the fact that an adult rat, when exposed to the same juvenile rat on two occasions, demonstrates that it has recognized the juvenile by a decrease in the amount of investigatory behavior on the second occasion. Absence of a decrease in investigatory behavior at the second occasion suggests that the adult rat has forgotten the juvenile.

This simple model of memory differs from those described above in that it is based on a natural behavioral tendency rather than the situations imposed on the animal by exposure to aversive stimulation or food deprivation.

PROCEDURE

An unfamiliar juvenile rat (40–50 g, 3 weeks old) is introduced into the individual home cage

(41 × 25 × 15 cm) of a mature adult rat (400–450 g, 3–4 months old) for 5 minutes. Following this first encounter (E1), the juvenile is returned to its isolation cage until a second encounter (E2) of 5 minutes with the same adult rat 30 minutes later.

Under such conditions, a mature adult rat recognizes the juvenile as familiar, as indicated by a marked reduction in the duration of social investigatory behavior at E2.

12 rats are studied per group. The test is performed blind.

The test substance is usually evaluated at 3 doses, administered p.o. 60 minutes before E1, and compared with a vehicle control group.

Scopolamine (0.5 mg/kg i.p.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes 5 groups.

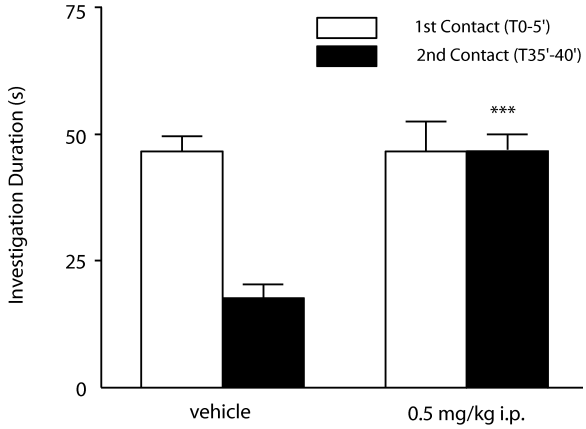

Fig. 12. Effects of scopolamine on social recognition memory in the rat. Note the clear decrease in social investigation in the vehicle control group between the first and the second exposure 30 minutes later (social memory) and the absence of effect of scopolamine on social investigation during the first exposure (absence of intrinsic effects of scopolamine on social investigation). In the scopolamine-treated animals there is no decrease in social investigation at the second exposure, demonstrating impairment of social memory.

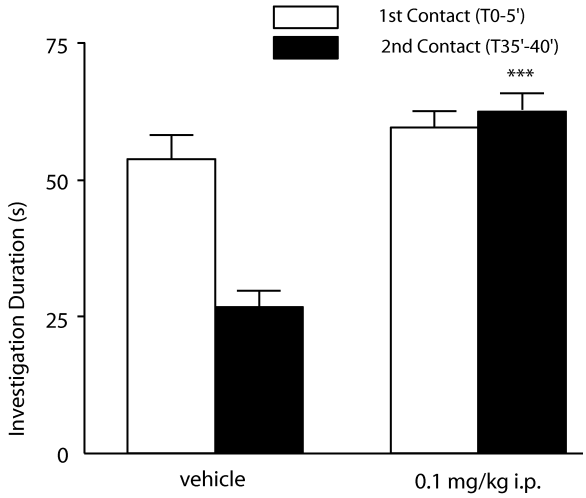

Fig. 13. Effects of triazolam on social recognition memory in the rat. Note the absence of effect of triazolam on social investigation during the first exposure (absence of intrinsic effects of triazolam on social investigation). In the triazolam-treated animals there is no decrease in social investigation at the second exposure, demonstrating impairment of social memory.

EVALUATION

The time the adult rat spends investigating (sniffing, grooming, closely following) the juvenile is recorded at each encounter.

These two measures permit the calculation of a recognition index which consists of the ratio between the durations of investigation at E1 and E2 (E2/E1). A recognition index of close to unity suggests that the mature adult has not remembered its previous encounter with the juvenile. A recognition index significantly less than unity suggests recognition of the juvenile by the mature adult.

These effects can be analyzed statistically by comparing treated groups and vehicle control at each test session using Student's t tests.

CRITICAL ASSESSMENT OF THE METHOD

Social exploration is a natural behavioral phenomenon which occurs when animals are confronted with one another. The behavior is not induced by the experimenter but occurs spontaneously. Such a method possesses therefore a certain face validity in that it appears to resemble memories for social encounters as they occur in man.

Caution has to be applied with such anthropomorphic interpretations however. Whereas the basis of human social recognition is mainly visual, the major component in rodent social recognition paradigms is more likely to be olfactory (Sawyer et al. 1984). Thus drugs which affect rodent social recognition may do so by mechanisms not pertinent to social recognition in man, for example by changing olfactory cues. Whatever the mechanism, it is clear that animals show memory in social recognition paradigms and that this memory is subject to disruption by drug treatment. The data obtained in a rodent social recognition paradigm may therefore usefully complement data obtained using the more classical approaches described above, where the learning/memory phenomena result from experimental manipulation of the animal's motivational state. With social memory procedures, no prior animal training or habituation

is required to obtain the sought after behavioral effects.

A problem with social recognition procedures is that, being based on spontaneous behavior, they are particularly susceptible to environmental changes. Thus, to obtain reproducible findings, it is essential that all environmental factors (temperature, lighting, ambient noise and temperature, cage size, bedding, age and weight of experimental animals and time of day) be kept as constant as possible.

Moreover, to ensure that the drug effects observed are not due to intrinsic effects of the test substance on social exploration, it is desirable to precede the main social recognition experiment by a prior dose-response experiment to ensure that the selected doses do not affect baseline social investigation.

MODIFICATIONS OF THE METHOD

The basic paradigm described above ensures conditions under which a high level of social memory is obtained under control conditions. Other approaches aim to attenuate the level of social memory with the hope of the test being sensitive both to memory impairment and enhancement. A decrease in social recognition under the same experimental conditions can readily be obtained by increasing the time interval between E1 and E2, for example to 120 minutes. Under these conditions, a vehicle-treated control group of normal mature adult rats will show less or no social recognition memory for the juvenile, whereas clear social recognition memory can be observed with a 30 min interval between E1 and E2 (Lemaire et al. 1994; Thor & Holloway 1982).

Another variant to social recognition, is an object recognition task (Castagné et al. 2004), where exposure to a juvenile rat is replaced by exposure to familiar or unfamiliar inanimate objects. Object recognition tests are less subject to olfactory cues and baseline values can be more stable as the objects can be placed in fixed positions at the start of a session, but also lose part of their initial face validity.

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I.C.3.1.5 Delayed Alternation Test

PURPOSE AND RATIONALE

One of the most powerful techniques for studying behavior under well controlled experimental conditions is operant behavior. The term operant behavior refers to a particular learned behavior whereby an animal obtains food reward or avoids punishment by pressing on a lever in a so-called Skinner Box. Operant techniques permit a wide range of psychopharmacological effects (e.g. antipsychotic, antianxiety, antidepressant, analgesic) to be studied in a quantified fashion, using a standardized environment with a high degree of automation.

The technique also lends itself readily to the study of memory function, in particular by use of delayed responding procedures. In delayed responding, the animal is required to retain information over a short period (usually seconds) and then to show, by pressing an appropriate lever, whether it has correctly remembered the information (Dunnett et al. 1988). In the procedure described below, a rat in a Skinner Box is presented with a lever, either on the left or the right side of the food dispenser. The rat presses the lever and the lever is withdrawn. Five seconds later, two levers are presented and the rat has to press the lever which was not presented previously to obtain a food reward (delayed alternation or delayed non-matching to sample). If the rat presses the same lever as that previously presented, the lever is withdrawn but no food is given. In this fashion, the animal can be trained to retain a piece of information (position of a lever), and thereby demonstrate its short-term memory capacity.

PROCEDURE

We use standard Skinner Boxes (Coulbourn Instruments, Allentown, PA 18106, USA) equipped with one or two retractable levers and a food distributor. The experiments are controlled and the data collected automatically using specialized software (MED Associates, St Albans, VT 05478, USA).

During the 7 days immediately preceding the experiment, the animals are placed on a restricted food regime (15 g standard diet per day) with free access to water. Before being given the standard diet each day they are also given several 45 mg food pellets (those used as re-

ward in the delayed alternation procedure) to habituate them to this novel food.

The delayed alternation experiment consists of two separate phases:

- Acquisition of lever-pressing (single lever)
- Acquisition of delayed alternation (two levers).

Acquisition of Lever-Pressing

The aim of this phase is to train animals, on the presentation of a single retractable lever, to press on it to receive a food pellet reward.

Animals are given daily 15 minute sessions for 10 days.

At the end of this phase between 80 and 100 % of the animals acquire the lever-press response. Animals which fail to learn are discarded from the experiments. If some animals are close to establishing steady lever-pressing behavior they are given extra training with the aim of attaining at least 10 animals per group.

Acquisition of Delayed Alternation (Drug Test)

Subsequent to lever-press acquisition, all animals are submitted to delayed alternation sessions over 10 days. During this phase the Skinner Boxes are fitted with two retractable levers one on each side of the food distributor.

A training session consists of 36 successive trials separated by 10 seconds. Each trial starts by presenting the animal with one lever (left or right). When the animal presses on the lever, it is given a food pellet, the lever is retracted and 5 seconds later two levers are presented. The animal has to learn to press on the lever not previously presented in order to gain a food reward (delayed alternation). If the animal does not lever-press within 20 seconds of a one- or two-lever presentation, the lever(s) are withdrawn and the next trial commences 10 seconds later.

The test substance is usually evaluated at 3 doses, administered p.o. 60 minutes before each session, and compared with a vehicle control group.

Scopolamine (8 mg/kg p.o.), administered under the same experimental conditions, is used as reference substance.

The basic experiment therefore includes 5 groups.

Drug administrations commence at the beginning of delayed alternation training and continue over the weekend between the two test weeks. The experiment therefore includes a total of 12 administrations.

EVALUATION

Three principal measures are taken:

- percent correct responses (the number of times the animal correctly alternates expressed as a percentage of the total number of trials
- simple reaction time (mean time taken to respond to a single lever presentation)
- choice reaction (time taken to respond to a two-lever presentation).

Response accuracy reflects the animal's capacity to remember the lever previously presented and therefore represents a measure of short-term memory. Simple reaction times reflect the animal's rapidity to respond to an unpredictable spatial stimulus and therefore represent a measure of attention. Choice reaction times reflect the animal's rapidity to choose between two levers and therefore represent a measure of decision taking or information processing speed. Performance on all parameters improves over the acquisition period and therefore indicates the animal's capacity to learn the new task.

These effects can be analyzed statistically by comparing performance between the treated groups and vehicle control at each learning session using Student's t tests. ANOVA with repeated measures (trials) provides a more sophisticated and sensitive assessment by including within the same analysis all the scores obtained per animal.

CRITICAL ASSESSMENT OF THE METHOD

The delayed alternation task provides a clear index of the animal's short-term memory capacity. Because the animal can be given numerous trials (lever presentations) within a single test session of 20 minutes, the procedure provides a very dense behavioral sampling from which to estimate the effects of a drug. Furthermore, measuring the animal's simple and choice reaction times provides additional information about drug effects on other aspects of cognitive performance more related to attention and information processing speed. The operant delayed alternation paradigm thereby provides a useful multiparameter measure of possible impairing effects of a test substance on cognitive function. Indeed results obtained in our laboratory suggest that different classes of test substance can differentially affect the three parameters measured (see example below), demonstrating the usefulness of the procedure for characterizing drug effects.

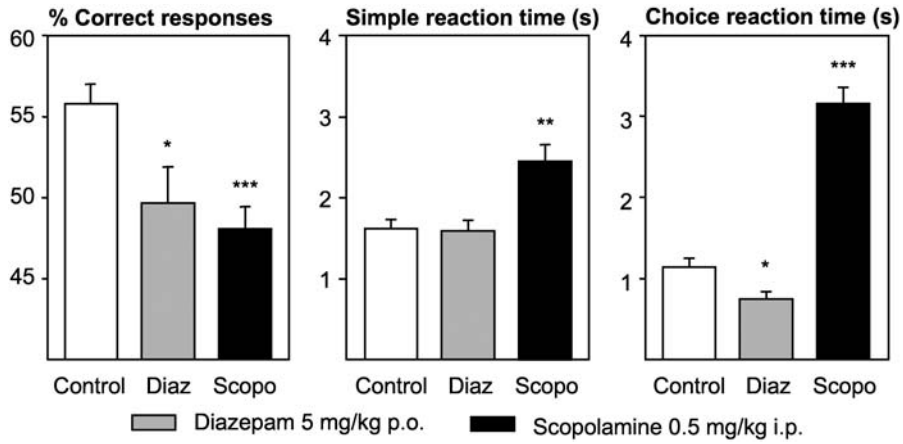

Fig. 14. Effects of diazepam and scopolamine on the 3 parameters measured during the acquisition of a delayed alternation task in the rat (mean performance over 10 sessions). Note that both diazepam and scopolamine significantly decrease the number of correct responses (impairment of learning/memory). Diazepam has no effect on simple reaction times whereas scopolamine significantly increases it (impairment of attention). Diazepam significantly decreases choice reaction times whereas choice reaction times are clearly increased by scopolamine. The increased choice reaction times with scopolamine probably reflect an impairment of information processing speed, whereas the decrease observed with diazepam probably reflects the disinhibitory effects of diazepam.

One of the principal qualities of operant behavior is the stability of the behavioral baselines between different executions of the procedure. Even with the present learning task, where the behavior is by definition labile, the possibility of obtaining comparable results on different occasions represents a major advantage of the procedure. The stable behavioral baselines undoubtedly result from the highly standardized experimental environment, where the behavior is measured automatically and there is a minimum of experimenter influence. Automation also permits numerous animals to be tested simultaneously by the same technician, thereby offsetting the cost of the apparatus and the time-consuming nature of the training procedures themselves.

Despite the stability of the behaviors observed on different occasions, the use of a learning task where the behavior evolves markedly during the course of the experiment constitutes a sensitive background against which to evaluate drug effects.

In view of the above, we recommend the delayed alternation procedure as being the most useful single procedure for evaluating possible deleterious effects of test substances on cognitive processes.

MODIFICATIONS OF THE METHOD

Delayed alternation behavior can be studied at two phases, during acquisition (as above) or when performance has been stabilized after continued training.

Delayed alternation acquisition is probably the most sensitive procedure for detecting potential adverse effects of different kinds, but lends itself less readily to unequivocal interpretation. A flattening of the learning curve could reflect either a drug effect on learning itself, or result from drug-induced impairment of the short-term memory necessary for correct performance of the task. If the animal cannot remember which lever was presented previously, it will have difficulty learning to alternate between the two levers.

This interpretational problem can be diminished by training the animals up to stabilized performance (Roux et al. 1994). Once stabilized, the behavior will more clearly reflect short-term memory because the learning component is no longer present. Indeed, once alternation performance is stabilized, it is possible to introduce delays of different lengths and thereby assess decreases in response accuracy as the retention delay is increased (delay-dependent forgetting). Thus, once animals have been trained up to stabilized performance, they can be used repeatedly to evaluate different drug treatments.

Another term for describing delayed alternation is "delayed non-matching to sample". This terminology refers to the first lever presentation (left or right) as a sample, and a subsequent response on the opposite lever (alternation) as a "non-matching to sample". Another variant of the procedure therefore is "delayed matching to sample" whereby the animal must always press on the same lever as that previously presented to obtain a food reward (Paule et al. 1998). Both procedures have been used in experimental studies of adverse drug action with essentially similar results. Our own experience in aged rats suggests that age-related deficits in response accuracy during acquisition and

stabilized performance are more readily demonstrated using a "non-matching to sample" procedure.

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I.C.3.2 EEG Studies

The Japanese guidelines suggested that electroencephalogram (EEG) studies should be conducted when necessary (Category B) whereas ICH S7A merely mentions electrophysiology examinations as part of the recommended follow-up studies. Electrophysiology covers a wide range of procedures from *in vitro* intracellular and extracellular studies to whole animal in vivo studies such as EEG. EEG itself is a very broad concept and refers to all aspects of the electrical activity of the brain, from superficial recordings outside the skull to depth electrodes recording from within the intracerebral structures at extra-cellular levels. As far as CNS follow-up safety pharmacology is concerned, two major EEG applications, the quantified EEG (QEEG) and the sleep/wake cycle, are of particular relevance, because they involve the conscious freely moving animal.

I.C.3.2.1 QEEG

PURPOSE AND RATIONALE

QEEG makes use of the Fourier theorem to analyze the electrical power in the different frequency bands of the EEG (Itil 1981). Animals, with electrodes fixed to the skull (cortical leads) and sometimes also implanted stereotaxically in selected structures (e.g. hippocampus, striatum), are exposed to brief measurement periods during which they are either left free to move spontaneously, or activated by means of a treadmill to ensure a stable heightened level of vigilance.

The EEG signals are continuously fed into a computer which divides them into short fragments which are decomposed by fast Fourier transformation (FFT) into a series of sine waves. The spectral power, a measure proportional to the wave amplitudes, is then estimated and integrated over predefined frequency ranges. The frequency ranges studied in the rat closely match those which are used to characterize the human EEG: 1.5–4 Hz (comparable with the delta-band in the human EEG), 5–8 Hz (theta-band), 8.5–12.5 Hz (alpha-band), 13–35 Hz (beta-band), 36–64 Hz (gamma-band). The total power 1.5–64 Hz is also calculated.

The protocol described below is for QEEG evaluation in the rat. Despite the considerable differences in function and structure between human and rat brain, psychoactive drugs showing effects in humans affect the rat brain similarly. Thus, the rat EEG is generally a reliable predictor for human CNS drug effects (Campi and Clarke 1995).

PROCEDURE

Animal Preparation

Rats are anesthetized (sodium pentobarbital 55 mg/kg i.p., plus supplementary doses of 5–10 mg/kg if necessary) and are implanted with 2 surface electrodes, consisting of miniature titanium screws, placed over the fronto-parietal cortex, and 2 depth electrodes, consisting of twisted platinum-iridium wires, placed stereotaxically into the hippocampus CA3 area (Paxinos and Watson coordinates interaural: AP + 5.0 mm, L ± 2.5 mm, V + 7.0 mm). The electrodes are connected to small plugs and the whole assembly is secured on the skull with acrylic dental cement. Following surgery, implanted animals are kept in individual macrolon cages (30 × 18 × 19 cm) and are allowed at least 10 days to recover.

In order to ensure a minimum of 6 animals completing the experimental treatments, a total of 12 rats are implanted.

Testing Procedure

Animals are placed individually on a stationary treadmill (30 × 10 cm) and are connected to a signal conditioning system (Coulbourn Instruments, Allentown, PA 18106, USA, Model V75-01) via a shielded multicore cable, connected at one end to the plug on the animal's head and at the other end to a swivel mounted 30 cm above the center of the treadmill. Animals are then given a 60 minute session with alternating periods of 10 minutes with the treadmill turned on (speed: 1.5 m/min) and off.

Recording sessions take place between 10 A.M. and 5 P.M. with several animals recorded in parallel.

Before beginning drug testing, all animals are submitted to at least one habituation session (without ad-

ministration) to ensure correct locomotion in the apparatus.

Each animal receives all the treatments in separate 60 minute sessions over periods of weeks. The testing procedure during each test week is as follows:

- Day 1: Control session (vehicle administration)
- Day 2: Drug test session (test substance administration)
- Day 3 to Day 7: Washout period.

Each animal is tested at the same time of day in the same treadmill during different test sessions.

8 rats are studied per test substance, and receive all the different treatments in an order balanced between the animals. The test is performed blind.

EVALUATION

EEG signals recorded by the signal conditioning system are filtered between 1 and 64 Hz and with a notch filter of 50 Hz. Amplification levels are adjusted to avoid saturation of the signal input to the computer. The differential output between the 2 cortical electrodes is digitized on-line at a sampling rate of 256 Hz/channel and the data stored in raw data files.

EEG is quantitatively analyzed by spectral analysis using a fast Fourier transform algorithm. Spectra are calculated from epochs of 4 sec duration. The mean total spectral power between 1.5 and 64 Hz and the power in 5 subfrequency bands (1.5–4, 5–8, 8.5–12.5, 13–35 and 36–64 Hz) are calculated for the periods during which the treadmill is on.

For each hour the mean relative power (% of total spectral power) in the subfrequency bands delta, theta, alpha, beta and gamma are estimated during treadmill-on phases. In addition, the ratios between fast (beta and gamma) and slow (delta, theta and alpha) frequencies for the same phases are calculated.

To take into account inter-individual variability in the EEG amplitudes, results are expressed as percentage change in the absolute power of corresponding spectra between vehicle and test substance.

The Wilcoxon signed rank test (two-tailed) is used to compare baseline recordings and recordings after substance administration.

Choice of Software

There are no generally available ready-made software systems for analyzing the rat EEG as described above. On the other hand, there are many comparable EEG acquisition/analysis software products that are commercially available for human EEG recording. Such software packages, for example Coherence32E, Scan4, Harmony, PolyviewPro, have the advantage of being immediately available, with upgrade versions usually being provided. They typically have a large number of channels, allowing the recording of several animals in parallel, but supplementary routines (treadmill control, artifact suppression, data reduction) have to be added in any case.

Creating custom application programs by means of higher programming languages for electrophysiology, for example Spike2 from Cambridge Electronic Design, or development of entirely self-made software requires considerable time and, furthermore, does not readily permit upgrades, which could cause problems if the hardware has to be replaced. Wholly or partially self-made systems also require a high level of inhouse programming expertise.

CRITICAL ASSESSMENT OF THE METHOD

Evaluations of drug effects on the EEG were already being performed in the early 1930s, by the discoverer of the human EEG himself, the psychiatrist Hans Berger. Visual analysis of EEG traces is nonetheless a time-consuming, painstaking procedure and therefore impractical on a routine basis for safety pharmacology. Apart from the subjective nature of visual trace analysis, subtle changes in EEG activity can often escape visual detection. With the advances in digital data processing, automatic EEG analysis (QEEG) has become possible and the potential usefulness of QEEG for drug screening has been recognized (Van Riezen and Glatt 1993). Drug-dependent QEEG effects have been shown in various species (Krijzer and van der Molen 1987). Although QEEG has frequently been characterized as an efficacy pharmacology procedure for identifying specific drug activity (Krijzer et al. 1993), QEEG also has undoubted usefulness in safety pharmacology. The same techniques are used for both, but the interpretation is different. Whereas the efficacy approach emphasizes the pattern of power changes in various brain structures, the main question for the safety approach is the presence or absence of such changes.

While there is debate as to whether QEEG, by virtue of the different profiles observed, is capable of identifying specific classes of psychotropic agent, there is general consensus that the QEEG can detect basic stimulant, sedative or even convulsant activity (van Riezen and Glatt 1993). This could have clear relevance for drug safety by corroborating in terms of brain activity, data obtained from behavioral observation. Furthermore, the QEEG can serve as a direct index of cerebral bioavailability, to determine up

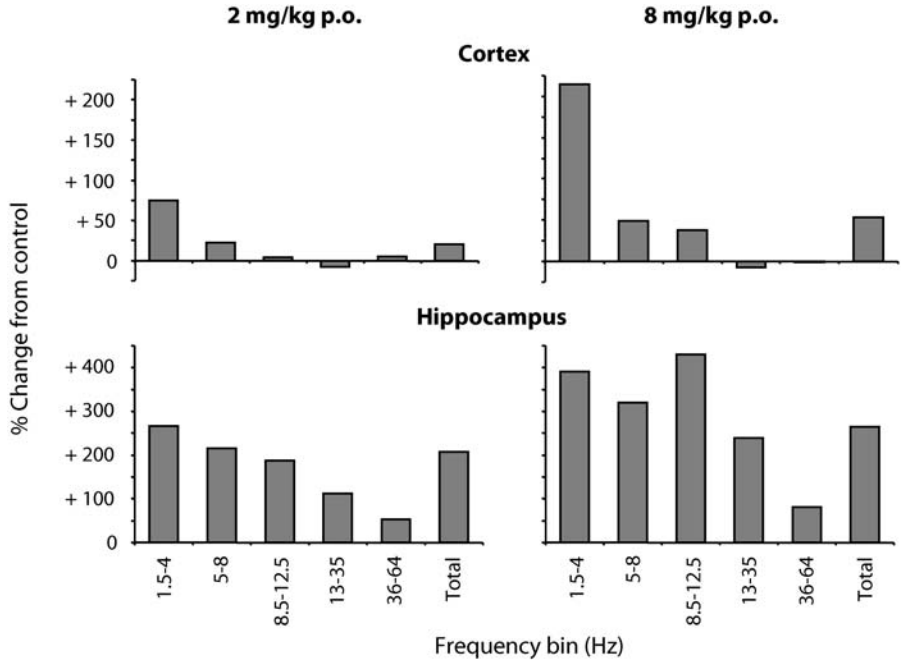

Fig. 15. Effects of d-amphetamine on the power spectrum of the quantified EEG. Amphetamine dose-dependently increases power over the whole frequency range, more markedly so at low frequencies and particularly in the hippocampus. Similar profiles are observed with other psychostimulants such as methylphenidate or nomifensine (data not shown).

to which dose a new drug, intended for a non-CNS application, is devoid of effects on the brain (Danhof and Visser 2002).

Analysis of the EEG traces can permit early detection of pathological changes in brain activity, frequently in the absence of overt effects on behavior. This appears to be particularly true for recordings from subcortical structures such as the hippocampus. Like other parts of the limbic system, the hippocampus has a lower threshold for convulsions than the cortex. Early signs of seizures, whether intentionally induced with a convulsant or as a side-effect of drug treatment, mostly appear first in the hippocampus. In man, the relevance of the hippocampus is demonstrated by its involvement in temporal lobe epilepsy (Quesney 1986). Moreover, the hippocampus is not only a key structure for pathological epileptic processes, but also for memory processing (Squire et al. 2004). Thus, for safety pharmacology purposes, the hippocampus constitutes a key structure for QEEG analysis. The results from such studies can be critical for deciding whether the development of a substance should be discontinued or shifted into another direction.

MODIFICATIONS OF THE METHOD

Since EEG activity varies with the level of vigilance, the maintenance of a constant vigilance level is essential for QEEG studies. Free-floating vigilance levels can lead to unwanted variability in the EEG signal.

Several procedures have been described to keep animals awake. One is to stimulate the animals' somatosensory system, using tactile or auditory stimuli (Sala et al. 1995). This method is effective for short recording periods, but is neither practical nor reliable for longer term recording because the animals habituate rapidly to sensory stimuli.

Another approach has been to invert the light/dark cycle and test the animals during their active period (Dimpfel et al. 1992). A persisting difficulty with an inverted light/dark cycle is that even nocturnal animals, such as the rat or the cat, will still show considerable sleep during the dark phase. Inversing the light/dark cycle is also time-consuming in that the animals require several days to habituate to the inversion, thereby increasing costs. It is also virtually impossible to eliminate all sources of disturbance, such as cage cleaning or ambient noise levels, without very sophisticated installations. A final disadvantage is that such inversions are not applied for other CNS safety pharmacology procedures, reducing the comparability of the data obtained.

For the above reasons, forcing the animals to remain active would appear to constitute the optimal procedure for maintaining a constant level of vigilance during

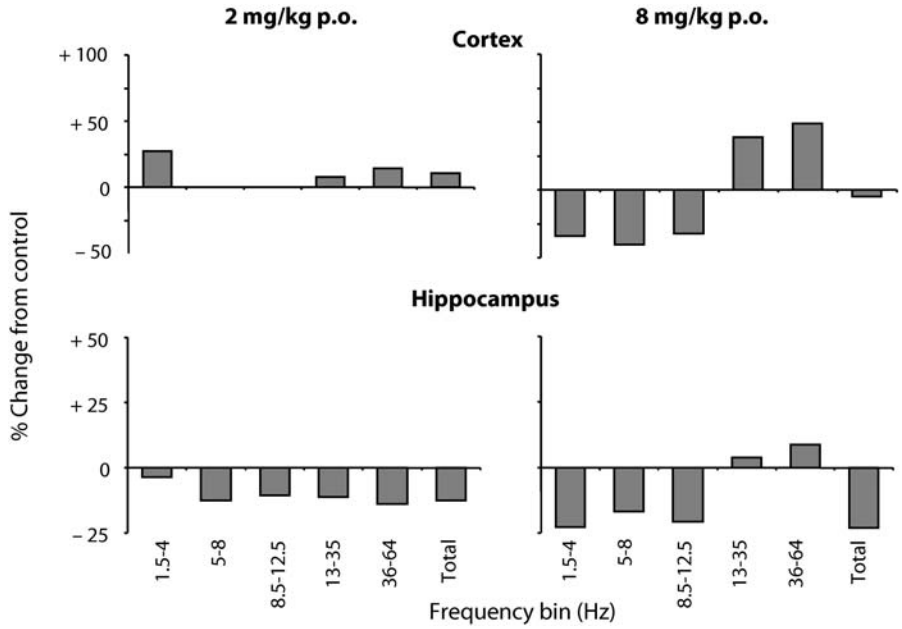

Fig. 16. Effects of diazepam on the power spectrum of the quantified EEG. Diazepam dose-dependently decreases power in the lower frequency range, with an inversion of this effect at high frequencies. The decreases in power are more marked in the hippocampus, whereas the increases are more marked in the cortex. Similar profiles are observed with other benzodiazepines and benzodiazepine-like substances such as zolpidem (data not shown).

QEEG studies. An equivalent approach to the treadmill is the use of a tread wheel (Glatt et al. 1983; Krijzer and van der Molen 1987). With both methods, animals can be kept awake for periods sufficiently long to permit adequate EEG sampling. We favor the treadmill approach because rats do not need any particular training, in contrast to the treadwheel approach where up to 30 days of habituation are necessary. Data obtained in our laboratory using a treadmill show clearly that spectral power is more variable during the periods of rest (data not shown).

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I.C.3.2.2 Sleep/Wake Cycle

PURPOSE AND RATIONALE

Studies of the sleep/wake cycle are to be distinguished from QEEG by the fact that the animals are studied over much longer periods of time (up to several days) (Jouvet 1969). In contrast to QEEG, the aim is to determine the effects of the test substance on spontaneous changes in sleep/waking activity in freely moving subjects, and in particular whether the test substance induces alterations

in the architecture of natural sleep. Although a power spectrum analysis can also be undertaken, the primary analysis is usually confined to changes occurring in the relative durations of the different phases of sleep, the hypnogram (Ruigt et al. 1989).

Although the rat is a nocturnally active animal with polyphasic sleep, experimental data show that sleep is similarly affected by drugs in both humans and rats (Lancel 1999). Thus, studies on the sleep-wake cycle in the rat offer the possibility to test drugs for their capacity to enhance sleep (efficacy pharmacology) or adversely affect the architecture of sleep (safety pharmacology). Unwanted sedative effects for non-hypnotics can be disclosed as well as sleep disturbance induced by both hypnotic and non-hypnotic drugs. From a safety pharmacology standpoint, the aim is to determine up to which dose the test substance is devoid of effects on sleep function.

PROCEDURE

Animal Preparation

Rats are anesthetized (sodium pentobarbital 55 mg/kg i.p., plus supplementary doses of 5–10 mg/kg if necessary) and are implanted with 2 surface electrodes, consisting of miniature titanium screws, placed over the fronto-parietal cortex, and 2 depth electrodes, consisting of twisted platinum-iridium wires, placed stereotaxically into the hippocampus CA3 area (Paxinos and Watson coordinates interaural: AP –5.0 mm L ± 2.5 mm, V + 7.0 mm). A further two straight platinum-iridium electrodes are implanted into the neck muscle for recording electromyographic activity. The electrodes are connected to small plugs and the whole assembly is secured on the skull with dental cement. Following surgery, implanted animals are kept in individual macrolon cages (30 × 18 × 19 cm) and are allowed at least 10 days to recover. One day after surgery, the rats are moved with their cages for adaptation into the recording environment, which is a sound-proof, ventilated, temperature-controlled Faraday room (1.8 × 2.4 × 2.3 m). This room is kept under a 12 h/12 h light/dark cycle with lights on at 10.00 A.M. Following recovery, rats are subjected to a 2–3 day habituation period in the recording cages (30 × 40 × 48 cm) made out of Plexiglas with sawdust covered floors, during which they get used to being connected via recording cables to the signal conditioning system (Grass Polygraph Model 7, installed with preamplifiers/amplifiers 7P5/DA and P511). The cables are connected at one end to the plug on the animal's head and at the other end to a turning commutator mounted on the top of the recording cage.

To ensure a minimum of 8 rats completing all the experimental treatments, a total of 10 rats is generally implanted.

Testing Procedure

After the habituation period, the test substance is generally evaluated at 2 doses administered p.o.

Recording sessions are performed as follows with 8 rats recorded in parallel:

Day 1: Baseline session 1 (treatment 1); without treatment

Day 2: Baseline session 2 (treatment 1) ; vehicle administration

Day 3: Drug session 1; treatment 1 administration

Day 4–Day 7: no testing

Day 8: Baseline session 1 (treatment 2); without treatment

Day 9: Baseline session 2 (treatment 2); vehicle administration

Day 10: Drug session 2; treatment 2 administration.

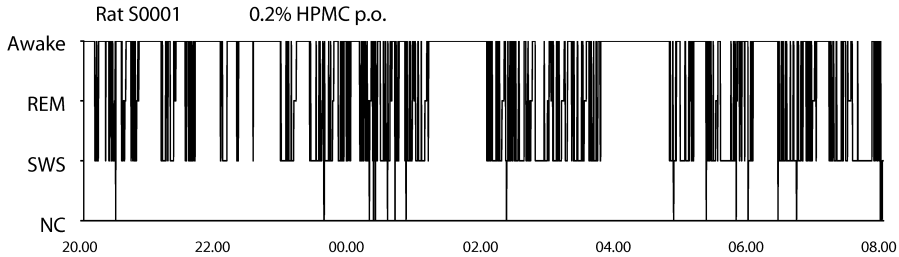
Administrations are performed at 10.00 A.M (immediately after switching on the lights) and recording starts 15 minutes later and lasts 23 hours. Half of the animals receive the high dose first, and the other half the low dose first (balanced cross-over). Each animal is tested in the same recording cage, with free access to food and water during the whole experimental period.

EVALUATION

EEG signals read into the signal conditioning system are filtered between 1 and 75 Hz for the cortex, 1 and 35 Hz for the hippocampus and 10 and 3000 Hz for the neck muscle and all signals are notch filtered at 50 Hz. Amplification levels are adjusted to avoid saturation of the signal input to the computer. The differential output between the 2 cortical sites, the 2 hippocampal sites and the muscle leads are digitized on-line at a sampling rate of 256 Hz/channel and the data stored in raw data files.

Sleep-wake cycle analysis is performed off-line using specialized commercially available software (Eclipse® and Rats®), and the statistical analysis and data presentation using SAS® software.

Sleep-wake cycle analysis is based on power spectral analysis. A fast Fourier transform algorithm calculates from the raw EEG signal total power and the power of subfrequency bands for each recorded channel from 512 data points. Single power spectra are then averaged to give a spectral point covering 20 sec of real time EEG. Under visual inspection of the spectral point-time curve, thresholds are placed



Proportion of trace by vigilance state:

	Time (min)	%
Awake	441	61.3
REM	35	4.8
Slow wave sleep	238	33.1
Non-classified	6	0.9

Fig. 17. Computer-generated hypnogram over a 12-hour measurement (dark period) where the rat is awake for two thirds of the time during this active phase of its diurnal cycle. Note the low level of non-classifiable signal (< 1 %).

manually between minima and maxima of the spectral amplitudes for the different frequency bands. The program then automatically assigns individual spectral points to vigilance stages. These values are then transferred into SAS.

In SAS, the delays to the first occurrence of SWS and REM are calculated. Further, the absolute time spent in sleep and wakefulness as well as the relative distribution of wake/SWS/REM and the proportion between total and active wake are calculated for the total recording sessions (23 hours) and for 5 time segments (0–4, 5–8, 9–12, 13–18 and 19–23 hours).

Differences between vehicle control and the test substance are calculated for all variables and for each animal, and the mean values per treatment group. Paired Student's t-tests are used for the statistical analysis of the variables.

CRITICAL ASSESSMENT OF THE METHOD

Human sleep is generally subdivided in 5 phases, phase 1–4 sleep and REM sleep. NonREM sleep in the rat has 2 phases, light sleep and deep sleep, corresponding to the human phases 1 and 2 and 3 and 4 respectively. Since it does not appear to be technically easy to implement an algorithm to differentiate between the two phases, rat sleep is rarely subdivided by computer programs. From a pharmacological point of view, such a differentiation may be of importance to show drug effects which deepen or lighten sleep without affecting other sleep parameters. To date, however, such drugs have not been described.

As with many other animal experimental models, most sleep-wake cycle studies are performed in normal rats. This is less of a problem for studies in safety than in efficacy pharmacology. In efficacy studies, a drug which may be useful to normalize sleep disturbances may not show effects when tested on the normal sleep-wake cycle. There are, on the other hand, sleep-wake cycle models which match human sleep disturbance. One example is sleep deprivation. Another is selective CNS dopamine-depletion, to model sleep disturbance related to Parkinson's disease.

Computerized analysis of the sleep-wake cycle by means of spectral analysis requires that each phase of the cycle expresses its unique activity pattern. Under physiological conditions this is the case, but may be less evident after certain drug administrations. In exceptional cases, computerized analysis may become impossible because of drug-induced abnormalities in the EEG. In these cases, however, visual analysis would also be impossible, because the spectral analysis approach uses the same information as visual analysis. In these cases measurement of more physiological parameters (see below) may be more helpful.

MODIFICATIONS OF THE METHOD

The signal transfer from the animal to a signal conditioning system can be done by telemetry instead of by the cable technique. No major differences have been reported in the quality of recording between the two approaches. Cable recording is generally preferred as it is the simpler and cheaper option.

In addition to the electrical brain and muscle activity used to differentiate between the sleep-wake cycle phases, other physiological parameters such as heart and respiratory rate, and body or brain temperature, can also be recorded. This may provide

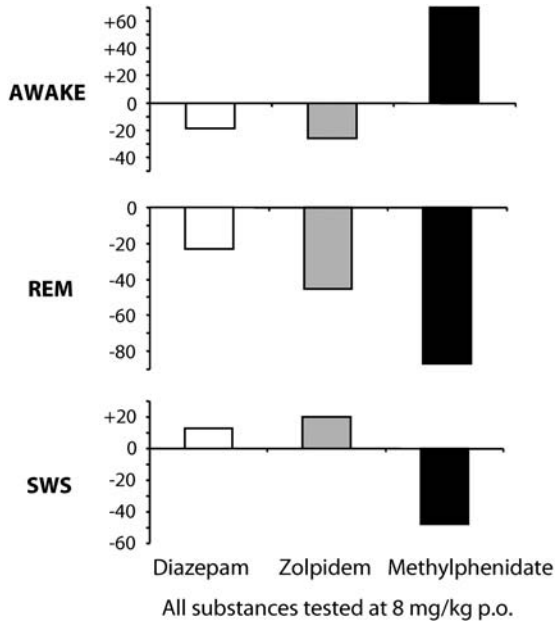


Fig. 18. Effects of diazepam, zolpidem and methylphenidate on the sleep/wake cycle in the rat during the first 4 hours after drug administration. Both diazepam and zolpidem decrease the amount of wakefulness and increase the amount of slow wave sleep, whereas opposite effects are observed with methylphenidate. All substances decrease the amount of REM sleep, with the most marked effects being observed on this parameter with methylphenidate.

supplementary information about sleep dynamics and approach techniques closer to those used in human polysomnography.

The drug administration time-point is an important variable for sleep EEG experiments. Some drug effects are seen only when the drugs are administered before the dark or before the light phase of the cycle (Bertorelli et al. 1996). Independent of pharmacokinetic considerations, most laboratories find that the "best" time for evaluating drug effects is several hours from the beginning of the light phase.

In recent years, a new mathematical approach, based on the theory of non-linear dynamics has been used to analyze EEG signals through the sleep-wake cycle (Thakor & Tong 2004). This kind of analysis has not been shown superior to the commonly used spectral analysis for pharmacological studies. This may rapidly change with the appearance of more powerful computers. In contrast to spectral analysis, which is basically not more than an automation of visual analysis, non-linear analysis could go well beyond the visual analysis of the EEG trace.

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I.C.3.3 Drug Dependence and Abuse

Another subject, clearly within the notion of safety, is the assessment of a novel substance's potential to be abused or to induce dependence. These topics are mentioned briefly in ICH S7A, "other organ systems". A much more detailed list of recommendations has been made for FDA purposes, but has never been officially adopted (Bigelow 1991).

Although dependence and abuse frequently occur together, they are not synonymous (Balster 1991). Drug dependence, as its name implies, refers to the inability of the dependent individual to function normally in the absence of drug. Dependence can be both physical and psychological, and is defined by the emergence of withdrawal symptoms either upon administration of an antagonist or upon discontinuation of drug treatment. Psychological dependence is indicated by drug-seeking behavior (e.g., craving) that can occur even after long periods of abstinence, whereas physical dependence is demonstrated more objectively by signs ranging from changes in body temperature to life-threatening conditions such as status epilepticus or delirium tremens. Abuse, on the other hand, refers to misuse or overuse of a drug and is indicated behaviorally by drug-seeking and drug-taking, many times in the absence of any evidence for physical dependence. The difference between dependence and abuse can be illustrated pharmacologically. Drugs such as heroin and alcohol are widely abused and often their abuse is associated with marked physical and psychological dependence. On the other hand, marihuana and LSD are commonly abused, but typically under conditions where neither physical nor psychological dependence is apparent.

Studies assessing dependence or abuse liability are, in principle, required only for drugs acting on the CNS. Candidates for particular attention are psycho-

stimulants, nicotinics, certain kinds of antidepressants, anxiolytics, sedative-hypnotics and analgesics. Drugs with psychotomimetic potential also require evaluation, whereas antipsychotic agents are virtually never abused. Safety studies for abuse/dependence liability are rare for substances without CNS activity, although peripherally acting analgesics might also have to come under scrutiny.

The following section describes protocols for evaluating both dependence and abuse. For reasons of homogeneity with the other procedures presented above, only protocols in the rat will be presented. Although results in the rat are generally similar to those obtained in non-human primates, the regulatory authorities, in particular the FDA, prefer primate studies for abuse evaluation because of a presumed increased predictability to man. The active doses and pharmacokinetics are likely to be closer between human and non-human primates, as are the kinds of overt behavioral effects observed.

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I.C.3.3.1
Drug Dependence

Tests for drug dependence consist mainly of repeated treatment studies, followed by drug withdrawal, where withdrawal symptoms occur either spontaneously (non-precipitated withdrawal) or after administration of specific antagonists, for example naloxone (opioids) or flumazenil (benzodiazepines). Precipitated withdrawal tests are usually the most sensitive and require only very short periods of drug administration for demonstrating withdrawal phenomena. Clear jumping can be induced in mice by administration of the μ opioid antagonist naloxone after as few as 5 pretest administrations of low doses of morphine (Saelens et al. 1971). Similarly, a decrease in the convulsive threshold can be induced by administration of the benzodiazepine antagonist flumazenil after as few as 2 benzodiazepine administrations (von Voigtlander and Lewis 1991). The relationship between these measures of "acute dependence and withdrawal" and the dependence and withdrawal that emerge after extended periods of drug treatment followed by discontinuation of treatment are far from clear. Precipitated withdrawal tests might be less indicative of dependence potential than of a particular mechanism of action or affinity for a receptor, and will not be discussed further here.

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I.C.3.3.1.1
Non-Precipitated Withdrawal Test

PURPOSE AND RATIONALE

The aim of non-precipitated withdrawal procedures is to evaluate whether sudden cessation of drug treatment is associated with the occurrence of identifiable withdrawal symptoms. The kinds of symptom examined are changes in food intake, body weight gain, body temperature and the occurrence of one or more overt behavioral and other symptoms, for example tremor, teeth chattering, wet dog shakes, diarrhea or piloerection. The occurrence of such signs is a first indication that the test substance induces drug dependence after repeated administration. An important advantage of non-precipitated withdrawal procedures is that they can be used to evaluate a wide variety of substances, including those for which specific antagonists are not available.

More elaborate procedures can be employed to assess whether drug withdrawal induces changes in fearfulness, pain sensitivity, convulsive threshold or even memory. On the other hand, it is frequently difficult to demonstrate effects on these parameters and the tests involved are particularly time-consuming. The procedure described below represents an initial screen which has been shown to be sensitive to several dependence-inducing drugs such as opioids and benzodiazepines (Goudie et al. 1993).

Whereas many CNS safety tests evaluate multiple doses, we recommend the inclusion of just two doses for non-precipitated withdrawal studies. The low dose should be close to the dose inducing clear effects in the test predictive of the substance's therapeutic indication. The high dose should be the maximally tolerated dose as determined, for example, from an Irwin test. If it can be shown that the test substance can be repeatedly administered at a maximally tolerated dose under conditions where similar treatment with an appropriately chosen reference substance induces

clear withdrawal signs, a reasonable conclusion would be that the test substance is unlikely to cause physical dependence.

PROCEDURE

Rats receive twice daily administrations of the test substance for 20 days and are then subjected to an 8 day observation period without drug treatment during which they are observed for changes in food consumption, body weight and rectal temperature. In addition they are observed for behavioral and physiological manifestations (e.g. jumping, tremor, hyperactivity, excessive grooming, and diarrhea). During the pretreatment phase, different groups of animals receive the test substance at 2 doses, and are compared with a vehicle-treated control group.

Twelve animals are studied per group. The test is performed blind.

Morphine (128 mg/kg p.o.), cocaine (64 mg/kg p.o.) or chlordiazepoxide (64 mg/kg p.o.) can be used as reference substances.

The experiment includes a control group pretreated with the vehicle twice daily for 20 days.

EVALUATION

Before assessing withdrawal effects in the absence of the test substance, it is important to assess the intrinsic effects of the test substance, to see whether repeated treatment itself influences the parameters observed. Body weight, food consumption, body temperature and the occurrence of behavioral and physiological manifestations are therefore observed during the last 3 days of drug treatment. Changes occurring after cessation of drug treatment can thus be more accurately assessed in relation to the drug effects themselves. Although we recommend doing most behavioral tests blind, this is particularly important for the present procedure where many of the behavioral parameters are assessed subjectively.

Differences from control are evaluated on a day-by-day basis using non-paired Student's t-tests.

CRITICAL ASSESSMENT OF THE METHOD

The non-precipitated withdrawal procedure represents a first screen for possible induction of drug dependence and has been shown to be sensitive to withdrawal effects with a variety of dependence-inducing agents including amphetamines, cocaine, opioids and benzodiazepines. It therefore possesses face validity. On the other hand, it is remarkably difficult, under the conditions of the protocol described, to show signs of withdrawal after treatment with agents such as nicotine or 9-tetrahydrocannabinol (THC) (unpublished data from our laboratory). While absence of withdrawal effects with THC may reflect the absence of proven drug dependence with cannabinoids in man, the human drug dependence data with nicotine suggest that nicotine dependence does occur.

A principal weakness of the procedure using one, two or three daily administrations is that the behavioral and physical signs induced are modest and very shortlived (2–3 days). Indeed under conditions of oral, i.p. or s.c. administration, there are no dramatic manifestations as have sometimes been described in the literature, particularly with opioids (Gianutsos et al. 1975). Administration by the i.v. route produces more rapid onset of drug action, and mirrors more closely human drug abuse behavior, but it is difficult to ensure repeated i.v. administrations or continuous i.v. slow infusion over longer periods (weeks) in rats to obtain the exposure necessary to induce signs of dependence. Certain authors recommend use of osmotic minipumps to ensure a more constant exposure to the test substance and a more abrupt discontinuation by surgical removal at the end of the exposure period (Kalinichev and Holtzman 2003). This has been reported with nicotine (Semba et al. 2004), although we have not been able to reproduce this data in our own laboratory. A major problem with osmotic minipumps is that they require the test substances to be soluble, and this is not always the case.

There is no easy way of ensuring that withdrawal from reference substances will induce dramatic behavioral changes. The experimenter is obliged to retain more subtle effects as indices of the dependence potential of the test substance. The method does possess the advantage that it is not mechanism-bound, and therefore may detect dependence-like phenomena with agents having no common neurobiological substrates. Absence of any signs of withdrawal after repeated treatment with maximally tolerated doses can be taken as a reasonable indicator of the absence of dependence liability.

MODIFICATIONS OF THE METHOD

The non-precipitated withdrawal procedure described above represents an open-ended screen for dependence liability. The procedure can be rendered more discriminating, by pre-exposing the animals to the same administration schedule and then testing them in procedures with more specific indications. For example, withdrawal-induced fearfulness could be assessed by using a plus-maze appropriately calibrated to detect anxiogenic activity. Similarly, a withdrawal-induced

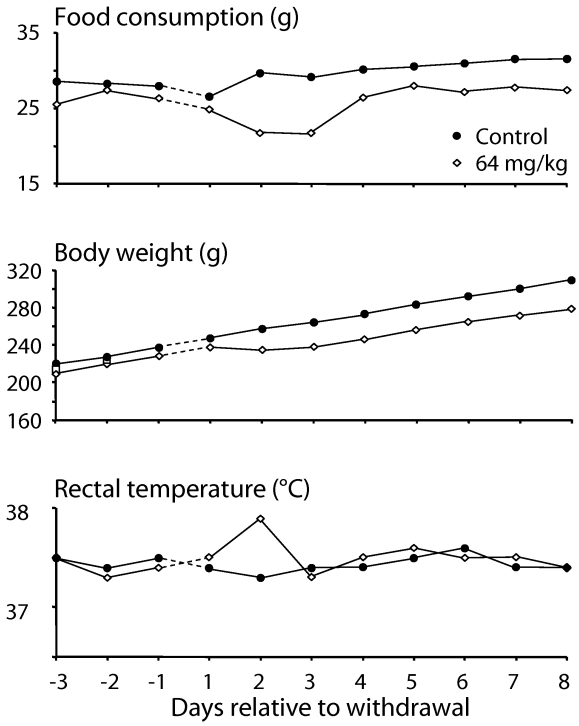

Fig. 19. Effects of drug withdrawal after 10 days twice daily treatment with chlordiazepoxide (p.o.) on food consumption, body weight gain and rectal temperature in the rat. Note a decrease in food consumption and an increase in rectal temperature during the first 2 days after drug withdrawal with recovery towards vehicle control values thereafter. Similar but less marked effects are observed on body weight.

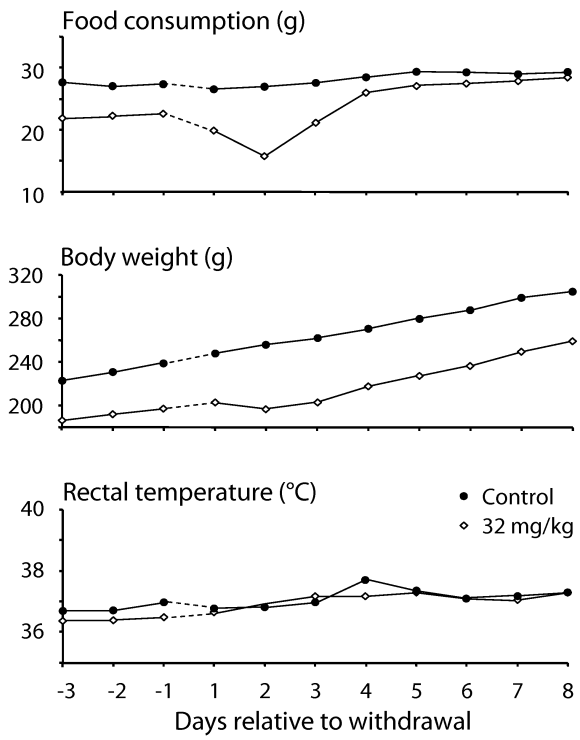

Fig. 20. Effects of drug withdrawal after 10 days twice daily treatment with morphine (i.p.) on food consumption, body weight gain and rectal temperature in the rat. Note the presence of a clear decrease in body weight as a result of the pretreatment with morphine. A further decrease in food consumption is observed during the first 2 days after drug withdrawal with recovery towards vehicle control values thereafter. Similar but less marked effects are observed on body weight.

decrease in the convulsive threshold could be evaluated using either an ECS or PTZ procedure.

More complex procedures for unmasking drug dependence liability could involve assessment of acquired positive reinforcing effects as a result of repeated treatment. Induction of conditioned place preference (see below) has been reported with different substances, for example nicotine (Shoaib et al. 1994), after the animals have been pre-exposed to repeated treatment with the substance, whereas no conditioned place preference can be induced with the same substances in the absence of pretreatment.

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I.C.3.3.2 Drug Abuse

Drug abuse represents a more serious social problem than drug dependence, because it involves a much greater variety of substances and because it can and does occur in the absence of dependence. Tests for abuse measure various aspects of drug taking and drug seeking behavior with the aim of establishing whether the test substance possesses positive reinforcing effects.

Indirect tests can assess whether the animal prefers to be in an environment associated with a substance with known positive reinforcing properties (condi-

tioned place preference procedures), or whether the test substance has stimulus effects that resemble a drug with known positive reinforcing properties (drug discrimination procedures). The most direct test of drug abuse liability, however, is to assess whether animals will work to receive administrations of the test substance (self-administration procedures). Examples of the three approaches are given below.

I.C.3.3.2.1
Conditioned Place Preference Test

PURPOSE AND RATIONALE

The principle of conditioned place preference is that an animal, repeatedly exposed to a distinct environment in the presence of a substance with abuse potential, will show preference for that environment when later given a choice because the environment has become associated with the rewarding propeprties of the test substance (Schechter and Calcagnetti 1993). If conditioned place preference can be established with a test substance, this suggests that the test substance possesses positive reinforcing effects and is therefore likely to be abused. If the test substance has no such effects or even induces a conditioned place aversion, where the animal avoids the environment previously associated with the test substance, this suggests that the test substance is devoid of positive reinforcing effects or may even be aversive and is, therefore, unlikely to be abused.

PROCEDURE

Rats are given 1 session per day (a.m.) over 8 days. During sessions, which last 30 minutes, they are allowed to explore 1 side of a 2 compartment box (35 × 35 × 70 cm). The 2 compartments are tactually and visually distinct (black/white striped walls with smooth floors versus grey walls with corrugated floors). The opening between the 2 compartments (12 × 12 cm) is closed by a guillotine door. Before each session the animals receive a p.o. administration of either the test substance or vehicle. The test substance is always associated with the grey compartment. Thus after 8 days, each animal will have been exposed to 4 pairings of test substance with the grey compartment and 4 pairings of vehicle with the striped compartment.

On the 9th day, in the absence of any treatment, the animals have free access to both compartments via the guillotine doorway which is left open and the session is monitored and recorded on videotape. The time spent by the animal in each of the two compartments is scored from the video records, and is broken down into 5 minute segments over the 20 minute test period. The number of crossings from one compartment to the other is also recorded.

Twelve animals are studied per group. The experiment is performed blind.

The test substance is usually evaluated at 2 doses and compared with a vehicle-treated control group.

Morphine (64 mg/kg p.o.) can be used as reference substance.

EVALUATION

Before commencing conditioning animals are generally given a 20-minute pre-test in the two compartments with the guillotine door open, and the time spent in each compartment is measured. These data provide an initial assessment of any natural preference for either compartment in the absence of drugs, and allows assignment of animals to treatment groups such that initial place preference is matched between the groups.

During the conditioning phase itself, where the animal in confined to one or the other compartment, no behavioral monitoring is undertaken.

The measure of time spent in each compartment on the test day (Day 9) in the absence of the test substance is an indication of the animal's preference for each compartment. Measures of the number of crossings provide an indication of the animal's spontaneous level of activity under the conditions of the test. It should be noted that crossings are not an indication of the effects of the test substance on locomotion, because the animal is not under the influence of the test substance during the test. Clear decreases in the number of crossings are, nonetheless, systematically observed on the test day after previous conditioning with substances such as morphine, and therefore appear to be part of the conditioned place preference process. On the other hand, no ready interpretation can be offered for these changes.

CRITICAL ASSESSMENT OF THE METHOD

Conditioned place preference appears to provide a fairly simple indication of whether a test substance exerts positive reinforcing effects. The existence of conditioned place preference with a novel substance therefore represents a clear first sign of abuse potential.

On the other hand, absence of conditioned place preference cannot be taken to indicate that the test substance is devoid of abuse potential. Indeed, the main weakness of the conditioned place preference paradigm is that several known substances of abuse

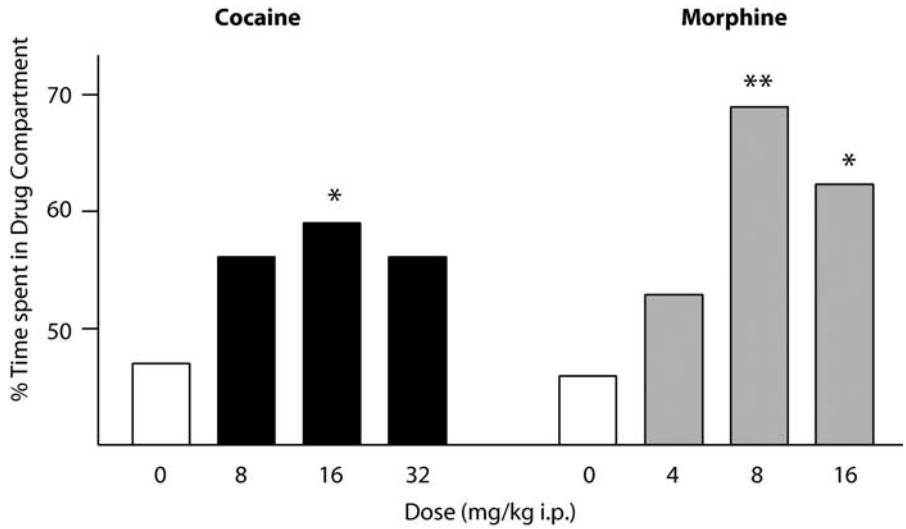

Fig. 21. Effects of cocaine and morphine (i.p.) in the Place Preference Test in the rat.

(amphetamine, THC, nicotine, alcohol) do not readily induce conditioned place preference, if at all. Moreover, even with substances such as cocaine, the conditioned place preference which occurs is considerably less robust than that observed with morphine. Cocaine place preference is generally observed only during the first 5–10 minutes of the test, in contrast to morphine where the effect is more clearly present over the whole test period. Even with morphine, the effect is observed only over a narrow dose-range, 64–128 mg/kg p.o., and is not clearly dose-dependent (our unpublished data).

Further problems are encountered with substances such as THC, where place preference can be observed over a narrow range of low doses (0.5–1 mg/kg i.p.), but where place aversion is observed at higher doses (Maldonado and Rodriguez de Fonseca 2002). Conditioned place preference has been reported with nicotine, but only after a short pretreatment with nicotine before commencing place preference conditioning (Shoaib et al. 1994). We have been unable to replicate this finding in our own laboratory (our unpublished data).

Taken together, existing data with conditioned place preference suggest that it is rather insensitive to several known drugs of abuse (false negatives). On the other hand, the demonstration of conditioned place preference with a particular substance represents a danger sign which should not be ignored.

MODIFICATIONS TO THE METHOD

The procedure described above gives individual conditioning sessions on separate days. This procedure, while time-consuming, ensures that the animals enter each conditioning session in the absence of drug effects from a preceding session.

Shorter procedures are available where the animals are given two conditioning sessions within the same day, mornings and afternoons (Bals-Kubik et al. 1993). With drugs such as morphine administered parenterally (i.v. or i.p.), the 5 hour interval between the two sessions is sufficient to enable adequate place preference conditioning to occur, presumably because the drug effect from the morning session is no longer present in the afternoon. This assumption cannot be made when the test substance has a long duration of action, or even when the test substance is administered by the p.o. or s.c.routes, where the drug effects typically last longer. If there is no clear pharmacological differentiation between two successive conditioning sessions, the absence of conditioned place preference could represent a "false negative" in that the animal is unable to discriminate the two compartments in terms of the presence and absence of the test substance. For test substances with a demonstrably short duration of action, the 2 sessions-per-day procedure represents a considerable economy in experimental time, and therefore cost.

Another time-consuming aspect of the paradigm described above is the off-line visual analysis of the video recordings, conducted after test completion. Considerable economies can be realized by use of commercially available automatic video image analysis systems. Once correctly validated, these

systems offer a viable alternative to visual analysis, providing cost-effective and objective data evaluations.

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I.C.3.3.2.2
Drug Discrimination

PURPOSE AND RATIONALE

Another indirect procedure for evaluating abuse liability is drug discrimination where animals, by pressing on one of two levers in a Skinner Box, can show that they can discriminate the presence of a particular drug of abuse (training drug) from vehicle. They are then given the new substance and can indicate by their choice of lever, whether the new substance resembles the training drug.

The basic assumption of drug discrimination procedures is that if a test substance is perceived as being subjectively similar to a drug of abuse, it is also likely to be abused. Many drugs of abuse are capable of being discriminated in a drug discrimination paradigm in rats, and include cocaine, amphetamine, morphine, phencyclidine, mescaline, THC, nicotine and benzodiazepines (Colpaert & Slangen 1982). It is important to note that the ability of a drug to serve as a discriminative stimulus does not necessarily indicate any potential for abuse since certain drugs that are not abused, for example flumazenil (Gerak and France 1999), can be established as a reliable discriminative stimulus in animals.

PROCEDURE

Training

Sessions are given in commercially available operant chambers (Coulbourn Instruments, Allentown, PA 18106, USA) equipped with two response levers, and a food hopper. Initially rats are trained to press levers for food in 2-hour sessions during which a single press of either lever will result in the delivery of a food pellet. Drug discrimination training commences as soon as rats receive at least 50 food pellets during a session (typically less than 3 sessions). Training sessions comprise a 15-minute timeout, during which the chamber is dark and lever presses have no programmed consequence, followed by a 15-minute response period, during which the chamber is illuminated and food is available as described below. Responding on one lever, for example the left lever, produces food on days when saline has been administered immediately before the time-out, and responding on the other lever (in this case the right lever) produces food on days when the comparator has been administered immediately prior to the timeout. The association of the left or the right lever with training drug or saline is constant for a particular rat but varies between rats. Training sessions proceed at first with a simple alternation (drug, saline, drug, saline) followed by a double alternation (drug, drug, saline, saline) across days. Simultaneously, the response requirement is increased by one response per session up to a maximum of 10 (called FR10 as the food reward is delivered according to a **F**ixed **R**atio of 10 lever-presses per food pellet) so long as rats receive at least 25 pellets in the session. If a rat receives fewer than 25 pellets in a training session the response requirement is decreased by 1 in the next training session. A maximum of 50 pellets is available in each daily 15-minute response period. Training continues until the following criteria are satisfied for 5 consecutive or for 6 of 7 consecutive training sessions: $\geq$ 80 % of total responses on the correct (injection appropriate) lever; and fewer than 10 (one FR) responses on the incorrect lever prior to the first food delivery. Thereafter drug tests are conducted every third day so long as satisfactory stimulus control is evident during intervening training sessions.

Morphine (4 mg/kg i.p.), diazepam (2 mg/kg i.p.) or d-amphetamine (0.6 mg/kg i.p.) can be used, among others, as comparator drugs.

To ensure that 8–10 animals can be retained for drug testing, training is commenced with 12 animals per comparator.

Drug Testing

Test sessions are identical to training sessions except that completion of the FR10 on either lever produces food.

The training dose of the comparator and saline are tested initially to confirm adequate stimulus control for testing. Thereafter, 4 different doses of the test substance are studied using a Latin Square design. On concluding test substance evaluation, the comparator and vehicle are tested again.

EVALUATION

Two basic measures are taken during drug discrimination procedures, the percent responses on the drug-associated lever, both prior to the first reinforcer and for the total session, and the total number of responses per session.

The first parameter (percent responses on the drug lever) provides an estimate of the degree of generalization between the test substance and the comparator (training drug). No responding on the drug lever (i.e. 100 % responding on the saline lever) would indicate that the test substance does not generalize to the training drug and that the test substance is therefore not recognized as being similar. Exclusive (100 %) responding on the drug lever would indicate complete generalization to the training drug and that the test substance is therefore recognized as resembling the training drug. In cases where clear generalization occurs, a dose-response effect is observed and the data lend themselves to calculations of ED50s which represent the dose levels at which the test substance generalizes 50 % to the training drug.

The second parameter (number of responses per session) provides an estimate of the effects of the test substance on operant performance. If the test substance exerts marked sedative effects, the number of responses would normally be decreased. Response rate could even be increased if the test substance possessed psychostimulant effects. Interpretation of drug effects on operant performance is, however, not simple, because other factors can contribute to effects of the test substance on response rate. In the present procedure, where the effect of the reinforcement schedule (FR10) is to produce a high rate of baseline responding, test substances with either sedative or psychostimulant effects will generally decrease the rate of responding.

Although the test substance may generalize partially or fully to the training drug up to some dose, further increasing the dose will eventually decrease or completely suppress responding. Partial generalization, accompanied by a suppression of responding at higher doses, could suggest that the test substance does not fully share discriminative stimulus effects with the training drug. Alternatively, such a result could indicate that rate-decreasing effects occur at comparatively smaller doses and, thereby, preclude measures of discriminative stimulus effects. Partial effects of this type must be evaluated with great caution. However, if complete generalization occurs before the test substance suppresses responding, this suggests a clear similarity between the test substance and the training drug, at least within that particular dose-range.

Differences from control are usually evaluated using paired Student's t-tests or ANOVA with repeated measures.

CRITICAL ASSESSMENT OF THE METHOD

Drug discrimination procedures provide a unique opportunity for assessing subjectively perceived drug effects using purely behavioral criteria and quantitative measures. Furthermore, under appropriate training conditions, the method has been shown to be exquisitely sensitive even to minor changes in pharmacological activity, for example between different benzodiazepines (Ator and Griffiths 1989) or even between different doses of the same drug (Walker et al. 2001). At the same time, the numerous published data show a high degree of selectivity within different substance classes. The perceived similarities between different psychotomimetics, for example PCP and MK-801 (France et al. 1991) or opioids (France et al. 1995) provide cogent behavioral evidence for a common mechanism of action, or even action at the same receptor subtype, whereas dissimilar discriminative stimulus effects provide suggestive evidence for action at different receptors or different actions at the same receptor. Thus, the greatest strength of drug discrimination is its pharmacological selectivity.

The interplay between generalization curves and drug effects on response rate can often provide behavioral evidence for the degree of similarity between a selected training drug and the test substance, or the dose-range in which such similarity exists, or of the kind of action occurring at a target receptor including agonism, antagonism, inverse agonism and the relative efficacy of those effects.

A further advantage of drug discrimination paradigms is that similar methodology can be used in a variety of species from mice (Shelton et al. 2004) to pigeons (Walker et al. 2001) to rats (Ator and Griffiths 1989) to monkeys (Gerak and France 1996) and even to humans (Foltin and Fischman 1992), thereby permitting direct extrapolations across species.

The primary disadvantage of drug discrimination procedures is that they are time-consuming. This becomes most apparent when the procedure is used to characterize the perceived pharmacological profile of a new test substance, where it may have to be evaluated in numerous independent groups trained to recognize a variety of known drugs of abuse. Thus characterization of test substances solely on the basis of drug discrimination procedures is likely to take considerable time.

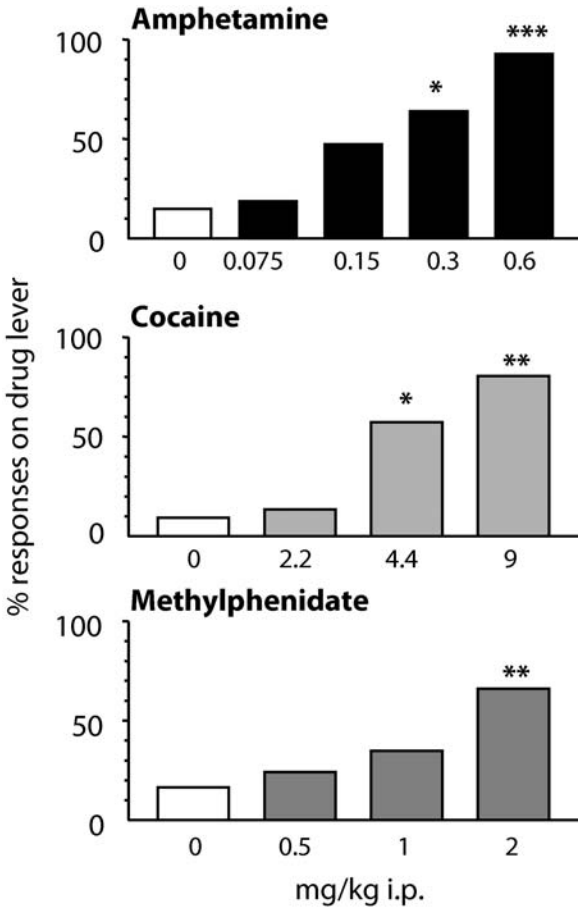

Fig. 22. Effects of d-amphetamine, cocaine and methylphenidate on drug discrimination in rats trained to discriminate 0.6 mg/kg i.p. d-amphetamine from saline. Note the dose-dependent generalization of lower doses of amphetamine towards responding on the lever associated with the training dose of amphetamine, with similar generalization curves for cocaine and methylphenidate.

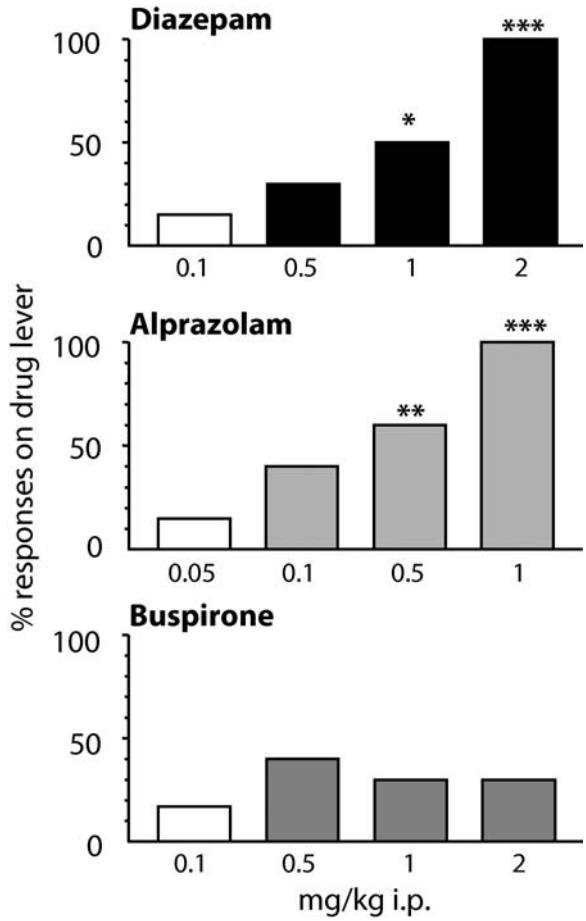

Fig. 23. Effects of diazepam, alprazolam and buspirone on drug discrimination in rats trained to discriminate 2 mg/kg i.p. diazepam from saline. Note the dose-dependent generalization of lower doses of diazepam towards responding on the lever associated with the training dose of diazepam, with a similar generalization curve for alprazolam. Buspirone does not generalize to diazepam.

The principal weakness of drug discrimination procedures for assessing abuse liability is that they provide only indirect evidence regarding abuse. If a test substance is discriminated as being similar to a known drug of abuse, this is taken to indicate that the test substance is likely to be abused in a similar manner. On the other hand, if a test substance does not share discriminative stimulus effects with any known drug of abuse, drug discrimination procedures alone provide no indication of whether the test substance is likely to be abused.

MODIFICATIONS OF THE METHOD

There are many variants in drug discrimination methodology whereby small procedural differences (fixed or variable interval instead of fixed or variable ratio reinforcement, probe trials with or without reinforcement to test for generalization, test sessions where either the chosen lever, i.e. that on which the animal emits say its first 10 responses, or both levers are reinforced) are practised in different laboratories without marked differences in the results obtained.

A more important difference is whether to score the level of generalization by the percentage responding on the drug-associated lever, or by the initial lever choice of the animal when exposed to the test substance. The former in theory permits a more quantified and graded estimate of the degree of generalization at the different doses, and should thereby permit a more powerful statistical analysis. The latter might provide a more unbiased measure of discriminative stimulus effects since responding prior to food delivery, unlike responding after food delivery, cannot be influenced by contingencies of reinforcement. However, simple

lever choice measures permit only quantal measures (proportion of animals choosing the drug-associated lever) at each dose investigated, with correspondingly less powerful statistical analysis. Again there are no marked differences in the kinds of results reported by laboratories using the two approaches. One reason is that the distribution of scores between the two levers is essentially bimodal, with little variation at the two extremes and considerable variation around the 50 % mark.

Another potentially useful drug discrimination approach is to establish whether the test substance itself can exert discriminative stimulus control as a training drug. If the test substance is poorly discriminable, more sessions will be required before achieving an adequate level of stimulus control, or abandoning training. Indeed, failure to establish discriminable control over many training sessions constitutes suggestive evidence that the substance has few CNS effects, assuming that it has been administered in an appropriate dose and at an appropriate time prior to training sessions. While inability to establish discrimination with a test substance provides presumptive evidence for a lack of CNS effects that could predict little or no abuse potential, the opposite result is not true. Rapid acquisition of discriminative control with a test substance simply indicates that the substance has stimulus effects, perhaps mediated in the CNS. Because many CNS-acting drugs that are not abused are reliably discriminated by non-humans, for example kappa opioid agonists (France et al. 1994), no predictions can be made regarding abuse potential based solely on ease of discrimination training.

Despite the time taken to train an animal to recognize a test substance, once the animals are sufficiently trained they can be used repeatedly with a variety of drugs of abuse with the aim of identifying which of these substances generalize to the training substance (in this case the test substance). In this fashion, it would be possible to establish the profile of perceived drug effects using the same animals tested with different drugs of abuse. This kind of procedure is rarely reported, but could represent a considerable economy in the number of animals required.

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I.C.3.3.2.3
Self-Administration

PURPOSE AND RATIONALE

The most direct tests of abuse potential are self-administration procedures, where the animals can, by pressing on a lever, receive an i.v. infusion of the test substance via a pump system connected to an indwelling catheter (Brady and Fischman 1985). Parallel methods have been used widely with rats and monkeys and provide substantially similar findings. More recently, self-administration procedures have also been developed using mice (Caine et al. 2002). Virtually all drugs which are abused in humans will induce or sustain self-administration behavior in animals. The self-administration paradigm is a clearly homologous animal model of human drug abuse with a consequently high level of face validity, construct validity and predictive validity.

PROCEDURE

Experiments are conducted in commercially available operant chambers (Coulbourn Instruments, Allentown, PA 18106, USA), equipped with two response levers, a food hopper and receptacle, stimulus lights and an exhaust fan. Male rats (225–250 g) are housed individually and maintained under a non-inverted 12/12 hr light/dark cycle with restricted access to food (15 g per day). Lever training begins under a continuous reinforcement schedule of food presentation. Daily sessions are terminated after 1 hour or 50 food presentations, whichever occurs first. Subsequently, the response requirement is increased by one response per daily session up to FR5. Once rats receive 50 food pellets in a single session under the FR5 schedule (typically within 5 days), food training is suspended and

a chronic indwelling i.v. jugular catheter is implanted. During all sessions, other than the first food-training session, only responding on one of the levers produces food or i.v. infusions (i.e. the active lever for individual subjects remains the same throughout a study). Responses on the second (inactive) lever are counted but have no programmed consequence. Beginning 5 days after surgery, daily sessions (typical maximum duration of 1 hour) are conducted during which rats can receive a maximum of 20 i.v. infusions of the baseline drug (cocaine, 0.25 mg/kg/infusion) under the FR5 schedule. Immediately prior to the beginning of daily sessions, rats receive a non-contingent (i.e. "priming") injection of the same solution that is delivered after appropriate responding during the session (cocaine, saline or test substance). A distinctive visual stimulus is displayed during periods when i.v. infusions are available. Each infusion is followed by a 30-second timeout, during which the chamber is dark and lever presses have no programmed consequence. The infusion duration is 4–6 seconds and the infusion volume is 74–110 μL, depending on the weight of the subject and the solubility of the test substance.

Self-Administration Testing

After responding for cocaine stabilizes (i.e. when the variability in the number of infusions delivered per session does not exceed ± 20 % over 4 consecutive sessions), saline is substituted for cocaine until the number of infusions per session is less than 8 for 4 consecutive sessions. Once this criterion is satisfied, a specified dose of the test substance is substituted for saline for 10 sessions or until the number of infusions per session either does not vary by more than ± 20 % or is less than 8 per session over 4 consecutive sessions. Next, saline is substituted for the test substance until the criteria listed above are satisfied. Finally, cocaine is substituted for saline until the criteria listed above are satisfied.

Each dose is studied in a minimum of 8 and a maximum of 12 rats with each subject used to study only a single dose of test substance.

The test substance is usually studied at 4 doses.

EVALUATION

The basic parameters measured during self-administration studies are the number of infusions taken and the response rate (responses per second) per daily session.

CRITICAL ASSESSMENT OF THE METHOD

The model described above is a so-called substitution procedure where the animal is first trained to respond for i.v. infusion of a known drug of abuse, in the present case cocaine. Once reliable self-administration is established, the test substance is substituted for cocaine to see whether the test substance continues to maintain self-administration behavior.

This procedure had several advantages. The first is that drug testing commences in an animal already shown to administer the reference drug. As such, the model possesses a high level of face validity in that the test substance evaluation is conducted in a drug-experienced animal. Moreover, even in the rat, there can be individual differences in the rate at which animals self-administer the reference drug, even after extensive training. Thus, the substitution procedure provides an assessment of the rate at which the test substance is self-administered compared with the reference drug, for individuals and for groups of subjects. Furthermore, evaluating the test substance in animals already shown to self-administer a reference drug of abuse decreases the possibility that a negative finding with the test substance is due either to the animal's low intrinsic tendency to self-administer or to other factors which might have prevented the animal from initiating self-administration. Thus, the substitution model represents a highly sensitive procedure for detecting positive reinforcing effects of test compounds which, in turn, have high predictive validity for abuse potential in humans. No self-administration behavior under these conditions suggests the absence of abuse potential.

A critical element in all self-administration procedures is the choice of the doses to be evaluated. If the doses are too high, it is possible that no self-administration will be observed because the high doses directly interfere with operant responding. Studying inappropriately high doses could therefore lead to the false conclusion that the test substance is devoid of abuse liability, whereas lower doses might clearly maintain self-administration. The best way to avoid overdosing is to conduct prior dose-finding experiments, using either an operant behavior schedule or a more simple measure of spontaneous locomotion, like the Activity Meter Test described above. The highest dose chosen for the subsequent self-administration experiment should be one just below that inducing a clear effect on operant or spontaneous behavior. The choice of the lowest dose represents less of a problem in that it should be close to that found active in procedures used to evaluate the potential therapeutic activity of the test substance.

Use of an appropriate self-administration paradigm, such as the substitution procedure described above,

is likely to be sensitive to a large variety of known drugs of abuse (Griffiths and Balster 1979; Griffiths et al. 1980) although certain substance classes, for example nicotine (Corrigall 1999) and benzodiazepines (Broadbear et al. 2004), while clearly positive reinforcers under a limited range of conditions, are not as readily self-administered as opioids or cocaine. Indeed, the different self-administration effects of these diverse substance classes probably reflects their real life abuse liability as indicated by "street use". On the other hand, the experimental literature describes self-administration in animals with several substances, for example modafinil (Gold and Balster 1996), nomifensine (Aspen and Winger 1997) and bupropion (Bergman et al. 1989), which have never been associated with significant problems of abuse in humans. Thus, self-administration procedures in non-humans can generate a certain number of apparent "false positives". Reasons for the absence of abuse in humans with some substances that maintain self-administration responding in animals could be related to the ease at which these substances can be put into an injectable formulation, pharmacokinetic factors such as their rapidity of absorption, their availability compared with other drugs, or to important differences between the self-administration doses and those which are active in the therapeutic indication.

MODIFICATIONS OF THE METHOD

The major variants of the self-administration paradigm, for the purposes of abuse potential studies, are substitution procedures and initiation procedures. With substitution procedures, animals are first trained to self-administer a reference drug and are then administered the test substance to see whether the test substance can substitute for the reference drug in maintaining self-administration behavior. The advantages of substitution procedures in CNS safety evaluation have been described above. Another advantage of substitution procedures is that they permit re-use of the same animals for assessing different test substances or different doses of the same test substance. This becomes highly important when primates are used because of the cost of the animals themselves and of ethical issues surrounding the use of primates. Reuse is less of an advantage with rats, because the practical lifespan of the assay (i.e. catheter patency) is significantly less with rodents.

Initiation procedures ask whether a drug-naïve animal will come to self-administer the test substance without any prior self administration training. If a test substance induces clear self-administration in a drug-naïve animal this constitutes even more compelling evidence of its abuse potential. On the other hand, initiation procedures for the same reason can be considered less sensitive, i.e. less likely to detect self-administration, and are therefore less appropriate for CNS safety pharmacology. Because there typically no positive control in this type of initiation study, the failure to establish self administration responding in naïve animals does not constitute adequate evidence for concluding there is no abuse potential.

Another important variant in self-administration procedures is the animal species used. The most frequently used animals are rats and non-human primates, usually macaques but also new world monkeys (capuchin, squirrel, marmoset) and baboons. More recent publications have described rapid self-administration procedures in the mouse (Caine et al. 2002) with consequent reductions in cost. There is no evidence that the data obtained between different species differ qualitatively in terms of the kinds of drugs showing positive reinforcing properties. On the other hand, primates, and in particular the macaque, are the species preferred by the drug registration authorities mainly because of their greater similarity to humans in terms of active doses and pharmacokinetics and because of the extensive literature on the behavioral effects of CNS-acting drugs in this species.

Analysis of self-administration behavior is mainly qualitative, indicating whether or not a test substance is a positive reinforcer in self-administration studies. Although there are clear differences between drug classes and even between individual animals, in the number of infusions taken during a test session, the number of infusions per session might not provide a clear reflection of the reinforcing effectiveness of a test substance because ongoing behavior can be affected by repeated drug delivery, potentially leading to suppression of responding or even lethality. The pharmacokinetic profile of a test substance is a major determinant of whether or not reinforcing effects are observed. In general, rapid onset drugs are more reinforcing than slow onset drugs. The number of reinforcers received throughout a test session also reflects overall reinforcing effectiveness. In general, long duration drugs can accumulate through the session, suppress responding and, thereby, appear to be less reinforcing than might be the case. One alternative to the typical repeated dosing self-administration procedure is a second-order schedule of self administration (e.g., Negus and Mello 2004). Under this schedule, non-drug stimuli (e.g., lights) that have been paired with drug delivery are presented under a schedule

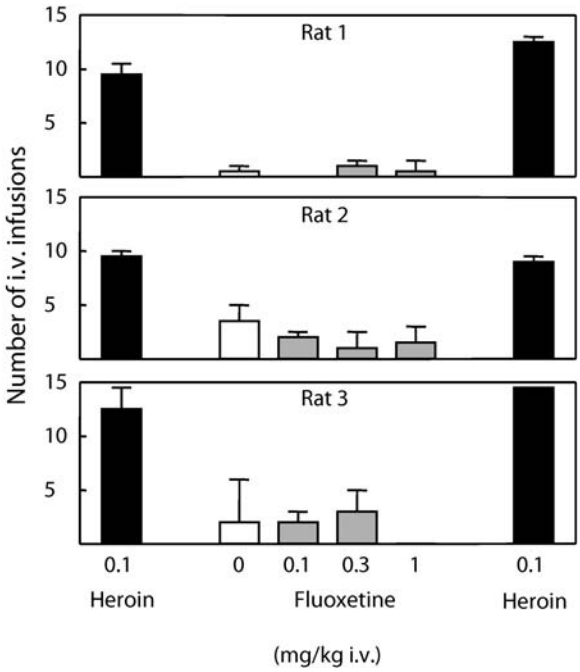

Fig. 24. Effects of heroin and fluoxetine in a self-administration procedure in the rat. Heroin induces clear self-administration at the beginning and the end of the experiment, in contrast to saline which induces a low level of self-administration. Fluoxetine, tested in rats showing clear self-administration of heroin, does not induce self-administration rates different from saline (absence of abuse potential). Note the clear differences between the rats in the number of self-administrations of heroin or saline.

of reinforcement throughout the session and only a single drug injection is administered at the end of the session. Thus, behavior throughout a session is not affected directly by drug delivery since drug is administered only once, at the end of the session. Second-order schedules have not been used widely in abuse potential studies, perhaps because they require extensive training and because behavior under these schedules tends to respond less rapidly to changes in dosing conditions.

Another indicator of the reinforcing effectiveness of a test substance can be the amount of work an animal will perform to obtain the test substance. This can be investigated with a progressive ratio schedule whereby the response requirement increases for every infusion (Solinas et al. 2004) or after a fixed period of time (Wilcox et al. 2000) until the animal ceases to lever-press for a pre-determined interval, defined as the breaking point. The presumption of progressive ratio studies is that the higher the breaking point the greater the reinforcing effectiveness, although the same potential complications regarding pharmacokinetics that were noted above for simple (FR) schedule of self administration also apply to progressive ratio schedules. Breaking point studies are usually undertaken as a supplementary investigation after a test substance has demonstrated positive reinforcing effects under other conditions.

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Chapter I.D
Methods in Cardiovascular Safety Pharmacology

Brian D. Guth

I.D.1 Background

The inclusion of pharmacological studies in the safety evaluation of new drugs is a well established practice (Zbinden 1966; Alder and Zbinden 1973). These studies contribute to the pharmacological profiles of possible new drugs and provide data that can be used for optimization of compounds and ultimate selection of compounds for clinical development. The emergence of safety pharmacology as a specialty area distinct from toxicology was facilitated by the appearance of the ICH S7A guideline in which the rationale for safety pharmacology studies was laid out and study types were defined (The European Agency for the Evaluation of Medicinal Products. Human

Medicine Evaluation Unit 2000). However, one topic in particular was instrumental in focusing attention on safety pharmacology studies, namely the concern about drugs causing severe ventricular arrhythmias, including torsade de pointes and, in some cases, sudden death. The association of this type of arrhythmia with the prolongation of ventricular repolarization, as seen with a prolongation of the QT interval of the electrocardiogram (Weissenburger 1993), identified a biomarker that can be best tested by safety pharmacological studies, as opposed to traditional toxicological studies. Regulatory authorities, led by the Committee for Proprietary Medical Products (The European Agency for the Evaluation of Medicinal Products. Human Medicines Evaluation Unit. Committee for Proprietary Medicinal Products. 1997), pointed out the need to assess the risk of new drugs for causing QT prolongation (as predictor of proarrhythmic activity) by conducting appropriate non clinical in vitro and in vivo studies. Interestingly, this particular topic was not fully addressed in the ICH S7A guideline on safety pharmacology, due to the difficulty in defining a generally acceptable study approach in this evolving specialty area. Instead, a secondary guideline (ICH S7B) has been prepared to describe preclinical studies for assessing the risk of arrhythmia due to prolongation of ventricular repolarization. Thus, while the cardiovascular safety pharmacological assessment has been in the forefront of regulatory interest, this field is still evolving. One must not forget, however, that the purpose of conducting cardiovascular safety pharmacology studies is not just to define a specific proarrhythmic risk, but to examine potential effects on the peripheral vasculature, the heart, or any other effect that may secondarily lead to an activation or depression of cardiovascular performance.

Numerous strategies for the conduct of cardiovascular safety studies have been suggested (Pourrias et al. 1999; Lacroix and Provost 2000; Champeroux et al. 2000; Gralinski 2000; Kinter and Valentin 2002; Guth et al. 2004) based on the anticipated regulatory requirements as well as the needs for efficient drug development. This chapter will present the methodologies common to most of these strategies.

I.D.1.1 General Considerations

The ICHS7A guidance on safety pharmacological studies for human pharmaceuticals (The European Agency for the Evaluation of Medicinal Products. Human Medicine Evaluation Unit 2000) states a preference for in vivo studies using experimental models with unanesthetized animals. This is a major deviation from previous general practice, based on the recommendations of the Japanese Guidelines for Nonclinical Studies of Drugs Manual (Yakuji Nippo Limited, Tokyo 1995) in which it was stated that anesthetized animal preparations are typically used for cardiovascular safety evaluations. The new regulatory preference for the use of conscious animals is based on the complicating effects that anesthetic agents can have on the cardiovascular system. For example, pentobarbital anesthesia is known to have direct effects on ventricular repolarization (Bachmann et al. 2002). Furthermore, non-optimized anesthesia can lead to non-physiological conditions that also may mask drug-induced effects. An example of this is the tachycardia often seen in pentobarbital-anesthetized animals. Thus, experimental models using conscious animals for measuring cardiovascular parameters will be highlighted. However, due to their great utility when carefully performed, cardiovascular models using anesthetized animals should still be included in the cardiovascular safety pharmacology portfolio and will therefore be addressed.

Conscious versus Anesthetized Animal Use

Despite the theoretical advantages, the use of conscious animals brings with it new considerations and, quite frankly difficulties, for an effective study design. For example, the cardiovascular system of a conscious animal, as compared to an anesthetized animal is much more variable, as the animal reacts to its environment. Subtle, drug-induced effects may not be readily apparent in animals that are reacting to external visual or acoustic stimuli in the laboratory environment. Furthermore, drug-induced central nervous system effects, or any other drug-induced effect that impacts the animals' well-being (e.g. anxiety, gastrointestinal disturbances, etc.) may result in a secondary effects on the cardiovascular system. These aspects can introduce a substantial amount of variability in the cardiovascular parameters measured such as heart rate. Given the reliance of the QT interval duration on heart rate, a variable heart rate can be a challenge for analysis and the assessment of proarrhythmic potential due to delayed ventricular repolarization. With the use of conscious animals, the amount of training needed to achieve the desired basal physiological conditions is associated with increased time, effort and consequently expense. Therefore, despite the preference for conscious animal studies for the safety pharmacological evaluation of new drugs, including

the cardiovascular system, these models are complex and need a considerable amount of time and effort to conduct them optimally. Models using anesthetized animals are usually simpler and highly stable models, in which a variety of invasive measurements (e.g. cardiac output, ventricular function, regional blood flow, etc.) can be made that are not easily accessible in the conscious animal. Thus, there still appears to be an important role for the use of anesthetized animals particularly in the study of cardiovascular effects of drugs such that this type of model is still included in this text.

Animal Reuse and Dose Selection

The use of conscious animals for conducting cardiovascular safety pharmacology studies introduces the possibility of reusing animals. Techniques are available for obtaining the basic cardiovascular data (heart rate, arterial blood pressure, ECG, and even left ventricular pressure and dP/dt) without affecting the health status of the animal and without the need to euthanize the animal at the end of the study. The possible reuse of animals is affected by the doses of test article selected for use in safety pharmacology studies. The ICHS7A guideline states that doses should include and exceed the therapeutic range with the goal of defining the dose-response relationship of any adverse effect observed. In practice, studies are typically designed to include therapeutically relevant doses of the test article and predefined multiples based on the expected side effect profile. This is also influenced by the intended therapeutic indication. The dose should be related to the actual drug exposure in terms of the plasma drug concentration. Since drug-induced effects are most likely to coincide with the peak plasma drug concentration reached, the Cmax becomes the most relevant pharmacokinetic parameter for safety pharmacological assessments. The duration of the exposure is of interest to correlate to the reversibility of a given effect. As an example, a maximal dose could be selected to achieve a 30-fold higher plasma drug level, than therapeutically needed, for a non-life threatening clinical indication. Alternatively, one may target a lesser maximal plasma drug level for compounds used to treat a life threatening disease, where side effects may be acceptable. Additionally, for cytotoxic agents one should seriously consider the use of an anesthetized animal model in which higher doses can be given without the fear of causing distress and in which the animal is euthanized after the study. The study design in conscious animals should avoid discomfort or pain for the animals used, as emphasized in the ICH S7A guideline. Furthermore, safety pharmacology studies are usually single administration studies in which effects, when seen, are usually fully reversible such that, after an appropriate washout of the test article, the animals are suitable for use in further studies. This results in a reduction in the number of animals needed for conducting safety pharmacology studies but does introduce the need to consider how to best monitor the health status of the animals to qualify them for further use.

Species Selection

The ICH S7A guideline proposes the use of relevant animal models without explicitly stating which animal species are most appropriate for use. For the cardiovascular evaluation of new drugs the dog has been the preferred species in the past and there is a wealth of comparative data available from studies performed using the dog (Gralinski 2003). As such, it is likely that the dog will remain the preferred species for such studies despite ethical pressure to limit the use of companion animals for drug testing. Use of the dog may be of particular advantage when the dog is also the species used for toxicological studies. Nevertheless, other animal species including the pig and non human primates appear to be appropriate models for cardiovascular safety pharmacology studies.

In contrast, the rat is not the best species for conducting cardiovascular safety pharmacology studies. The evaluation of the electrocardiogram is an essential element of a safety pharmacology cardiovascular assessment. The rat is not a suitable species for testing the effects of a drug on ventricular repolarization since its repolarization is not dependent upon I_{Kr} and I_{Ks}, as in larger mammalian species, but rather on I_{to}. Despite this limitation, the rat still is an attractive cardiovascular model for detecting effects on arterial blood pressure and heart rate, or even other types of arrhythmia. Technology is available for studying rats in the conscious state and they may also be reused, as mentioned above in conjunction with dogs or other larger animals. The use of the rat as a cardiovascular model is probably best suited for early pharmacological testing of drug candidates and may provide useful data for lead optimization at a time when amounts of a given test article are not adequate to perform studies in the larger species used as cardiovascular models. Finally, since the rat is still a standard species for toxicological studies, there is interest in knowing the cardiovascular effects of a test article in that species. Therefore, the conscious rat model is included

in this text as being a useful cardiovascular safety pharmacology model.

In vitro Studies for Electrophysiological Effects

The reason the ICH S7A guideline was unable to address the risk assessment for proarrhythmia associated with QT interval prolongation was that there was no consensus as to how to best approach this assessment experimentally. Indeed, even with the appearance of the ICH S7B guidance, there is still much controversy as to what types of non clinical studies are most useful in predicting the clinical risk of QT-based arrhythmia. Several study types have emerged as being the most frequently used and appear to be routinely requested by regulatory authorities. There is now a clear association between drug-induced QT prolongation and arrhythmia and the blockade of myocardial I_{Kr}. This suggests the measurement of drug effects on I_{Kr} or a model mimicking I_{Kr} in studies in vitro. Thus, the minimal requirement appears to be two study types: 1) effects on I_{Kr}, the specific myocardial membrane current thought to be mechanistically responsible for most drug-induced QT prolongation and 2) effects in vivo on the QT interval of the electrocardiogram, the currently available biomarker for proarrhythmic risk.

The CPMP "points to consider" paper of 1997 (The European Agency for the Evaluation of Medicinal Products. Human Medicines Evaluation Unit. Committee for Proprietary Medicinal Products 1997) pointed out that a thorough electrophysiological characterization of a drug requires evaluation on multiple levels of physiological complexity. In particular, the assessment of the effects of a drug on the myocardial action potential is an important link between the possible effects on a single ion channel type (such as I_{Kr}) and the consequent effects seen in the electrocardiogram (such as QT prolongation). Models using isolated purkinje fibers, papillary muscles, myocardial wedge preparations or even isolated hearts were proposed to allow one to assess the effects of a drug on the integrated electrophysiology of the heart. These types of models have been shown to detect the action potential prolonging effects of many of the known proarrhythmic agents. However, comparative evaluations, most notably that organized by ILSI (International Life Sciences Institute), provided data suggesting that not all of the know proarrhymic agents produced effects that could be easily detected in the dog Purkinje fiber model. It was then suggested that the lack of sensitivity should lead to dropping this model as a mandatory study type. Nevertheless, the usefulness of this type of model is still acknowledged and these studies may play an important role for early drug discovery to provide a more thorough risk assessment at a time when in vivo studies are still not feasible due the lack of adequate amounts of a test article. Thus, both in vitro study types (I_{Kr} and similar models, and the action potential models) are included in this text although the action potential models appear not to be an absolute regulatory requirement.

Models of Repolarization-Dependent Arrhythmia

Perhaps the most convincing evidence that a given drug can be proarrhythmic would be to show a dose-dependent incidence of a torsade de pointe-like arrhythmia in an animal model. Indeed, the ICH S7B guidance also acknowledges the theoretical interest in the use of this type of model. However, due to the complexity of both these experimental models and their unclear relationship to a proarrhythmic activity clinically, there is no requirement to provide such data at present. Rather, there is simply the call for a further development of these types of models to determine their predictive value for risk in humans. There are a few models that appear to be useful, should one choose to evaluate proarrhythmic action of a drug directly and these will be presented in this text. Whereas these models are not considered to be part of the basic cardiovascular safety pharmacology evaluation of a drug, they may be useful as follow-up studies (see below).

Follow-Up Cardiovascular Safety Pharmacology Studies

Follow-up studies are those that provide a greater depth of understanding and thereby augment the core battery safety pharmacology studies. The need for follow-up studies for cardiovascular effects and the specific study design of such studies must therefore be considered on a case-by-case basis. Due to the complexity of the cardiovascular system and the multiple possible pharmacological factors that can affect cardiovascular endpoints, the list of possible cardiovascular follow-up studies is almost limitless. Nevertheless, several experimental models are included in this text that have proven of use in past experience for the in depth profiling of drugs. This is not, however, meant to be an exhaustive list.

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I.D.2
In vivo Experimental Models for Cardiovascular Safety Pharmacology

I.D.2.1
Cardiovascular Safety Studies in Conscious Dogs and Other Species

PURPOSE AND RATIONALE

The preferred model for performing safety pharmacology studies on the cardiovascular system according to the ICH S7A guideline is the conscious animal under unstressed, physiological conditions (The European Agency for the Evaluation of Medicinal Products. Human Medicine Evaluation Unit 2000). This includes the evaluation of drug-induced effects on systemic arterial hemodynamics and the electrocardiogram. Important cardiovascular parameters are, however, only accessible through invasive measurement techniques that can interfere with the physiological status of the animal subject. Telemetry-based systems allow one to monitor hemodynamic parameters including heart rate, arterial blood pressure the ECG (and, with some systems, left ventricular blood pressure and left ventricular dP/dt) with the animals in their home cage thereby serving to reduce the stress associated with the collection of data. It should be noted that the use of telemetry is not essential for the performance of this type of study. For example, animals can be trained to the laboratory environment such that they can be examined using light restraint (e.g. sling or chair) under physiological conditions.

PROCEDURE

There are two commonly used full-implant telemetry systems employed for this type of study (Data Science International (DSI), Arden Hills, MN, USA and Integrated Telemetry Services (ITS), Pinkney, MI, USA). They typically measure arterial blood pressure and a single electrocardiogram, but they can also be configured for the measurement of left ventricular pressure or multiple ECG leads. A single ECG lead with clearly definable wave forms is usually sufficient for detecting drug-induced effects, however. Beagles are frequently used for these studies but larger dogs have the advantage of a simplified implantation procedure and, in general, lower heart rates at the time of the subsequent studies. There is some evidence that drug-induced effects on the QT interval duration are more pronounced in females. For the dog this difference, if present, appears to be very small and can be disregarded such that animals of both sexes can be included in these studies. All animals should be adequately trained for adaptation to their home cage and laboratory environment prior to their being instrumented.

Instrumentation

The transducers of the telemetry implant are calibrated prior to implantation and the unit is sterilized using a low pressure ethylene oxide process.

Dogs are anaesthetized with a combination of Rompun (xylazine hydrochloride, 1 ml/10 kg, i.v.) and Ketavet (ketamine hydrochloride, 0.7 ml/10 kg, i.v.) after premedication with Temgesic (Buprenorphin, 0.2 mg/kg i.m.) and ventilated with 66 % N_2O and 33 % O_2 and 1 % Isofluorane. All procedures are performed under aseptic conditions using sterilized equipment.

For this example, the implantation of a T27 unit from ITS is described. The dog is placed in a lateral recumbancy with the left side facing the surgeon. An incision is made in the 5th intercostal space, beginning from the dorsal tip of the scapula for approximately 20 cm. The latissimus dorsi, serratus dorsalis and iliocostalis thoracis muscles are divided using a scalpel. The incisions are then covered with saline-soaked gauze. The battery and transmitter of the implant are positioned by making an 8 cm incision right-angled on an imaginary line between the sternum and the end of the costa fluctuans. A small pocket is opened between the external and internal oblique abdominal muscle layers, just large enough to place the battery (dorsal) and the transmitter (ventral) inside this "pocket". The pocket is closed using sutures of 2–0 vicryl (V517H) stitches. A pocket is opened for the switch-on antenna that is placed under the skin. The cables with both pressure transducers and ECG leads extending from the ventrally implanted transmitter are guided subcutaneously to the lateral incision. The antenna used to send the telemetry signals is guided subcutaneously from the ventral transmitter location dorsally towards the spine and then runs parallel to the spine for ~25 cm. The distal end is fixed in place with a suture. The small incisions required to place the antenna are then closed and the initial ventral incisions required for battery and transmitter placement are closed.

The aortic pressure transducer is implanted next. The intercostal muscles are incised. A rib retractor is then inserted and gently and gradually opened to give the required access. Lung lobes are packed away with saline-soaked gauze to expose the aorta. The 5 cm segment of the descending aorta is then isolated to allow implantation of the aortic pressure transducer. The isolated segment is partly clamped proximally and distally to maintain blood flow underneath the clamp. A micro scalpel is used to make a hole in the aortic wall and the pressure transducer is introduced via this hole into the aorta using a Russian forceps. The transducer is sutured into place and blood flow is restored.

The left ventricular pressure transducer is then implanted. The pericardium is opened so that the apex of the heart is exposed. The heart is gently held in one hand and two stitches in the ventricular wall for fixation are made. A purse-string suture is made around the apex. A sharp spike is then placed into the apex of the left ventricle and the transducer is placed into the ventricle and sutured firmly in place.

The lung is then inflated and the intercostal muscles are sutured closed. The ECG lead is then fixed close to the sternum in the sixth intercostal space. The muscle layers are then closed and the lungs are inflated once more to displace all residual air. The skin is then closed.

The gas anesthesia is then turned off, all incisions are treated with topical antibiotics and dressed, and dogs are allowed to wake up. Animals are extubated when they can swallow. Analgesics and antibiotics (Temgesic and Tardomycel, Benzylpenicillin-benzatin and Benzylpenicillin-procain) are administered for 7 days following the procedure. Dogs are allowed to recover for 14–21 days before experiments using test substances are initiated.

Studies are conducted with the animals in their home cages that have been equipped with antennae to pick up the transmitted physiological signals. Drugs can be treated orally, intravenously, subcutaneously or inhalatively (Markert et al. 2004). After treatment, dogs are returned to their home cages for the duration of the study that can last up to 24 hours if needed. This is usually based on the duration of the expected drug exposure or possible pharmacological activity of the test article. Longer studies should allow for feeding of the animal and water should be available ad libidum.

Experimental Design

Studies can be conducted as group comparisons if sufficient numbers of animals are available. Alternatively, the use of a latin square cross-over experimental design allows for studies with fewer animals (eg. N = 4).

The experiment starts after an equilibrium period of 60–120 min to allow the dogs to acclimate to the measurement pens. The administration of the test compound is started after a 30–45 min control period. Experiments involving intravenous infusions or frequent blood sampling are performed in smaller cages to allow close monitoring of indwelling catheters. Continuous measurements should not, however, exceed 6–8 hours without allowing for a short pause for exercise. Continuous measurements for up to 24 hours are acceptable if the animals can be kept in larger cages.

EVALUATION

The hemodynamic and ECG parameters include: systolic, diastolic and mean aortic pressure, peak systolic and end-diastolic left ventricular pressure, LV dP/dt max and dP/dt min, heart rate; PQ-, QRS- and QT intervals. NOTOCORD-software (or equivalent) is used for acquisition of data whereas EXCEL (or equivalent) is used for data analysis. Data are summarized at predefined time points by calculating median values+ SD. Whereas all physiological parameters are routinely averaged over predefined time intervals, it has been proposed that for the ECG only a few beats

are required to detect effects (Hamlin et al. 2004). Of particular interest are time points corresponding to the time of Cmax.

Correction of QT Interval for Heart Rate

The QT interval duration is heart rate dependent. The use of correction formulae derived from clinical data are not appropriate for use with dog ECGs. Algorhythms designed specifically for the dog are required and historical data from the dogs actually used in a given study is the preferred way to derive any QT correction (Meyners and Markert, 2004). Still better, if no drug-induced effect on heart rate is observed, no correction of the QT interval should be undertaken.

Drug Exposure

It is essential to relate any drug-induced effect to the plasma drug levels achieved. It is possible to take blood samples during this type of study, but it must be accepted that this disrupts the hemodynamic status of the animals for up to 30 min. An alternative approach is to conduct pharmacokinetic studies in the same animals (or other similar animals) on another day.

Animal Reuse

Animals instrumented for this type of study may be reused. Since the instrumentation is fully implanted without externalization of wires or catheters, there is little risk of developing sepsis after successful implantation. The batteries are designed to provide long life well in excess of a year. After the completion of a given study and an appropriate washout time, further studies can be conducted. This is facilitated by the fact that studies are typically single administration studies using doses that are not intended to cause irreversible effects. The qualification of animals for subsequent use should be based on the health status of the animals. Given the ease of obtaining hemodynamic data from these animals, one can maintain historical data on heart rate and blood pressure, parameters that are sensitive to the overall well-being of the animal. Additionally, clinical chemistry parameters can be monitored to detect possible effects on kidney and liver function.

MODIFICATIONS OF THE METHOD

Species

Similar telemetry-based cardiovascular safety pharmacology studies can be conducted in pigs and monkeys (normally macaca mulatta (Rhesus) or macaca fascicularis (Cynomulgus)). These two alternative species are of interest due to their use in toxicology studies where the dog is deemed inappropriate. Whereas rats are also used in toxicological studies, they are not appropriate for use in cardiovascular safety pharmacology studies since they are not sensitive to drug-induced effects on ventricular repolarization dependent upon a blockade of the myocardial rectifying current I_{Kr}. Rats may nevertheless prove useful for detecting hemodynamic effects of drugs, particularly early in drug research when amounts of test article may be limited (see below).

The guinea pig has been shown to demonstrate drug-induced effects on the QT interval duration (Hamlin et al. 2003) and is suitable for use with telemetry-based systems. However, their intolerance of arterial catheters limits their use for simultaneously measuring arterial blood pressure.

Critique of the Method

The telemetric assessment of cardiovascular effects of drugs is considered the gold standard model for safety pharmacology studies. Animals can be studied in optimized, physiological conditions with neither anesthesia-dependent effects nor with the possible interference from other external influences. This is thought to best mimic a clinical setting and thereby best demonstrate possible drug-induced effects. The reuse of animals instrumented for the telemetric assessment of cardiovascular parameters also allows for a reduction in the numbers of animals needed for such studies. The disadvantages of this experimental approach relate primarily to the costs for establishing and maintaining such a system, as well as the dedicated laboratory space required. Furthermore, a high level of training is required particularly for the successful implantation of the telemetric equipment. Do to the fact that data can be collected continuously for long periods of time, attention has to be paid to the manner in which the collected data is collected, reduced, managed and statistically evaluated.

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I.D.2.2 Cardiovascular Safety Pharmacology Studies in Anesthetized Dogs and Other Species

PURPOSE AND RATIONALE

The basic parameters needed for a safety pharmacology evaluation of possible cardiovascular effects of drugs includes heart rate, arterial blood pressure and the electrocardiogram. These parameters can also be measured in anesthetized dogs or other appropriate animal species. One must recognize, however, the potential influence of the anesthesia on the parameters measured. The use of an anesthetized animal allows for more invasive techniques to be applied, thereby giving a more in-depth evaluation of possible drug effects on heart and vascular function in a variety of perfusion beds. A well-performed study using an anesthetized animal model is characterized by a highly stable hemodynamic state with very low variability of the measured parameters. This translates into a high sensitivity for detecting possible drug-induced effects. The oral route is, however, not accessible in the anesthetized animals although intraduodenal administration may provide a reasonable alternative administration route if intravenous administration is not feasible or desirable.

Measurements possible in this model include end-diastolic and systolic pressure of the left ventricle, contractility of the heart (usually using peak positive LVdP/dt or LVdP/dt at a developed pressure of 40 mm Hg), heart rate, cardiac output and arterial blood flow in a given local perfusion bed. Test compounds can be classified in terms of a variety of pharmacological actions:

- Positive and negative inotropic effects
- Arrhythmogenic effects
- Hyper- or hypotensive effects
- Tachycardic or bradycardic effects

In summary, despite the preference for studies in conscious animals stated in the ICHS7A guideline, the use of anesthetized animals for assessing cardiovascular effects of drugs is a valid and useful approach with a variety of practical advantages in comparison to conscious animals.

PROCEDURE

Male or female dogs weighing between 15 and 25 kg are used. Various anesthesia regimens are appropriate for conducting this type of study including injectable anesthetics (e.g. pentobarbital, alpha-chloralose) as well as inhalative anesthetics (e.g. isoflurane). Animals are ventilated mechanically and blood gas parameters should be monitored to confirm appropriate ventilation rate and depth. Venous and arterial catheters are placed either in the neck (A. carotis, V. jugularis) or groin (A. femoralis, V. femoralis) for vascular access and the measurement of arterial blood pressure (using external pressure transducer), blood sampling and intravenous administration of test compounds.

Left ventricular pressure can be measured by use of a catheter-tip micromanometer placed in the left ventricle through the carotid artery. Alternatively left ventricular pressure can be measured with a pressure transducer placed directly into the ventricle through the apex and secured with a purse-string suture; this approach requires a thoracotomy. The use of a solid state manometer, as opposed to a fluid-filled catheter is essential for the measurement of left ventricular pressure when one also wants to assess myocardial contractility using LV dP/dt, the derivative of the left ventricular pressure signal. Fluid-filled catheter systems do not have a frequency response capable of capturing subtle changes in LV dP/dt and give damped measurements.

Cardiac output can be measured using dye- or thermal-dilution techniques or by placement of an electromagnetic flow probe around the pulmonary artery. Insertion of a pressure transducer or fluid-filled catheter into the pulmonary artery and pulmonary vein allows for the calculation of pulmonary vascular resistance. The measurement of flow through the proximal aorta may also be useful but does not include coronary blood flow and as such is not equivalent to

total cardiac output. Selective arterial blood flow can be measured using either electromagnetic or Doppler flow probes around the vessel of interest. Commonly measured regional flows include coronary (circumflex or anterior descending arteries), femoral artery or renal artery blood flow.

The electrocardiogram can be obtaining using standard limb leads and/or precordial leads. A lead should be selected that is stable over time and that has a sharp demarcation at the end of the T wave to facilitate the measurement of the QT interval duration. One can also position a monophasic action potential electrode catheter through the femoral or carotid artery to obtain endocardial monophasic action potentials (see below Modification of the Method).

Experimental Course

It is imperative to allow sufficient time after the instrumentation is complete to achieve a stable, steady-state hemodynamic condition prior to administering a test compound. Continuous monitoring of arterial blood pressure and heart rate should demonstrate no changes in the parameters measured for at least 20 minutes. A test article can then be applied by the chosen route (intravenous, intraduodenal, inhalative) in doses appropriate for the compound. Sufficient time is then allowed for the development of any hemodynamic effects, if any (usually at least an additional 20–30 min), and further higher doses of the test article can then be administered. It is advisable to take blood samples at the end of each dosing interval for the determination of drug levels. The overall duration of the study can last for hours if care is taken to keep the animal's body temperature in a normal range and to replace fluids through the intravenous administration of warmed saline or other suitable volume replacement. Whereas possible drug induced effects can be assessed by comparing data at each dosing interval to the pretreatment measurements, it is advisable to include a vehicle-treatment arm in which animals are treated identically but with an equal volume of the vehicle used.

Parameters Measured

The following parameters should be measured in all studies:

1. Heart rate (HR, beats/min)
2. Arterial blood pressure (BP, diastolic and systolic, mean) (mm Hg)
3. Electrocardiogram (PR interval, QRS duration, QT interval) (ms).

If used, the following additional measurements can yield additional insight into possible drug actions:

1. Left ventricular end-diastolic (LVEDP) and peak systolic pressure (mm Hg)
2. LV dP/dt or LV dP/dt_{40} (mm Hg/s)
3. Cardiac output (CO, ml/min)
4. Regional arterial blood flow (ml/min).

Other parameters can be calculated from those measured including:

1. Stroke volume (SV = CO/HR) (ml)
2. Total peripheral resistance (TPR = BPm/CO × 79.9) (dyn s/cm^5)
3. Left ventricular stroke work (LVSW = (BPm–LVEDP) × SV × 0.333 × 10^{-3})
4. Left ventricular minute work (LVMW = LVSW × HR).

Studies are typically conducted with at least 4 animals per treatment group (active vs vehicle). Statistical analysis can be done using a analysis of variance for repeated measures.

Critique of the Method

Until recently, this experimental approach was the most common for studying drug-induced effects on the cardiovascular system and was specifically mentioned in the Japanese guidelines for general pharmacology as the standard test. As such, there is a vast amount of experience with this type of study and a large amount of comparable data available. The primary reason why anesthetized animal models lost their primary role in pharmacological studies was due to the recognition of the possible effects of particularly pentobarbital anesthesia on ventricular repolarization and therefore on drug-induced effects on the measured QT interval duration (Bachmann et al. 2002; Weissenburger et al. 2000). Nevertheless, a well performed study using anesthetized animals, as supported recently by the Japanese PRODACT investigators, can provide a useful and sensitive model of detecting drug-induced effects on the QT interval duration. Furthermore, the utility of this model for providing a sensitive model for detecting effects on arterial blood pressure, heart rate and ventricular contractility is well established.

MODIFICATIONS OF THE METHOD

Pigs (juvenile farm pigs or minipigs) may be used for this type of study and is an attractive alternative if the dog is not suitable for any reason. Adult domestic pigs are difficult to use due to their size, such that if

adult animals are preferred, one of several breeds of mini- or micropigs may be used. The induction of anesthesia is different than with the dog and typically an intramuscular sedative is administered first (e.g. ketamine) followed by the anesthesia used for the remainder of the study. Halothane anesthesia should not be used in pigs due to a high incidence of hyperthermic reactions.

Hey et al. (1996) analyzed the ECG wave in anesthetized guinea pigs to determine QT interval, QTc interval, PR interval QRS interval and heart rate after the administration of the second generation antihistamines ebastine and terfenadine. In separate studies in conscious guinea pigs, the effect of oral ketoconazole on the ECG parameters after oral ebastine and terfenadine was studied.

Measurement of the monophasic action potential (MAP) in anesthetized dogs for evaluation of arrhythmogenic activity of drugs was recommended by Usui et al. (1998), Weissenburger et al. (2000).

A quad-polar electrode catheter is inserted through the left femoral artery and positioned at the noncoronary cup of the aortic valve to record a His bundle electrogram. A bidirectional steerable monophasic action potential (MAP) recording/pacing combination catheter is inserted through the left femoral vein and positioned at the endocardium to obtain MAP signals. The signals are amplified with a DC amplifier.

The amplitude of MAP is measured as the distance from the diastolic baseline to the crest of the MAP plateau phase. The duration of the MAP signal is measured as an interval from the MAP upstroke to the desired repolarization level along a line horizontal to the diastolic baseline. The interval [ms] at 90 % repolarization is defined as MAP_{90}. The heart is driven electrically through the pacing electrodes of the combination catheter. Stimulation pulses are rectangular in shape, about twice the threshold voltage (1.5–2.2 V) and of 1 ms duration. The MAP_{90} is measured during sinus rhythm (MAP_{90}(sinus)), and at a pacing cycle length of 400 ms (MAP_{90}(400)) and 300 ms (MAP_{90}(300)).

The effective refractory period (ERP) of the right ventricle is assessed with a programmed electrical stimulator. The pacing protocol consists of 8 beats of basal stimuli in a cycle length of 400 ms followed by an extra stimulus of various coupling intervals. Starting in late diastole, the coupling interval is shortened by 5–10 ms steps until refractoriness occurs. The difference ERP–MAP_{90}(400) is calculated to predict the vulnerability of the myocardium.

The amplified MAP signals together with systemic blood pressure, left ventricular pressure, heart rate and ECG are continuously monitored using a polygraph system. Each value of ECG and MAP should represent the mean of at least three consecutive complexes.

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I.D.2.3 Cardiovascular General Pharmacology Studies in Conscious Rats

PURPOSE AND RATIONALE

There is a clear preference for performing safety pharmacology studies for drug effects on the cardiovascular system in a non-rodent species in order to capture possible effects on ventricular repolarization, effects that have received great attention recently from regulatory agencies world-wide. Nevertheless, it must be acknowledged that there may be great value in conducting studies to examine drug effects on the cardiovascular system in particularly rats. One reason is that toxicity studies are conducted in rats and drug effects on cardiovascular parameters in rats is of relevance to such studies. Furthermore, cardiovascular studies in non-rodents (usually the dog) require substantially more compound than studies in rats. Therefore, as part of the lead optimization, selection and development process, cardiovascular studies in rats can be invaluable.

Telemetry System

Cardiovascular parameters such as arterial blood pressure and heart rate can be measured in conscious, free-moving rats using commercially available radio telemetry systems (Deveney et al. 1998). Such systems

include: 1) an implantable transmitter with battery capable of sending the pressure signal from a fluid-filled catheter implanted into the abdominal aorta, 2) a receiver unit that detects the transmitted signal and converts it into a digital format, 3) a pressure reference module that adjusts the measured aortic pressure for atmospheric pressure, and 4) data acquisition software for data storage and computation.

The rats are anesthetized and undergo an aseptic procedure to implant the aortic catheter distal to the renal arteries. The catheter is secured using cyanoacrylate glue. The transmitter and battery unit are placed in the abdomen and attached to the abdominal musculature using a suture. After closure of the surgical wound, the animals are giving sufficient time to recover from the procedure before being used in a study, usually at least one week.

Experimental Procedure

Rats can be studied while still in their home cage. However, since the transmitter frequency used is the same for all animals, they must be held alone during the study. This means that in most cases the rats are moved from multiple housing cages to single cages for the duration of a study. The cages are placed directly on the receiver units to provide a close contact between the receiver and the animal. The transmitters are turned on before starting the study; the transmitter units can be turned on and off using a magnet thereby lengthening battery life. A control period of up to an hour allows the animals to acclimate to the environment and to provide a steady state prior to administration of a test article. The oral administration of compounds is most convenient in rats, using gavage, but intravenous and intraperitoneal administration is also feasible. Inhalative administration of compounds is also possible by nebulizing the compound into a small exposure box or through the use of an application system bringing the nebulized compound to the nose of the animal. The initial 15–30 min following the administration of a compound is affected by the excitement inherent with the administration process. Usually within a half hour the animals have returned to a relaxed hemodynamic steady state.

Study Design

Studies can be conducted with a group comparison or with cross-over design. Individual parameters can be averaged over predefined time periods but it may not always be possible to predict the time of drug-induced effects. Also, in freely moving animals, drug-induced changes may not be continuous but there may be overall changes that may be missed if one assesses only a short time periods. An alternative approach is to calculate areas under the parameter-time curve and test for drug-induced effects. In this way, the analysis is not restricted to a given time point. With cross-over design an appropriately long washout phase is needed between treatments. A treatment arm with vehicle is also useful to demonstrate the stability of the system. Measurements can be made continuously or at regular intervals as long as desired. Water is usually provided ad libidum and food can be given should the measurements be needed over an extended time period.

Instrumented animals may be reused after appropriate washout periods on the assumption that the compounds in the doses tested cause no reversible damage. Since the cardiovascular parameters measured are sensitive to the overall well being of the animals, changes in baseline conditions should be examined for detecting possible compound-related toxicities. Assuming such toxicities are not observed, animals can be maintained for over one year with appropriate care.

MODIFICATIONS OF THE METHOD

Due to the fact that whole body plethysmographs for rats are constructed out of plexiglass, they can be used in conjunction with telemetry systems without interfering with the transmitted signals. Therefore, this offers the opportunity to combine studies for determining cardiovascular function using telemetry, with whole body plethysmograph studies for simultaneously measuring respiratory function (Schierok et al. 2000).

Critique of the Method

The most important limitation of the rat as cardiovascular model for safety pharmacology studies is its lack of utility for detecting ventricular repolarization-related drug effects. Whereas this is a substantial limitation, there are nevertheless other drug-induced effects that can be observed in the rat model including effects are arterial blood pressure, heart rate and contractile ventricular function. The rat, due to its rather small size, requires substantially less compound to perform a study in comparison to that what is needed for larger animals. Thus, this model can be used at an earlier time during drug optimization and selection when amounts of a given test compound are limited. One should keep in mind however, that a rat instrumented for telemetric collection of cardiovascular data, if kept for longer periods of time, grows considerably in comparison to the "typical" laboratory rat and can grow to over 800 grams.

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I.D.3 In vitro Cardiovascular Safety Pharmacology Models

I.D.3.1 "High throughput" hERG Assays

PURPOSE AND RATIONALE

The pivotal role of a blockade of the myocardial membrane current I_{Kr} in most instances of drug-induced arrhythmia associated with QT interval duration prolongation is now well established. Recent work has highlighted the K^+ channel encoded by the human ether-à-go-go related gene (hERG) as the molecular target for a wide range of drugs whose administration is associated with an increased risk of an unusual life-threatening form of arrhythmia known as torsade de pointes (Vandenberg et al. 2001). This suggests the need to screen for this activity at an early time in drug development. Indeed, this could be even addressed by screening drug libraries to identify compounds having this potential. Alternatively, one might consider using this activity as an early secondary screen of hits emerging from a first high throughput assay. In either case, experimental approaches are needed that allow the testing of large numbers of compounds thereby necessitating a high degree of automation. Several test systems have emerged as potential high-throughput approaches for detecting activity on I_{Kr}, a current mediated by the channel coded by the KCNH2 gene and known often as the hERG channel. It must be recognized, however, that these relatively new technologies are still in a development stage and have not yet gained acceptance by regulatory authorities. Nevertheless, they are mentioned in this text, at least briefly, since they may still provide useful approaches for early drug discovery. Such techniques have been reviewed and critically assessed previously (Netzer et al. 2001, 2003).

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I.D.3.1.1 Binding Competition Assays

PURPOSE AND RATIONALE

The purpose of this type of study is to determine the competitive binding of a test compound in comparison to a radio-labeled compound known to be a high affinity antagonist of the hERG channel. Radio-labeled, potent blockers of hERG such as [^{3}H]-dofetilide (Finlayson et al. 2001a and 2001b) or [^{35}S]MK-499 (Wang et al. 2003) can be used in conjunction with myocardial cells, stably transfected cell lines or membrane preparations. With this approach, the hERG channels are fully blocked with excess, for example, [^{3}H]-labeled dofetilide. The test compound is then incubated with the hERG-dofetilide system and can compete for the binding sites initially occupied by dofetilide as a function its potency for binding the same site.

Critique of the Method

The test system has relatively low costs and can provide a high throughput. However, compounds that can block hERG-mediated currents but do not compete for the dofetilide binding site will not be detected. Due to the heterogeneity of chemical structure classes known to block hERG it is thought that many binding sites may exist. This test system detects binding and does not demonstrate any change in the electrophysiological action of the cells used. Indeed, there are differences to results of electrophysiological assessments of drug activity. Using a [^{35}S]MK-499-based test system, astemizole and terfenadine produce IC_{50} results that compare favorably with electrophysiological recordings (Wang et al. 2003). In contrast, the measured value for cisapride using this assay is 3-fold higher than electrophysiological studies and MK-499 is 30-fold lower than an electrophysiological approach (Wang et al. 2003). Such discrepancies also appear using a [^{3}H]-dofetilide-based system and may even be more pronounced. Thus, the specificity of the binding site for the radio-labeled compound, versus the test compound, may limit the usefulness of this approach.

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I.D.3.1.2 Rubidium Flux Assays

PURPOSE AND RATIONALE

Rubidium exhibits a very high flux through potassium ion channels. Upon depolarization, this flux can be quantified as a measure of channel integrity (Tang et al. 2001; Chang et al. 2002). Fluorometric assays have been developed that can detect drug-induced effects on hERG channels.

The test system utilizes cells expressing hERG channels that are incubated with rubidium that effectively replaces potassium in the cells. The cells are then incubated together with a given test compound and then depolarized with a high concentration of potassium. The supernatant is then removed after several minutes of incubation and the distribution of rubidium between the supernatant and that remaining in the cells is determined. Accordingly, a hERG blockade prevents the efflux of rubidium from the cells whereas the lack of blockade allows rubidium to leave the cells. Radio-labeled rubidium can be measured using scintillation counting (Weir and Weston 1986) or with atomic absorption spectroscopy (Terstappen 1999).

CRITICAL ASSESSMENT OF THE METHOD

This experimental approach assesses the patency of the hERG potassium channel and is therefore a functional assessment and not a mere binding assay. A good correlation between results with this assay system and electrophysiological measurements has been reported (Tang et al. 2001; Chang et al. 2002) but this may be dependent upon the specific type of blocker being tested. For example, a poorer correlation may be found with voltage-dependent blockers (Chang et al. 2002). The radioactive waste generated with the use of this technique should also be considered when selecting a test system. The use of atomic absorption spectroscopy avoids this problem, but has lower throughput.

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I.D.3.1.3 Fluorescence Ion Channels Assays Using Voltage-Sensitive Dyes

PURPOSE AND RATIONALE

Fluorescent, voltage-sensitive dyes can be employed to investigate the activity of ion channels by reflecting the cellular membrane potential (Epps et al. 1994; Plasek and Sigler 1996). This approach is suitable for use in conjunction with different measurement systems including Fluorometric imaging plate readers (FLIPR), Voltage/Ion probe readers (VIPR) or conventional fluorescence readers.

The FLIPR technique, from Molecular Devices Corporation (USA), utilizes an oxonol-derivative dye in a patented test kit (FMP-kit, (Baxter et al. 2002)). Use of this approach for the measurement of IC_{50} values for known hERG blocking drugs has been reported (Tang et al. 2001), but there are apparent discrepancies between such values and those obtained using electrophysiological studies. This may, at least in part, be due to the rather slow kinetics of the dye making steady-state measurements preferable using this system. An approach yielding a higher time resolution uses the fluorescence resonance energy transfer between two dyes. This can be used in conjunction with voltage/ion probe readers configured as a high throughput system (Gonzalez and Maher 2002). Preliminary data has been reported using this system for detecting drug effects on hERG channels (Zlokarnik 2002), but the reported IC_{50} values vary substantially from those reported using electrophysiological methods. Conventional fluorescence readers can also be employed to make equilibrium measurements with potential-sensitive oxonol dyes (Netzer et al. 2001; Netzer 2003) using a technique developed by Evotec OAI (Germany). The reported IC_{50} values generated with this approach appear to correlate well with electrophysiological measurements (Netzer 2003) with similar absolute values.

CRITICAL ASSESSMENT OF THE METHOD

The use of membrane potential-sensitive dyes allows a high throughput approach for detecting drug effects on hERG potassium channels. Independently from the exact measurement system chosen, they suffer from potential fluorescence or quenching artifacts. The membrane potential is also an indirect measurement of a drugs effect on the hERG potassium channel. The start up costs for the necessary instrumentation (e.g. FLIPR of VIPR) may also present a constraint to this approach (Netzer et al. 2001).

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I.D.3.1.4
Automated Patch Clamp Systems

PURPOSE AND RATIONALE

Whole cell patch clamp techniques are still considered to be the gold standard for determining the effects of drugs on the hERG potassium channel (inhibition of I_{Kr}). New systems are currently in development that attempt to incorporate the patch clamping approach into an automated and, thereby, higher throughput format. Whereas these systems cannot be described as being truly high throughput, their goal is to speed the study process through automation while preserving the manner in which the study is conducted and the measurements that are made. Thus, cells transfected with hERG and expressing current resembling I_{Kr} are used and a whole cell patch clamp methodology is applied. Patching of the cells and addition of the test compound is, however, done on an automated basis.

CRITICAL ASSESSMENT OF THE METHOD

A critical step to the performance of a whole cell patch clamp experiment is the formation of a tight, high resistance seal ("gigaohm seal") between the cell membrane and the glass micropipette of hole in a planar chip. The formation of this seal remains the limiting factor with this experimental approach with success rates on the order of 50 %. The use of a so-called "perforated patch" using pore-forming agents to form less tight seals that do not need a negative pressure on the cell, but yield lower resistance seals reported to be approximately 100 megaohms (Kiss et al. 2003; Schroeder et al. 2003). While increasing seal success rate, the resultant seals may not be optimal for the electrophysiological recordings. As with standard patch-clamp studies, the stimulation protocol and bathing solutions may influence the results.

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I.D.3.2
Voltage Clamp Studies on Potassium Channels

I.D.3.2.1
General Characteristics of the Voltage Clamp Technique

PURPOSE AND RATIONALE

The introduction of the patch clamp technique (Neher and Sakmann 1976) revolutionized the study of cellular physiology by providing a high-resolution method of observing the function of individual ionic channels in a variety of normal and pathological cell types. By the use of variations of the basic recording methodology, cellular function and regulation can be studied at a molecular level by observing currents through individual ionic channels (Liem et al. 1995; Sakmann and Neher 1995).

The most commonly used method is called the "on-cell" or "cell-attached" configuration, because ion channels can be recorded on an intact cell (Jackson 1993). This mode is well suited for investigation of ion channels, which are activated by hormonal stimulation and triggered by intracellular second messengers.

Another versatile mode is the "cell-excised" configuration (Hamill 1993). It is obtained by suddenly removing the patch-pipette from the cell, so that the membrane patch is pulled off the cell. This mode easily allows to expose the channel proteins to drugs by changing the bath solution. The single channel currents are recorded on a videotape and are analyzed off-line by a computer system. Various parameters are evaluated, such as the single channel conductance, open-and closed-times of the channel, and the open-state probability, which is the percentage of time the channel stays in its open state.

Besides these modes, which enable the recording of single channel currents, it is also possible to measure the current flowing through the entire cell. This "whole-cell mode" is obtained by rupturing the membrane patch in the cell-attached mode (Hamill et al. 1981; Dietzel et al. 1993). This is achieved by applying suction to the interior of the patch-pipette. The "whole-cell mode" not only allows to record the electrical current, but also to measure the cell potential. Moreover, the cell interior is dialyzed by the electrolyte solution filled into the patch-pipette.

The fabrication of patch clamp pipettes has been described (Sakmann and Neher 1983; Corey and Stevens 1983; Cavalié et al. 1993).

Variations of the patch-clamp technique have been used for studying neurotransmitter transduction mechanisms (Smith 1995).

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I.D.3.2.2
Voltage Clamp Studies on Potassium Channels

PURPOSE AND RATIONALE

Potassium channels represent a very large and diverse collection of membrane proteins which participate in important cellular functions regulating neuronal and cardial electrical patterns, release of neurotransmitters, muscle contractility, hormone secretion, secretion of fluids, and modulation of signal transduction pathways. Main categories of potassium channels are gated by voltage or an increase of intracellular calcium concentration (Escande and Henry 1993; Kaczorowski and Garcia 1999; Alexander et al. 2001).

The delayed outward potassium current in heart muscle cells of several species in made up of a rapidly (I_{Kr}) and a slowly (I_{Ks}) activating component (Sanguinetti and Jurkiewicz 1990; Wang et al. 1994; Gintant 1996; Lei and Brown 1996; Carmeliet and Mubagawa 1998). Several potent and selective blockers for I_{Kr} have been shown to prolong the effective refractory period, but have a reverse rate-dependent activity with both normal and elevated extracellular potassium concentrations (Colatsky et al. 1990). Using the whole-cell configuration of the patch-clamp technique, Grissmer et al. (1994) analyzed the biophysical and pharmacological properties of five cloned voltage-gated K^+ channels stably expressed in mammalian cell lines.

Inhibitors of the slow component I_{Ks} were developed in order to circumvent the negative rate dependence of I_{Kr} blockers on the effective refractory period (Busch et al. 1996; Suessbrich et al. 1996, 1997; Bosch et al. 1998). Gögelein et al. (2000) studied the effects of a potent inhibitor of I_{Ks} channels in *Xenopus* oocytes and guinea pig ventricular myocytes.

PROCEDURE

Studies in *Xenopus* oocytes are performed with the two microelectrode voltage clamp method. For isolation of the oocytes, the toads are anesthetized using a 1 g/l solution of 3-aminobenzoic acid ethyl ester and placed on ice. A small incision is made to retrieve sacs of oocytes and is subsequently closed with absorbable surgical suture. On waking up, the toads are placed back into the aquarium. The ovaries are cut up into small pieces and the oocytes are washed in Ca^{2+}-free Or-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES; pH 7.4) and subsequently col-

lagenized in Or-2 containing collagenase A (1 mg/ml, Worthington, type II) until follicles are not longer detectable on the oocyte surface. The oocytes are stored at 18 °C in recording solution ND-96 (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM, pH 7.4) with additions of sodium pyruvate (275 mg/l), theophylline (90 mg/l) and gentamycin (50 mg/l).

For electrophysiological recordings, the two-microelectrode voltage-clamp configuration is used to record ion currents from *Xenopus* oocytes. Injection of cRNA is performed according to Methfessel et al. (1986) or Golding (1992). Oocytes are injected individually with cRNA encoding for the human protein minK, guinea pig Kir2.1, human hERG, human Kv1.5, mouse Kv1.3, or human HNC2. In the case of minK the functional potassium channel is a heteromultimer composed of the endogenous (*Xenopus*) KvLQT1 and the injected human minK. This heteromultimeric potassium current in then called I_{Ks} (Barhanin et al. 1996; Sanguinetti et al. 1996).

The electrophysiological recordings are performed at room temperature, using a Geneclamp amplifier (Axon Instruments, Foster City, CA, USA), and MacLab D/A converter. The amplitudes of the recorded currents are measured at the end of the test voltage steps. The control ND-96 solution contains (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, (pH 7.4). To amplify the inward potassium current through Kir2.1 and HNC2, the external potassium concentration is raised to 10 mM KCl and the NaCl concentration lowered to 88 mM (ND-88). The microelectrodes are filled with 3 M KCl and have a resistance between 0.5 MW and 1 MW. During the recordings the oocytes are continuously perfused with ND-96 (or ND-88 in the case of Kir2.1 and HCN2). The test compounds are dissolved in DMSO and added to the buffer ND-96 or ND-88. The current amplitude is determined after 5 min of wash-in time.

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I.D.3.2.3 Voltage Clamp Studies on hERG Potassium Channels in Heterologous Cell Systems

PURPOSE AND RATIONALE

The delayed rectifying current in ventricular myocytes that participates in the repolarization of the membrane potential has two components, a "rapid" and a "slow" current, known as I_{Kr} and I_{Ks}, respectively. hERG is

a potassium channel alpha subunit that, as a tetramere, makes up the ion conducting pore of the channel responsible for I_{Kr} (Tagliatela et al. 2000). Drug-induced prolongation of the QT interval duration of the ECG has been attributed in most all cases to a blockade of this channel (Vandenberg et al. 2001). Thus, the assessment of drug action on this current is useful in drug development. The purpose of this type of study is to examine drug effects on model systems for the ventricular membrane current I_{Kr} by the use of cells transfected with, and functionally expressing, hERG channels. The activity of these voltage-gated channels is measured using voltage clamp techniques.

PROCEDURE

Various cells can be used to conduct electrophysiological experiments to determine drug effects on hERG-mediated potassium current. This includes cells that naturally have hERG current including isolated ventricular cardiomyocytes (Jurkiewicz and Sanguinetti 1993), atrial cells lines (Busch et al. 1998) or neuroblastoma cell lines (Arcangeli et al. 1998). Alternatively, heterologous expression can be used to study drug effects on hERG channels in other cells selected to ease the measurement of the hERG-mediated current. This can be further categorized to either transient expression systems using, for example, Xenopus oocytes (Wagner et al. 2000), or stably transfected mammalian cells lines with HEK293 and CHO cells being the most commonly used cell types. Such heterologous expression systems, particularly the mammalian cell-based systems, have become the most widely used and accepted models for hERG-channel testing.

Xenopus Oocytes

Xenopus oocytes are a useful heterologous system for measuring hERG current due to their robust nature and their expressing a comparatively large and well defined hERG current. Their large size also makes the technical aspects of the study easier than with small cell types. The use of *Xenopus* oocytes for defining drug effects on hERG activity has the disadvantage, however, that most drugs show lower potency for hERG blockade in *Xenopus* oocytes in comparison to mammalian cell lines (Po et al. 1999; Weerapura et al. 2002; Witchel et al. 2002). This may be due to absorption of drug in yolk particles within the cells thereby lowering the free drug intracellular concentration. Whereas the absolute IC_{50} values may be 3–10 fold higher in *Xenopus* oocytes than in mammalian cells, their rank order of potency appears to be comparable to that seen in mammalian cells. Nevertheless, there are examples of compounds (e.g. sotalol and erythromycin) shown to block hERG in mammalian cells that do not show a blockade in *Xenopus* oocytes, suggesting a greater risk of a false negative assessment when using oocytes in comparison to mammalian cells.

Stably Transfected HEK293 Cells

HEK293 cells are plated on coverslips placed at the bottom of a 2 ml perfusion chamber mounted on the stage of an inverted microscope (e.g. Diaphot, Nikon, Japan). Cells are superfused with a bath solution containing (mM): NaCl (137), KCl (4.0), $MgCl_2$ (1.0), $CaCl_2$ (1.8), Glucose (10), HEPES (10) at pH 7.4. The microelectrodes are positioned using a micromanipulator. They were made from borosilicate glass tubing (Hilgenberg, Malsfeld, FRG) with an electrode Puller (DMZ-Universal Puller, Zeitz-Instrumente, Augsburg, FRG) and filled with pipette solution containing (mM): K-Aspartate (130), $MgCl_2$ (5.0), EGTA (5.0), ATP-K2 (4.0), HEPES (10.0), pH 7.2. Resistance of the microelectrodes should be in the range between 2 and 4 MΩ. The measured membrane currents are filtered at 10 kHz before being digitized at 5 kHz using an amplifier (e.g. EPC-7 HEKA Electronics, Lambrecht, FRG).

For investigating effects on the hERG-mediated potassium channel, HEK293 cells were clamped at a holding potential of 0 mV and measured using a pulse pattern with fixed amplitudes (hyperpolarization: –80 mV for 25 ms; depolarization: +40 mV for 80 ms) repeated at 10 s intervals. (This protocol is shown as one example; other pulse protocols have also been used successfully in the past.) Experiments can be performed with multiple concentrations of test article and usually three different cells are used for each concentration tested. A steady state level of current is measured for at least 2 min before applying the test article for 5 min. Drug effects on current amplitude can be measured 3 ms after the step to +40 mV after 5 min in the presence of compound. Compounds are typically dissolved in DMSO to yield a 10 mM stock solution and dilutions are prepared freshly before starting the experiments.

Data are recorded using e.g. TIDA software (HEKA Electronics, Lambrecht, Germany) and the results are typically expressed as fraction of baseline current. Concentration-response data can be fitted to an equation of the following form: $I/I0 = 1/(1 + ([compound]/IC_{50}))$ such that the IC_{50} can be calculated with a sigmoidal dose-response curve model.

CRITICAL ASSESSMENT OF THE METHOD

A preference for a mammalian cell line for measuring drug effects on hERG current is based on the perception that the results may be more representative of what may happen in patients than results from a *Xenopus* oocyte-baed model. The relatively small size of the HEK293 cell and the lack of a yolk provide a system in which the measured blockade is usually more potent than in *Xenopus* oocytes. HEK293 and CHO cells are most commonly used for this purpose. HEK293 cells may have an endogenous transient outward potassium current (I_{to}) (Snyders & Chaudhary 1996) whereas CHO cells do not have such a current (Teschemacher et al. 1999). The amount of I_{to} in HEK293 cells may be variable between batches of cells and could interfere with hERG measurements making CHO cells in this regard superior. HEK293 cells may, however, be more consistent in their level of heterologous expression in comparison to CHO cells (Witchel et al. 2002).

HEK293 cells stably transfected with hERG cDNA and expressing the hERG-mediated potassium channel can be obtained commercially or the cells can be transfected in house if the necessary experience is available. Issues relating to the use of stably versus transient transfection have been reviewed (Witchel et al. 200). Membrane currents can be recorded from HEK293 cells at room temperature (20–22 °C) or at physiological temperature (37 °C), using the whole-cell patch-clamp technique. Although it is attractive to conduct such studies at 37 °C to have the current at a more physiological condition, there are some practical limitations that suggest conducting the studies at room temperature, at least for routine use (Witchel et al. 2002). This includes not having to have preheated perfusion media and the observation that cells last substantially longer when studied at room temperature. The measured current is larger at 37 °C in comparison to room temperature and with faster kinetics. Also, at 37 °C currents measured during strongly depolarizing pulses may diminish during the course of the pulse, a phenomenon not seen at room temperature.

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I.D.3.2.4 Studies on Potassium Channels in Isolated Ventricular Myocytes

PURPOSE AND RATIONALE

Patch-clamp techniques using isolated ventricular myocytes may be used measure potassium channel function to clarify the mechanisms underlying the development of torsade de pointes ventricular arrhythmias (Salata et al. 1995; Drolet et al. 1999; Kalifa et al. 1999).

PROCEDURE

Preparation of Single Cells

For the isolation of *ventricular myocytes*, guinea pigs (weight about 400 g) (Jurkiewicz et al. 1993) or Sprague-Dawley rats of either sex are sacrificed by cervical dislocation. The hearts are dissected and perfused retrogradely via the aorta at 37 °C: first with nominally Ca^{2+}-free Tyrode solution (in mmol/l): 143 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.25 NaH_2PO_4, 10 glucose, 5 HEPES, pH 7.2, then with Tyrode solution containing 20 mmol/l Ca^{2+} and 3 mg/ml collagenase type CLS II (Biochrom, Berlin Germany). After 5–10 min collagenase treatment the ventricles are cut up into small pieces in the storage solution (in mmol/l): 50 L-glutamic acid monopotassium salt, 40 KCl, 20 taurine, 20 KH_2PO_4, 1 $MgCl_2$, 10 glucose, 0.2 EGTA, pH 7.2. The myocytes are then dispersed by gentle shaking followed by filtration through a nylon mesh (365 mm). The cells are finally washed twice by

centrifugation at 90 g for 5 min and kept in the storage solution at room temperature.

Voltage-Clamp Studies

Whole cell currents are recorded in the tight-seal whole-cell mode of the patch-clamp technique, using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Patch pipettes are pulled from borosilicate glass capillaries (wall thickness 0.3 mm, outer diameter 1.5 mm) and their tips are fire-polished and filled with 0.5 M K^+ gluconate, 25 mM KCl, 5 mM K_2ATP (Sanguinetti and Jurkiewicz 1990). Series resistance is in the range of 1–10 MW and 50 % compensated by means of the EPC's compensation circuit.

The I_{Ks}, I_{Kr}, and I_{K1} currents are investigated in guinea pig ventricular myocytes. The voltage pulses for recording the current components are as follows: I_{Ks} current: holding potential –80 mV to –50 mV (200 ms) to +60 mV (3 s) to –40 mV (2 s) to –80 mV; I_{Kr} current: holding potential –80 mV to –50 mV (200 ms) to –10 mV (3 s) to –40 mV (2 s) to –80 mV. I_{Kr} is evaluated as the tail current evoked by a voltage pulse from –10 mV to –40 mV; I_{K1}: holding potential –80 mV to –120 mV (200 ms) to –80 mV. In order to suppress the L-type Ca^{2+} current, 5 mmol/l nifedipine is added to the bath solution.

Concentration-response relations are determined by measuring action potentials of currents in each cell during control conditions and during superfusion with two successively increasing concentrations of a given drug. Concentration-response curves are fit to a logistic equation, $Y = (a–d)/[1 + (X/c)^b]+d$, using a Marquardt-Levenburg algorithm for least-squares nonlinear regression analysis. Using this equation, a and d are maximal and minimal responses estimated for infinite and zero concentrations, respectively; c is the inflection point that estimates the 50 % effective concentration (IC_{50}); and b is the slope factor (Hill coefficient). During the time required (4 to 8 min) to achieve steady-state drug effects, I_{Ktail} measured after a voltage step from t50 to t10 mV, which is used to quantify I_{Kr}, decreases by an average of 5 % even in the absence of drug or vehicle. Because of this "rundown" of the current the concentration-response curves are fit with a minimal inhibition of 5 %.

MODIFICATIONS OF THE METHOD

Carlsson et al. (1997) performed voltage clamp studies in isolated ventricular myocytes from **rabbits**.

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I.D.3.3 Myocardial Action Potential Configuration

Studies to assess the potential of a drug to affect ventricular repolarization, and therefore a prolongation of the QT interval duration, focus on the pivotal role of hERG-mediated potassium current in mediating this effect. Nevertheless, it is well established that even a potent blockade of the hERG channel does not necessarily lead to a QT prolongation. For example, the calcium channel blocker verapamil does not lead to QT interval prolongation despite an IC_{50} on hERG in the range of 100–200 nM. The reason for this apparent discrepancy is simply that the myocardial action potential configuration is the net result of the concerted activity of numerous ion channels and effects on a given channel can be masked by the activities of other competing channels; other examples are also apparent (Yuill KH et al. 2004). It is this recognition that suggests that drug profiling should examine the effects of a compound not only on hERG channels, but on the overall myocardial action potential. This allows one to assess the physiological relevance of any activity on hERG channels that may be present (The European Agency for the Evaluation of Medicinal Products. Human Medicines Evaluation Unit. Committee for Proprietary Medicinal Products 1997; Haverkamp et

al. 2000). In other words, a compound that shows both potent inhibition of hERG channels and prolongs the myocardial action potential in low concentrations will present a higher risk for clinical QT prolongation than the compound that blocks hERG potently, but shows no effect on the overall action potential. Whereas in vivo data will also add significantly to this preclinical risk assessment, performance of hERG channel assays together with action potential assays may provide an early risk assessment with a better correlation to the in vivo and clinical setting than with just one of these assays alone (Guth et al. 2004).

Drug effects on the myocardial action potential can be measured in myocardial tissue in vitro and typically using Purkinje fibers (Gintant et al. 2001), papillary muscles, ventricular wedge preparations (albeit less commonly) or the entire isolated heart (Franz 1991). The focus of all these approaches is to assess the action potential duration, measured usually in terms of the time to a give percentage of repolarization, e.g. APD_{90} or the time to 90 % repolarization. Additionally, some of these models can respond to hERG-blocking drugs with early after depolarizations (EAD), thought to be a substrate for arrhythmia. The use of the myocardial wedge preparation also addresses the important issue of potential transmural differences in drug-induced effects on repolarization (Antzelevitch et al. 1999), an aspect not addressed by Purkinje fibers or papillary muscles.

Measurement of the Concentration of Test Article in in vitro Systems

The results from studies designed to assess potential drug effects on hERG channels or on the action potential in vitro are used as an early risk assessment for clinically relevant effects on the QT interval duration and even proarrhythmic activity. The use of this type of data for the estimation of safety margins necessitates an accurate measurement of the drug concentrations present in the test system. The perfusion baths used are typically protein-free meaning that in comparison to the in vivo situation, one needs to consider potential protein binding of a compound. Furthermore, compounds may adhere to glass or plastic used in the experimental setups with the possibility that the intended drug concentrations are not reached, particularly in the lower concentration ranges. Multiple aspects of this important issue have been reviewed recently (Herron et al. 2004).

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I.D.3.3.1 Studies in Isolated Purkinje Fibers

PURPOSE AND RATIONALE

Studies in isolated Purkinje fibers can detect compound-induced effects on the action potential configuration and possible early after depolarizations. This type of study is a logical adjunct to studies examining effect on hERG-mediated current since it examines the potential relevance of any potential hERG blocking activity on the overall myocardial action potential. Purkinje fibers from the dog or the rabbit have been used typically, while other species, including the pig (Gintant et al. 2001) may also be appropriate models.

PROCEDURE

Purkinje fibers are excised from the isolated heart and placed in a superfusion chamber with warmed (37 °C) Tyrode's solution containing 4 mM potassium and a flow rate of 8–10 ml/min. The fibers are stimulated using platinum electrodes at 2 times threshold for 1–2 ms using a biphasic wave form. The fibers are then

impaled with a microelectrode having a resistance of 10–30 M-ohm and filled with 3 M KCL. The electrical activity can then be monitored using a high-input impedance electrometer. The transmembrane potential recordings are usually displayed using a storage oscilloscope and saved to a computer, as desired.

An initial equilibration period of at least 20 min is used to insure the stability of the preparation. Additionally, the recording should demonstrate a resting membrane potential of at least –80 mV and an action potential duration of between 300 and 500 ms when stimulated with a cycle length of 2 sec. The rate-dependency of the action potential can be determined by recording at stimulation rates of 2 s, 800 ms and 400 ms, each for at least 2 min. A test article can then be added to the perfusion bath and sufficient time should be allowed to obtain a maximal drug effect; this may be up to 30 min or longer. The rate-dependency of the action potential could be determined again, prior to applying a higher concentration of test article.

EVALUATION

The primary evaluation involves the determination of the action potential duration. Typically, the time from depolarization to a predefined % repolarization is used to describe the repolarization dynamics of the tissue. That is, time to 10 % (APD10), 30 % (APD30), 50 % (APD50), 70 % (APD70) and 90 % (APD90) can be used to quantify the repolarization curve. For hERG-dependent effects on the action potential, the APD90 is most commonly reported. Most compounds known to block hERG-mediated potassium current can be shown to lead to action potential prolongation (e.g. prolongation of the APD90) in this model. The model is capable of detecting effects on the action potential including prolongation and shortening of the action potential and it should not be forgotten that other drug-induced effects on electrophysiological function can be detected, including effects on sodium (e.g. effects on the rate of depolarization) and calcium currents (e.g. effects on the plateau phase of the action potential).

CRITICAL ASSESSMENT OF THE METHOD

The Purkinje fiber is a non-contractile tissue, which facilitates electrode positioning and stability. In comparison to the papillary muscle or the monophasic action potential in the intact heart, drug-induced effects on the action potential duration are considerably larger. Whereas this is not necessarily a disadvantage, the model has been viewed as possibly being too sensitive for drug-induced effects on repolarization.

A clear disadvantage of the Purkinje fiber model (as well as the guinea pig papillary model, see below) is that not all hERG-blocking compounds produce the expected prolongation of the action potential. One notable example is terfenadine (Gintant et al. 2001), that in both canine and porcine Purkinje fibers, even in supratherapeutic concentrations failed to prolong the action potential duration. The reason for this is still unclear but has lead to these types of studies receiving secondary status from regulatory authorities due to the perception of a risk for false negative results. It must be emphasized, however, that most hERG-blocking drugs do indeed lead to an action potential prolongation in this model and that exceptions are rare.

MODIFICATIONS OF THE METHOD

The basic experimental approach is suitable using Purkinje fibers from a variety of species including in addition to the dog, the rabbit and pig. The use of primate tissue for such studies carries with it some ethical and financial considerations, as does, in fact the use of dog purkinje fibers in certain countries.

Studies in isolated rabbit Purkinje fibers were recommended to assess the risk of QT interval prolongation by drugs (Adamantidis et al. 1995, 1998; Cavero et al. 1999; Dumotier et al. 1999; Champeroux et al. 2000).

New Zealand white rabbits of either sex weighing 1.5–2 kg are sacrificed by cervical, dislocation and exsanguinated. Their hearts are quickly excised and placed in potassium- and glucose-enriched Tyrode solution (in mM: NaCl 108.2; KCl 27; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 1.8; $NaHCO_3$ 25; glucose 55; pH 7.35 ± 0.05) oxygenated (95 % O_2/5 % CO_2) at a temperature of about 32 °C. The left ventricle is opened through an incision into the anterior intraventricular groove. Purkinje fibers still attached to the ventricular muscle are carefully dissected and pinned to the silicone base of the experimental chamber. The pins are exclusively placed in ventricular muscle to avoid stretching of the Purkinje fibers. The preparations are superfused for 30 min, at a flow rate of 2 ml/min with the above-described solution maintained at 36.5 ± 0.5 °C. Then the superfusate is switched to normal Tyrode's solution (in mM: NaCl 108.2; KCl 4; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 1.8; $NaHCO_3$ 25; glucose 11). The preparations are electrically stimulated by rectangular pulses of 1 ms with a frequency of 120 pulses per min and an intensity 1.5 times the diastolic threshold. After 30 min at least, the stimulation frequency is turned to 60 pulses per min.

Transmembrane action potentials are recorded using 3M KCl glass microelectrodes with a tip resistance of 10–15 MΩ, which are coupled with an Ag-AgCl bath electrode and connected to an impedance amplifier, and are displayed on an oscilloscope with numerical memory, analyzed by an external computer system and stored on a digital magnetic type recorder which allows display on paper recordings of the occurrence and development of electrical abnormalities. The following parameters are measured: testing membrane potential, action potential and overshoot, maximal rate of depolarization and action potential duration at 50 % and 90 % of repolarization.

The studied drug is added to the Tyrode's solution in increasing cumulative concentrations, each concentration being tested for 30 min. The stimulation frequency is 60 pulses per min except between the 20th and 22nd min, when the frequency is reduced to 12 and then switched back to 60 pulses per min. Action potentials are measured after 15 and 30 min of each concentration superfusion and at the end of the 2-min stimulation of 12 pulses per min.

The results are expressed as means ± SEM. Comparisons vs. controls are performed statistically using an analysis of variance for repeated measures completed by the corrected Dunnett's t-est.

Kondo et al. (1999) studied the antagonism of potassium channel openers against the effect of class III antiarrhythmic agents in Purkinje fibers of dogs.

Studenik et al. (1999) used isolated spontaneously beating Purkinje fibers from guinea pigs to study the proarrhythmic potential of antidepressant and neuroleptic drugs.

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I.D.3.3.2 Studies in Isolated Guinea Pig Papillary Muscles

PURPOSE AND RATIONALE

Studies in isolated guinea pig papillary muscles can detect compound-induced effects on the action potential configuration. This type of study is a logical adjunct to studies examining effect on hERG-mediated current since it examines the potential relevance of any potential hERG blocking activity on the overall myocardial action potential. The most commonly used model is the guinea pig papillary muscle, due to its appropriate size; use of papillary muscle from large species may be compromised by ischemia in the middle of the muscle during the experiment.

PROCEDURE

Guinea pigs are euthanized and the hearts were rapidly excised and placed in a dish filled with buffer solution containing (millimolar) NaCl (140), KCl (4.7), $NaHCO_3$ (20), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (1.8), glucose (10). After opening the right heart a suture loop is tied around the cordae tendinae and the base of the papillary muscle is cut. The papillary muscle is placed in a temperature controlled dish filled with the same solution as above but additionally containing 1 % DMSO. The buffer is bubbled with 95 % O_2 and 5 % CO_2 and the temperature of the solution is kept constant at 35 °C; careful control of the temperature is essential for a successful preparation as changes in temperature lead to substantial changes in muscle function and the action potential. The base of the papillary muscle is tied with suture and connected to a tension loading transducer (e.g. model Q11, Hottinger Baldwin Messtechnik; Darmstadt, Germany).

The muscle is stimulated with a monophasic rectangular stimulus of 3 ms duration and at 1.5 times the stimulation threshold using a 1831A stimulator

(e.g. WP Instruments, Inc.; Longmont, CO, USA). Stimulation frequency is 1 Hz for an equilibrium period of one hour and decreased 0.33 Hz (20 cycles/min) during the experiments. Rate-dependency of an effect can easily be incorporated into the study protocol by manipulating the stimulation rate.

Microelectrodes are fabricated from borosilicate glass tubing with a DMZ-Universal Puller (e.g. Zeitz-Instrumente, Augsburg, Germany) and filled with 3 N KCl. The resistance of the microelectrodes should be in the range between 15 and 20 MΩ. A WPI S7071A amplifier (WP Instruments, Inc.; Longmont, CO, USA) or similar is used and the signals are digitized at a rate of 1 kHz using a 16 bit AD/DA converter and recorded and analyzed with a commercially available computer program (e.g. HEM 3.0, Notocord, Paris, France). Control measurements are taken immediately before adding the test substance and during each subsequent steady state at increasing concentration with continuous stimulation at 0.33 Hz. Compounds were typically dissolved in DMSO at a concentration of 10–2 mol/L and added to the bath solution at a ratio of 1:1000. The final DMSO concentration will therefore not exceed 1.5 % even in concentrations of up to 10–30 μM. Two groups of experiments are usually performed: one group (n = 5) receives a given test article in cumulative concentrations of e.g. 0.1, 0.3, 1.0, 3.0, and 10.0 μM, the other group (n = 5) serves as a control group and receives equivalent concentrations of the solvent. Measurements in the control group are taken at baseline and times corresponding the measurements in the group receiving the test article.

EVALUATION

The primary evaluation involves the determination of the action potential duration. Typically, the time from depolarization to a predefined % repolarization is used to describe the repolarization dynamics of the tissue. That is, time to 10 % (APD10), 30 % (APD30), 50 % (APD50), 70 % (APD70) and 90 % (APD90) can be used to quantify the repolarization curve. For hERG-dependent effects on the action potential, the APD90 is most commonly reported. Most compounds known to block hERG-mediated potassium current can be shown to lead to action potential prolongation (prolongation of the APD90) in this model. The model is capable of detecting effects on the action potential including prolongation and shortening of the action potential and it should not be forgotten that other drug-induced effects on electrophysiological function can be detected, including effects on sodium (e.g. effects on the rate of depolarization) and calcium currents (e.g. effects on the plateau phase of the action potential). Thus, the parameters of V_{max} (maximum depolarization velocity), resting membrane potential, action potential amplitude and overshoot amplitude are also useful to describe the effects on the action potential configuration. Additionally, effects on contractile function can be expressed as relative changes in contractile force.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to the Purkinje fiber, the papillary muscle is a contractile tissue lending it also for the measurement of contractile force, together with the action potential configuration. Since effects on the inotropic state of the myocardium can also affect the action potential, this is a useful secondary measurement to have to interpret possible drug-induced effects. However, the contraction of the muscle makes the placement of the electrode more difficult and changes in the contractile function can lead to loss of the electrode placement in the course of a study.

As with the Purkinje fiber model, some compounds known to block hERG channels potently and to cause QT prolongation in the clinic do not demonstrate the expected action potential prolongation; this includes astemizole, bepridil, pimozide and terfenadine. In the case of the guinea pig papillary muscle, it has been suggested recently in conjunction with the PRODACT evaluation in Japan, that the way in which the data are evaluated may be key for detecting the drug-induced effect on the action potential configuration. In conjunction with these studies, the investigators employed a new parameter, the APD30-90, that is the difference between the APD90 and the APD30, as a parameter that should reflect specifically effects on I_{Kr}. With this approach, these investigators were able to demonstrate effects of astimizole, bepridil and pimozide in the isolated guinea pig papillary muscle. Nevertheless, terefenadine did not show an effect even on the APD30-90, such that it was suggested that other limitation must prevent terfenadine from demonstrating its expected APD prolongation in this in vitro setting.

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I.D.3.3.3
Arterially Perfused Wedge of Canine Left Ventricle

PURPOSE AND RATIONALE

The M cell is a unique myocardial cell type found in the deeper layers of the ventricular wall (Antzelevitch et al. 1999). These cells respond more sensitively to agents that block hERG channels and, as such, contribute to possible drug-induced transmural heterogeneity of ventricular repolarization and thereby the proarrhythmic potential. The perfused myocardial wedge preparation is design to allow the study of transmural differences in drug action on the action potential and may therefore provide a better assessment of possible proarrhythmic potential of a test article.

PROCEDURE

The methodology was first described by Antzelevitch et al. in 1996. Dogs are anticoagulated with heparin and then anesthetized. The heart is removed through a thoracotomy and placed in cold (4 °C) cardioplegic solution containing 8.5 mmol/l K^+. Transmural wedges are then dissected from the left ventricular wall having a height of 0.8–0.9 cm, length of 0.9–2 cm and wall thickness of 0.8–1.1 cm. The tissue is then perfused with cardioplegic solution by cannulating a small branch of the coronary artery having a diameter of 100–150 μm. This procedure should take no more than 4 min. The tissue preparation is then placed in a bath and perfused with Tyrode's solution having (mmol/l): NaCl 129, KCl 4, NaH_2PO_4 0.9, $NaHCO_3$ 20, $CaCl_2$ 1.8, $MgSo_4$ 0.5, glucose 5.5 and 1 U/liter of insulin buffered with 95 % oxygen and 5 % CO_2 at 36 °C. Obviously non-perfused tissue is removed with a razor blade. Perfusion pressure is maintained between 40 and 50 mm Hg by adjusting the perfusion pump supplying the coronary artery. One hour equilibration is allowed during stimulation at BCLs ranging from 500 to 5,000 ms using bipolar silver electrodes insulated except at the tips and applied to the endocardial surface.

A transmural electrocardiogram is recorded using extracellular silver chloride electrodes placed near the epicardial and endocardial surfaces of the preparation. Transmembrane action potentials were simultaneously recorded from the epicardial, endocardial and M regions using three separate intracellular floating microelectrodes filled with 2.7 KCl and connected to a high input impedance amplifier. Impalements are obtained from the cut surface of the preparation at positions approximating the transmural axis of the ECG recording.

EVALUATION

The evaluation is based on the measurement of the QT interval of the electrocardiogram, together with the action potential configuration measured using the transmurally located electrodes. The action potential duration is typically measured using the time to either 50 % or 90 % repolarization. The action potential duration of the M cells (midwall measurement) is expected to be longer than either endocardial or epicardial cells and drug-induced effects on these cells is also greater, for compounds known to prolong the action potential through a blockade of the delayed rectifying current. The rate dependency of a given drug effect is also amplified in the M cells and may importantly contribute to the proarrhythmic potential of a drug.

CRITICAL ASSESSMENT OF THE METHOD

The measurement of a transmural ECG together with local action potentials from across the ventricular wall of the dog provides one of the most sophisticated in vitro approaches for determining drug-induced effects on repolarization, as well as having implications for proarrhythmic potential. This is due to the fact that the M cells of the midmycardial wall are most sensitive to drug induced effects on repolarization and this contributes to a transmural heterogeneity of repolarization that provides a proarrhythmic substrate. Thus, objectively assessed, this model is perhaps the best in vitro model to examine drug-induced effects on repolarization of the heart. Its main disadvantage is the experimental complexity which makes it accessible to the specialty laboratory and may require extensive training to master the technical preparation. A further complicating factor is the use of dogs for studies in which only small portions of the excised heart are utilized, which has both ethical and financial aspects that need to be considered.

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I.D.4
Models for Proarrhythmic Potential

The goal of pharmacological profiling of drugs for effects on hERG-channels, the action potential or the

ECG is ultimately to predict the potential for proarrhythmic activity. Such models are useful in detecting drugs with potential proarrhythmic potential but none of these approaches are fully predictive since even potent hERG-blocking drugs that produce a prolongation of the action potential duration in vitro and QT interval in vivo do not necessarily lead to an increased rate of arrhythmia. It would therefore appear very useful to assess the proarrhythmic potential of a drug in a model designed to detect arrythmias, as opposed to surrogate markers. Nevertheless, the available proarrhythmia models failed to win the confidence of regulatory agencies and the use of them has neither been mandated nor widely recommended. Such models could, however, prove useful in cases where the results from other types of tests (hERG, action potential, in vivo) are not conclusive.

I.D.4.1 Studies of Arrhythmogenic Effects in Isolated Heart Preparations

PURPOSE AND RATIONALE

The evaluation of arrhythmogenic, anti-arrhythmic and antifibrillatory effects in the LANGENDORFF heart preparation is described in Sect. A.3.1.2 of the book Vogel HG (ed.) (2002) Drug Discovery and Evaluation – Pharmacological Assays. Second edition, Springer-Verlag, Berlin, Heidelberg

Eckardt et al. (1998), Johna et al. (1998) proposed the isolated perfused rabbit heart as a model to study proarrhythmia induced by class III antiarrhythmic drugs.

PROCEDURE

Male New Zealand white rabbits weighing 2.5–3.0 kg are anti-coagulated with heparin sodium (2000 IU) and anesthetized with thiopental sodium via a marginal ear vein. After transecting the carotid arteries, the chest is opened and the heart rapidly excised. A cannula is inserted into the aortic stump, the pulmonary artery incised, and the spontaneously beating heart perfused through the coronary arteries at 37 °C in a non-circulating system at a constant pressure of 70 mm Hg with Krebs-Henseleit buffer containing (in mM): $CaCl_2$ 2.52; KCl 4.70; KH_2PO_4 1.18; $MgSO_4$ 1.66; NaCl 118; $NaHCO_3$ 24.88, Na-pyruvate 2.00; glucose 5.55 (= solution I). The buffer is gassed with 95 % O_2 and 5 % CO_2 yielding a pH of 7.4. Solution II contains KCl for a total of 2.0 mM K^+ and Mg^{2+} 0.5 mM; the other components are left unchanged. The heart is immersed in an organ bath at 37 °C. The coronary flow is estimated by collecting the outflow of the bath in a bowl placed on an electronic balance from which the values are read out by a computer at 10 s intervals. Electrocardiograms are recorded from the bath via Ag/AgCl electrodes (filter settings: 0.1–300 Hz) in two perpendicular directions in the horizontal plane. QT intervals are measured from the beginning of the QRS complex to the intersection of the descending part of the T wave with the baseline in either ECG lead. The larger value is used for further analysis. Monophasic action potentials (MAP) are obtained using a commercially available Franz combination catheter (Franz 1994) (EP Technologies Inc., Sunnyvale, CA, USA) placed epicardially on the left ventricular posterior wall. Isovolumetric left ventricular pressure (LVP) is measured via a pressure transducer connected to a water-filled latex balloon attached to a rigid catheter and inserted through the left atrium into the ventricle (preload 5–10 mm Hg). ECGs, MAPs, and LVP are registered with a strip chart recorder, digitized at a rate of 1 kHz with 12 bit resolution and stored on discs for further analysis. Drugs are infused through an injection port above the aorta.

After a baseline period of 10 min, the test drug or a standard drug (Clofilium, a class III antiarrhythmic drug, Steinberg and Molloy 1979; Friedrichs et al. 1994) is infused for 20 min and MAP recordings are started. Following 5 more min of solution I (K^+ 5.88 mM, Mg^{2+} 1.66 mM) the perfusion is switched to solution II (K^+ 2.0 mM, Mg^{2+} 0.5 mM). During the subsequent 5 min the cardiac rhythm is described in consecutive 5 s windows by means of the ECG. The following patterns are distinguished: normal sinus rhythm, bigeminal pattern, monomorphic ventricular activity, ventricular rhythm with two alternating QRS morphologies and polymorphic ventricular tachyarrhythmias of the TdP type with undulating peaks of sequential QRS complexes (Jackman et al. 1988). MAP durations are measured at the 50 %, 70 % and 90 % repolarization level. Subsequently, the hearts are again perfused with solution I.

EVALUATION

All values are expressed as mean ± SD. Statistical evaluation is carried out by two-tailed t-test for paired or unpaired observations.

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I.D.4.2 Langendorff Rabbit Heart (Screenit System)

PURPOSE AND RATIONALE

The purpose of this model is, in addition to assessing drug-induced effects on the action potential duration, to assess specifically the proarrhythmic potential of a drug. This assessment is based on a more indepth analysis of drug-induced effects on the action potential including the action potential duration, but also including instability of the action potential prolonging effects, reverse use-dependency of the effects and triangulation, a description of the manner in which an action potential may be prolonged (Hondeghem 1994, Valentin et al. 2004). This information is synthesized into a proarrhythmic assessment that is claimed to discriminate between compounds that are proarrhythmic and those that are not, independently from their effects on the action potential duration per se. In fact, it is claimed that compounds that prolong the action potential but without being instable, reverse-use-dependent, nor causing triangulation are actually antiarrhythmic (Hondeghem et al. 2001).

PROCEDURE

It should be noted that this technique is used routinely only in a single laboratory, where it was developed. Hearts are removed from albino rabbits (2.5 kg) of either sex and perfused at 80 cm H_2O with a bicarbonate buffer (mmol/L): NaCl 118, KCl 3.5, $NaHCO_3$ 22, $MgCl_2$ 1l1, NaH_2PO_4 0.4, $CaCl_2$ 1.8, dextrose 5, pyruvate 2, and creatine 0.038, with 95 % O_2 and 5 % CO_2, with pH being adjusted to 7.4 at 34 °C. The HIS bundle is cut and a stimulating electrode is sutured on each side of the distal HIS bundle. A recording electrode is advanced until it reaches the left ventricular subendocardium of the septum. A reference and an epicardial recording electrode are positioned on the left venticualr epicardium. The reference electrode is perfused at 1 mL/min with isotonic KCl, with 1.8 mmol/L $CaCl_2$ and grounded (Hondeghem 1994). Hearts are automatically stimulated at 1.5 times threshold. If automaticity and escape cycle length are > 1000 ms, threshold stimulation current $< 300\,\mu$A, coronary perfusion > 17 mL/min, ectopic rate < 8 bpm, and the cardiac activation time < 60 ms, then the preparation is stimulated until instability (determined by the "best easy systematic method", described below) of the last 20 trains became < 10 ms. Preparations that did not achieve these criteria should not used further.

In a "brief protocol" the action potentials of a 10-beat train at 1000 ms and 300 ms are recorded together with a train of 30 action potentials stimulated at a cycle length of 1000 ms. In a "large protocol", automaticity, escape cycle lengths, conduction times and action potential durations for cycle lengths at 2000, 1500, 1000, 750, 500, 300 and 250 ms are determined. Prior to drug administration, the "brief protocol" is repeated 10 times and the "large protocol" is then carried out. Drug is then infused into the perfusion system for 10 min at each of 5 concentrations and the "brief protocol" is conducted each minute. If an effect on the action potential duration is detected (defined as shortening by 40 ms or lengthening by 80 ms) the given concentration is continued for 8 min followed by the "large protocol". Exposure times may be lengthened, if needed. Septal and epicardial monophasic action potentials are digitized at 1 kHz and for the conduction date, sampling is done at 10 kHz.

EVALUATION

Action potential duration to various times to repolarization (10, 20, 30 %, etc.) are measured from the midpoint of the upstroke. As "APD30 to APD90" prolongs, the action potential takes on a more triangular shape. "Triangulation" is defined as the repolarization time from APD30 to APD90. Reverse use dependence is measured as the difference between the APD60 of the first 10 and the last 20 action potentials of a 30-pulse train.

Action potentials with upstrokes not within 80 ms of a given stimulus are considered to be ectopic. The number of such ectopic beats is assessed each minute during the last 3 minutes of drug exposure.

CRITICAL ASSESSMENT OF THE METHOD

Whereas the measured characteristics of instability, triangulation and reverse use-dependency contribute to proarrhythmic activity of a drug, their relative

importance is unclear. This may differ from drug to drug and synergistic actions are likely. Nevertheless, drugs that lead to instability are considered to be the most problematic. Triangulation is, in general, a better predictor of proarrhythmic activity than reverse use-dependency, making reverse use-dependency the least predictive parameter of the three. The use of the rabbit is based on its having I_{Kr} as the main repolarizing current in the heart, but it has little I_{Ks} activity (Nattel 1999) and is therefore not sensitive for drugs that potentially affect this current. Futhermore, as with all in vitro test systems, effects due to metabolites of drugs are not detected. Nevertheless, the performance of this test system for differentiating between proarrhymic drugs and non-proarrhythmic drugs has been impressive (Valentin et al. 2004). The model has not been reproduced outside of the laboratory that developed it thereby limiting its availability and its acceptance.

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I.D.4.3 Methoxamine-Induced Arrhythmia in Rabbits

PURPOSE AND RATIONALE

The purpose of this model is to produce a setting in which proarrhythmic drugs may exacerbate the occurrence of a torsade-like ventricular tachycardia. This is achieved using anesthetized rabbits receiving an intravenous infusion of the alpha-adrenergic agonist methoxamine as described by Carlsson et al. (1990).

PROCEDURE

Rabbits are anesthetized with methohexital and alpha-chloralose and ventilated mechanically. After establishing an intravenous port, the animals receive the $alpha_1$-agonist methoxamine at 15 μg/kg/min, a dose that should have only a slight effect on systemic arterial blood pressure. The ECG is measured using standard limb leads and an monophasic action potential can be measured using a bipolar suction electrode attached to the epicardial surface through a thoracotomy.

EVALUATION

The evaluation is based on the incidence of either an overt tachyarrhythmia or the appearance of early after depolarizations in the monophasic action potential.

In studies by Carlsson (1990) the subsequent administration of clofilium (20.8 μmol/kg) lead to a prolongation of the QTU interval and the monophasic action potential with the appearance of early after depolarizations. This was followed by ventricular arrhythmias in all rabbits. Pretreatment with prazosin (1 mg/kg iv) was able to attenuate the arrhythmia.

CRITICAL ASSESSMENT OF THE METHOD

Although this model has been established for some time, the sensitivity and selectivity of the model for detecting drugs that can induce clinically relevant arrhythmias is not established. For instance, in a slightly modified model (fentanyl and pentobarbital anesthesia), Weissenburger et al. (Weissenburger et al. 1993) were able to reproduce the finding with clofilium in terms of effects on the QT duration. However, they found a QT lengthening and bradycardia with sotalol (9 mg/kg) but without arrhythmias.

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I.D.4.4 Drug-Induced Proarrhythmic Effects in Dogs with Chronic AV Ablation

PURPOSE AND RATIONALE

The purpose of this type of study is to assess the proarrhyhmic activity of drugs in a model susceptible to arrhythmias triggered by drugs that prolong ventricular repolarization. This is accomplished by producing a chronic AV nodal ablation in dogs and then allowing them time to recover, which entails a bradycardia-induced ventricular hypertrophy and increased sensitivity to ventricular tachyarrhythmias due to electrical remodeling (Verduyn et al. 1997).

PROCEDURE

Male and female dogs > 20 kg are used for these studies. In an initial aseptic surgical procedure, a complete

AV block is created either by the injection of formalin into the AV node (Verduyn et al. 1997) or by AV nodal ablation (Rodriguez et al. 1998). Pacing electrodes are inserted epicardially into the basal portion of the right ventricle and the apex of the left ventricle and the wires are exteriorized at the back of the neck. The AV disruption leads to both ventricular hypertrophy and electrical remodeling over the ensuing 4–5 weeks, although the processes are not mutually dependent (Schoenmakers et al. 2003). Dogs are selected for susceptibility for developing torsade de pointes by using a known proarrhythmic drug, such as dofetilide (25 μg/kg/5 min, iv), and these are used in further studies.

Dogs are anesthetized and equipped with electrode patches on both sides of the chest attached to an electric defibrillilator. Programmed electrical stimulation is then performed using an epicardial electrode attached to a programmable stimulator capable of pacing synchronously to the QRS complexes. Unipolar stimuli are given using a pulse of 2 ms and a stimulus strength of 2-fold the diastolic threshold. An indifferent electrode is placed through the skin. Six surface ECG leads and one local ECG are measured throughout the study.

Pacing is done in four modes representing up to 3 cycle length changes. The pacing mode for the one cycle length change consists of three stimuli (NVS = 3) and continuous pacing for 30 seconds. Both are performed with equal interstimulus intervals and shortened from 1200 to 400 ms in 100 ms steps for NVS = 3, whereas continuous pacing is started just below the cycle length of the idioventricular rhythm and reduced to 1200, 900, 600 and 400 ms. The pacing modes with more than one frequency change are a basic train of eight stimuli followed by an extrastimulus (8 + 1) and a short/long/short sequence. The extrastimulus is shortened from 350 ms in 10 ms steps until the effective refractory period is reached, this being defined as the longest stimulus interval that is not followed by a ventricular complex. The effective refractory period is determined during idioventricular rhythm and at paced rates similar to the continuous pacing protocol. A short/long/shot sequence consists of (1) four to eight paced beats with an interval of 600 ms followed by a beat with an interval of 1200 ms and finally an extra stimulus (4 to 8 × 600/1200/extra), (2) 400/800/extra, and/or (3) 2 × 300/900/extra. The pacing protocol requires 30 to 40 minutes to complete and the different pacing modes are applied randomly.

EVALUATION

The evaluation involves determining incidence of arrhythmia or early after depolarizations after drug administration. A torsade de pointes arrhythmia is defined as a polymorphic ventricular tachycardia having 5 or more beats twisting around the baseline having a rate of more than 200 bpm and occurring in the setting of a prolonged QT duration. The arrhythmias either revert spontaneously or degenerate into ventricular fibrillation, which can then be terminated using cardioversion (60–70 J) if it persists longer than 10 s. No more than 6 defibrillations are performed per study.

Using an endocardial monophasic action potential recording, the incidence of early after depolarizations can be determined. The catheter (e.g. Franz combination catheter, EPT No. 1650) can be placed through the jugular vein or carotid artery under fluoroscopic guidance.

CRITICAL ASSESSMENT OF THE METHOD

This experimental model has the advantage of allowing one to study the arrhythmogenesis of a given drug directly, as opposed to surrogate endpoints (e.g. QT prolongation) that may suggest proarrhythmic activity. Whereas the chronic AV-block dog model is sensitive to drug-induced prolongation of the QT and arrhythmia, the relationship between this model and patients with long QT syndrome is unclear. Indeed, the incidence of torsade de pointes in this model is thought to be considerably higher than in most clinical settings (Weissenburger et al. 1993) such that a quantitative assessment of proarryhmic potential is not possible. Nevertheless, comparison of new drugs to reference drugs is possible.

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I.D.5 Supplemental and/or Follow-Up Studies

Several in depth pharmacological studies may be useful to characterize the safety range of a new drug, such as determination of cardiac output, ventricular contractility, vascular resistance, the effects of endogenous and/or exogenous substances on the cardiovascular responses, etc. They are described in detail in the second edition of the book: H.G. Vogel (ed) "Drug Discovery and Evaluation – Pharmacological Assays" Springer Verlag 2002.

I.D.5.1 In-Depth Hemodynamic Analysis in Anesthetized Dogs

PURPOSE AND RATIONALE

The hemodynamic effects of compounds supposed to affect the cardiovascular system are evaluated by measuring preload and afterload of the heart, contractility, heart rate, cardiac output and peripheral or coronary flow. To measure these cardiovascular parameters accurately, the use of larger animals such as dogs or pigs is necessary. This experimental model allows the classification of test drugs according to their action as having:

1. positive inotropic effects
2. negative inotropic effects (Ca^{2+}-antagonist, antiarrhythmic?)
3. hypertensive effects
4. hypotensive effects
5. coronary-dilating effects
6. β-Adrenoceptor blocking effects
7. α-Adrenoceptor blocking effects
8. anti-anginal effects
9. peripheral-vasodilating effects.

PROCEDURE

Male or female inbred Beagle or Labrador-Harrier dogs weighing between 15 and 25 kg are used. They are anesthetized with a bolus injection of 35–40 mg/kg pentobarbital, and continued with an infusion of 4–6 mg/kg/h. A catheter is placed into the cephalic vein for intravenous injections. Another catheter is placed into the duodenum for enteral administration. Respiration is maintained with room air through a tracheal tube using a positive pressure respirator, e.g., Bird-Mark-7-respirator. Blood gas analyses are performed at regular time intervals. Oxygen is supplied via the respirator as needed.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannula inserted into the left femoral artery and connected to a Statham pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left common carotid artery into the left ventricle. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dP/dt_{max} is differentiated and heart rate is counted. The LVP-signal also triggers a cardiotachometer.

Cardiac output, pulmonary artery pressure (PAP) and stroke volume are measured by a thermodilution technique using a Cardiac Output Computer (Gould/Statham SP 1245) and a balloon-tip triple lumen catheter (Gould SP 5105, 5F) with the thermistor positioned in the pulmonary artery via the jugular vein.

Myocardial oxygen consumption (MVO_2) is calculated as pressure-work-index according to Rooke and Feigl (1982).

Femoral blood flow and coronary flow are measured with electromagnetic flow probes attached to the femoral artery and the circumflex branch of the left coronary artery (LCX), respectively.

Experimental Course

When stable hemodynamic conditions and blood gas values of $pO_2 > 100$ mm Hg and $pCO_2 < 35$ mm Hg are achieved for at least 20 min (control values), the test substance is administered through a catheter inserted into a cephalic vein in doses of 0.1, 0.3, 1.0, and 3.0 mg/kg or into the duodenum in doses of 0.3, 1.0, 3.0, and 10.0 mg/kg.

All parameters are recorded continuously during the whole experiment.

Characteristics:

- blood pressure
  - systolic, BPs
  - diastolic, BPd
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure, LVEDP
- maximal rate of pressure rise, dP/dt_{max}
- heart rate, HR
- peripheral blood flow in A. femoralis, PF
- blood pressure A. pulmonalis, PAP
- coronary flow, CF
- cardiac output, CO

- stroke volume, SV
- total peripheral resistance, TPR
- left ventricular stroke work, LVSW
- left ventricular minute work, LVMW
- left ventricular myocardial oxygen consumption, MVO_2

EVALUATION

Besides the different directly measured hemodynamic parameters, the following data are calculated according to the respective formulae

1. stroke volume [ml/beat]

$$SV = \frac{CO}{HR}$$

2. total peripheral resistance [dyns/cm^5]

$$TPR = \frac{BPm}{CO} \times 79.9$$

3. left ventricle stroke work [J/beat]

$$LVSW = (BPm - LVEDP) \times SV \times 0.333 \times 10^{-3}$$

4. left ventricular minute work [J/min]

$$LVMW = LVSW \times HR$$

5. left ventricular myocardial oxygen consumption [ml O_2/min/100 g]

$$MVO_2 = K_1(BPs \times HR) + K_2 \times \frac{(0.8BPs + 0.2BPd) \times HR \times SV}{BW} + 1.43$$

$K_1 = 4.08 \times 10^{-4}$
$K_2 = 3.25 \times 10^{-4}$
BPs = systolic blood pressure [mm Hg]
BPd = diastolic blood pressure [mm Hg]
BPm = mean blood pressure [mm Hg]
HR = heart rate [beats/min]
CO = cardiac output [ml/min]
SV = stroke volume [ml/beat]
LVEDP = left ventricular enddiastolic pressure [mm Hg]
BW = body weight [kg]

Changes in parameters measured after drug administration are compared to control values obtained during the 20 min pre-drug period.

Results are presented as mean ±SEM with $n > 3$.

Statistical significance is assessed by means of the paired t-test.

MODIFICATIONS OF THE METHOD

Instead of dogs, pigs (German landrace) weighing between 20–35 kg can be used. They are pretreated with ketamine 500 mg/5 ml i.m., methomidate hydrochloride 200 mg/4 ml i.p., xylazine 60 mg/3 ml i.m., and anaesthetized with 15–20 mg/kg pentobarbital sodium, followed by continuous infusion of 12 mg/kg/h. The parameters are evaluated similarly to the experiments in dogs.

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I.D.5.2 Measurement of Heart Dimensions in Anesthetized Dogs

PURPOSE AND RATIONALE

The measurement of the heart dimensions allows to localize the effect of a drug on the activity of the heart. An ultrasonic technique is used for continuous measurement of left ventricular dimensions. Compounds are tested with potential anti-anginal activity due to the reduction of left ventricular diameter. The test is used to evaluate the influence of drugs on left ventricular external and internal diameter in anesthetized dogs.

PROCEDURE

Male or female Beagle or Labrador-Harrier dogs weighing 15–25 kg are used for the test. The dog is anesthetized by intravenous injection of 35–40 mg/kg pentobarbital sodium followed by subcutaneous injection of 2 mg/kg morphine. Respiration is maintained through a tracheal tube with N_2O/O_2 (3:1) using a positive pressure respirator.

Implantation of Ultrasonic Transducers

Ultrasonic transducers are constructed and implanted as described by Stinson et al. (1974).

To measure left ventricular external diameter (LVED), two ultrasonic transducers are fixed to the left ventricular wall. One crystal is sutured to the posterior wall within the rectangular area formed by the left circumflex coronary artery and the left posterior descending artery. The other one is placed near the first diagonal branch of the left anterior descending coronary artery. Exact positioning is assured with an oscilloscope.

To measure left ventricular internal diameter (LVID), the transducers are placed in the same anatomical area as for the epicardial crystals. However, they are pushed through the wall of the left ventricle through stab wound incisions. The crystals are positioned across the greatest transverse diameter of the left ventricle, one on the anterior and the other on the posterior endocardial wall.

Bleeding during the implantation procedure is controlled by umbilical tapes around the cranial and caudal veins and by purse string sutures at the implantation sites. The pericardial incision and the chest is closed by sutures and the transducer wires are connected to the recording equipment.

In each dog, either LVED or LVID is measured together with the other hemodynamic parameters.

Preparation for Hemodynamic Measurements

Blood pressure is recorded through a cannulated femoral artery by a pressure transducer (Statham P 23 DB).

For determination of LVP, a Millar microtip catheter (type PC 350) is inserted via the left A. carotis communis. LVEDP is measured on a high-sensitivity scale. From the pressure curve, dP/dt_{max} is differentiated and heart rate is calculated.

Hemodynamic parameters are recorded continuously during the whole experiment.

Experimental Course

When stable hemodynamic conditions are achieved for at least 30 min (control values), the test substance is administered by intravenous or intraduodenal injection.

Readings are taken at times 0, 15, 30, 45, 60, 75, 90 and 120 after drug administration. Left ventricular dimensions are measured at the end of the diastole and systole.

Characteristics:

- blood pressure
  - systolic blood pressure
  - diastolic blood pressure
- left ventricular pressure, LVP
- left ventricular enddiastolic pressure
- left ventricular contractility, dP/dt
- heart rate, HR
- left ventricular external diameter, LVED
- left ventricular internal diameter, LVID

EVALUATION

Hemodynamic parameters, LVED and LVID [mm] are determined.

Changes in parameters after drug administration are compared to control values obtained during the 30 min pre-drug period.

Statistical significance is assessed by means of the paired t-test.

Since a change in the diameter of the left ventricle is a reasonable accurate index of left ventricular volume, a reduction of LVED or LVID with no change in dP/dt and HR can be considered as a strong indicator for "venous pooling" and thus an anti-anginal activity of a compound.

Scores are allotted relative to the efficacy of standard compounds assessing the intensity as well as the duration of the effect.

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I.D.5.3 Baroreceptor Reflexes

I.D.5.3.1 Influence on Orthostatic Hypotension

PURPOSE AND RATIONALE

Orthostatic hypotension with dizziness up to unconsciousness is a syndrome occurring in many human

individuals. Moreover, several drugs are known to cause orthostatic hypotension. In several animal species, such as rabbit, cat and dog, this syndrome can be evoked by changing the usual horizontal position into a vertical position with the head upwards using a tilting table.

PROCEDURE

Cats of either sex weighing 2.0–3.0 kg are temporarily anesthetized with ether. Anesthesia is maintained by intravenous injection of 70 mg/kg chloralose. The animal is fixed with its legs on a heated operating table which can be tilted by 90 degrees. The carotid artery is cannulated for measuring blood pressure through a Statham P 23 Db transducer on a 6 channel Hellige recorder. The femoral vein is cannulated for injection of the test compound. After the blood pressure is stabilized for 30 min, the animal is quickly tilted to a vertical position for 1 minute. Due to the change of position and gravitational force, there is a rapid fall in blood pressure which recovers as soon as the animal is restored to its original position. After taking the control reading, the test compound is administered intravenously and the same procedure is repeated. The fall in blood pressure is recorded.

EVALUATION

A significant increase in postural hypotension with respect to the control would indicate that the test compound may produce orthostatic hypotension in human. Moreover, some compounds, like sympathomimetics, can reduce or prevent postural hypotension.

MODIFICATIONS OF THE METHOD

Methods for evaluating postural hypotension were described in conscious dogs (Baum et al. 1981), conscious rabbits (Sponer et al. 1981), in rats (Lee et al. 1982) and in cynomolgus monkeys during ketamine anesthesia (Pals and Orley 1983).

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I.D.5.3.2 Bezold–Jarisch Reflex

PURPOSE AND RATIONALE

The circulatory collapse after intravenous injection of veratrine has been first described in cats and is known as BEZOLD–JARISCH reflex (Bezold and Hirt 1867; Jarisch and Richter 1939a,b; Jarisch 1940). Fleckenstein et al. (1950) recommended this as a suitable animal model of shock. The original observation was a triphasic blood pressure response in cats or dogs characterized by a short lasting fall in blood pressure accompanied by bradycardia, followed by a short lasting increase and than a long-lasting decrease of blood pressure after intravenous injection of veratridin or other veratrum alkaloids. Kalkman et al. (1984) showed that three distinct subtypes of serotonergic receptors mediate the triphasic blood pressure response to serotonin observed in the Bezold–Jarisch reflex. In cats and dogs, the Bezold-Jarisch reflex was elicited by veratrine and veratridine, but also by capsaicin and the 5-HT_3 receptor agonists 2-methyl-5-HT, phenylbiguanide, chlorophenylbiguanide and serotonin itself. In rats, mostly 5-HT or 2-methyl-5-HT were used as stimuli to characterize 5-HT_3 receptor antagonists.

PROCEDURE

Male Sprague Dawley rats weighing 250–380 g are given food and water ad libitum, except those used for intraduodenal drug administration; these rats are deprived of food overnight. The animals are anesthetized by intraperitoneal injection of 1.5 g/kg urethane. Body temperature is maintained at 37 °C by placing the animal on a heating pad. The left jugular vein or duodenum, trachea and left femoral vein are cannulated for drug administration (i.v. or i.d.), facilitation of respiration and injection of 2-methyl 5-HT, respectively. Heart rate is derived from a limb lead II ECG monitored via subdermal platinum electrodes and is recorded with amplifiers on a polygraph. A dose-response curve to 2-methyl 5-HT (5–100 μg/kg, i.v.) is constructed in each rat to establish a submaximal dose (usually 10 or 20 μg/kg, i.v.) which elicits a reproducible bradycardic response. Each rat receives then a single dose of test drug or standard and is then challenged with 2-methyl 5-HT at 5, 15, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min post dosing. A separate group of rats receiving

vehicle (saline for i.v., deionized water for i.d.) is similarly tested in each study.

EVALUATION

Duration of action of the compounds is assessed by determining the period of time for which the inhibitory effects remain significantly different from vehicle controls. Statistical analysis of the data is performed by a repeated measure analysis of variance (ANOVA) followed by pairwise comparisons against control at each time period using Fisher's LSD multiple comparison test.

MODIFICATIONS OF THE METHOD

Harron and Kobinger (1984) used capsaicin to elicit the Bezold-Jarisch reflex in anesthetized artificially respirated dogs pretreated with a beta-adrenoceptor antagonist to evaluate the activity of clonidine-like drugs on central alpha$_2$ adrenoceptors after intracisternal administration.

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I.D.5.4 Measurement of Cardiac Output and Regional Blood Flow with Microspheres

PURPOSE AND RATIONALE

The microsphere technique allows the measurement of cardiac output and regional blood flow. Using different radionuclides, repeated determinations are possible. The method is applicable not only for dogs, cats, and minipigs (Hof et al. 1980) but also for rats (McDevitt and Nies 1976; Bonnacrossi et al. 1978; Ishise et al. 1980; Stanek et al. 1985) using microspheres of appropriate size.

PROCEDURE

Male Sprague-Dawley rats weighing 265–375 g are anesthetized with 35 mg/kg i.p. pentobarbital. The right carotid and right femoral arteries are cannulated. Using pressure monitoring, a carotid cannula is manipulated into the left ventricle. Carbonized microspheres (15 ± 5 μ diameter) labelled with ^{85}Sr are drawn into a glass injection chamber and suspended in 0.3 ml 6 % dextran so that each chamber contains 60 000 to 80 000 microspheres. The radioactivity in each chamber is determined by gamma scintillation counting before and after microsphere injection, the difference being the amount of radioactivity injected. The microspheres are injected into the left ventricle in a total volume of 0.8 ml 6 % dextran over 20 s. Simultaneously, arterial blood from the femoral artery is withdrawn at 0.8 ml/min for 90 s with a syringe withdrawal pump.

EVALUATION

This reference blood sample is used to calculate the cardiac output by the formula:

$$\text{cardiac output} = \text{counts injected} \times \frac{\text{reference sample withdrawal rate}}{\text{reference sample counts}}$$

After obtaining the reference sample, the animals are sacrificed with pentobarbital and the organs dissected, placed in counting vials, and counted for 5 min. Regional distribution of the cardiac output is calculated by comparing the radioactivity in each organ with the total injected radioactivity. Organ flow is determined by multiplying the cardiac output by the fractional distribution of the cardiac output to the organ.

CRITICAL ASSESSMENT OF THE METHOD

Problems associated with the microsphere technique in rats are the hemodynamic effects of the solutions used to inject the microspheres and the effects of blood withdrawal after repeated determinations (Stanek et al. 1985).

MODIFICATIONS OF THE METHOD

For repeated determinations, other nuclides have been used, such as ^{46}Sc, ^{51}Cr, ^{141}Ce, ^{125}I (Hof et al. 1980).

Kovách et al. (1992) used up to 5 radiolabelled microspheres (^{57}Co, ^{113}Sn, ^{85}Sr, ^{95}Nb and ^{46}Sc) for measurement of regional cerebral blood flow in cats.

Faraci and Heistad (1992) measured blood flow with radioactive microspheres (15 μ diameter) labeled with ^{46}Sc, ^{95}Nb, ^{153}Gd, ^{85}Sr, and ^{141}Ce in anesthetized rabbits.

Kowallik et al. (1991) measured regional myocardial blood flow with multiple colored microspheres. The method yielded values very similar to those obtained with radioactive microspheres.

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Chapter I.E
Safety Pharmacology of Drugs for the Urinary Tract

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Based on the second edition of "Drug Discovery and Evaluation – Pharmacological Assays" edited by H.G. Vogel, Springer-Verlag 2002, with contributions by H.G. Vogel, R. Gregor, H. Gögelein, M. Hropot and M. Bleich.

I.E.1 General Functional Assessments

I.E.1.1 In vitro and in situ Assessments

I.E.1.1.1 Carbonic Anhydrase Inhibition in vitro

PURPOSE AND RATIONALE

Acetazolamide (Diamox) was one of the first synthetic non-mercurial diuretics. The mode of action was found to be inhibition of carbonic anhydrase. Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reversible hydration (or hydroxylation) of CO_2 to form H_2CO_3 which dissociates non-enzymatically into HCO_3^- and H^+. At least three isoenzymes, designated as I, II and II or A, B and C, are known to exist. The chemistry, physiology and pharmacology of carbonic anhydrase has been extensively reviewed by Maren 1967. In spite of the fact that newer diuretics are based on other modes of action, the test for inhibition of carbonic anhydrase should be performed for evaluation of a new compound. Moreover, the specific use of carbonic anhydrase inhibitors as antiglaucoma drugs has been described (Friedland and Maren 1984; Caprioli 1985). The mechanism by which carbonic anhydrase inhibitors lower intraocular pressure is through a reduction in aqueous humor formation, by affecting electrolyte and water balance in the nonpigmented ciliary epithelium (Friedland and Maren 1984; Caprioli 1985). Although many methods to measure carbonic anhydrase activity have been developed (Philpot and Philpot 1936), the micro method described by Maren (1960) is relatively simple, sensitive and reliable. The enzyme source is erythrocytes, a rich source of the same isoenzymes found in the eye (Maren 1967; Armstrong et al. 1966; Wistrand et al. 1986; Wistrand and Knuutila 1980).

PROCEDURE

Materials and Solutions

- phenol red indicator solution:
- 2.6 mM $NaHCO_3$, pH 8.3 + 218 mM Na_2CO_3
- 1 M sodium carbonate/bicarbonate buffer, pH 9.8
- Enzyme: Carbonic anhydr 12.5 mg phenol red/literase from dog blood; Blood is collected into a heparinized tube and diluted 1:100 with deionized water
- Equipment:
- Reaction vessel – custom made by Labglass Inc., Vineland, NJ, USA
- Monostat bench mounted flowmeter
- 30 % CO_2 – M&G Gases, Branchburg, NJ, USA.

Assay

CO_2 flow rate is adjusted to 30(45) ml/min. The following solutions are added to the reaction vessel:

- 400 µl: phenol red indicator solution
- 100 µl: enzyme
- 200 µl: H_2O or appropriate drug concentration after 3min for equilibration:
- 100 µl: carbonate/bicarbonate buffer is added.

The following parameters are determined in duplicate samples:

T_u = (uncatalyzed time) = time for the color change to occur in the absence of enzyme

T_e = (catalyzed time) = time for the color change to occur in the presence of the enzyme

$T_u - T_e$ = enzyme rate

T_i = enzyme rate in the presence of various concentrations of inhibitor.

EVALUATION

Percent inhibition of carbonic anhydrase is calculated according to the following formula:

Standard data:

Compound: IC_{50} [M]

Acetazolamide: 9.0×10^{-9}

Chlorothiazide: 9.0×10^{-7}

LIMITATIONS OF THE METHOD

Determination of carbonic anhydrase inhibition is of value to characterize the activity spectrum of sulfonamide diuretics.

MODIFICATIONS OF THE METHOD

Landolfi et al. (1997) reported a modified procedure for the measurement of carbonic anhydrase activity. The measure of carbonic anhydrase activity is based on the rate of CO_2 hydration by the enzyme. Such transformation was monitored by a procedure which consists of the measure of time necessary for the pH of an appropriate buffer to decrease from 8 to 7.5 in the presence of a constant CO_2 flow: this time period is dose-dependently reduced by the addition of the enzyme and further modified in the presence of carbonic anhydrase inhibitory compounds.

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I.E.1.1.2
Inhibition of Xanthine Oxidase in vitro Indicating Hypouricemic Activity

PURPOSE AND RATIONALE

Xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to uric acid. Xanthine oxidase is a complex metalloflavoprotein containing one molybdenum, one FAD and two iron-sulfur centers of the ferredoxine type in each of its two independent subunits. Usually, the enzyme is isolated from cow's milk. The enzyme is inhibited by allopurinol and related compounds. The production of uric acid from the substrate (xanthine) can be determined by measuring the change in optical density in the UV range.

PROCEDURE

The test compound is incubated with xanthine oxidase (usually derived from milk, sometimes derived from rat liver or small intestine), EDTA and phosphate buffer solution (pH 7.8) at 37 °C. Control solutions without test compound are incubated under identical conditions. Following addition of xanthine, the change in absorbance is determined.

Assay conditions:

- wavelength: 293 nm
- line path: 10 mm
- final volume: 1.0 ml.

EVALUATION

The percent inhibition of xanthine oxidase is determined relative to control solutions.

IC_{50} values of test compounds are calculated.

Standard data:

Allopurinol: IC_{50}: ca. 10–8 mol/l

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I.E.1.1.3
Urate Uptake in Brush Border Membrane Vesicles

PURPOSE AND RATIONALE

The urate-anion exchanger system in brush border membrane vesicle, which mediates hydroxyl ion gradient-dependent urate uptake, is the most likely route for the mediation of urate transport in the first step of urate reabsorption in the proximal tubules. Luminal drugs which inhibit urate reabsorption are inhibiting the transport of urate by blocking the urate/anion exchanger.

PROCEDURE

Male Sprague Dawley rats weighing 320–380 g are euthanatized by exsanguination under anesthesia and the kidneys are removed immediately. All steps for the preparation of brush border membrane vesicles are carried out at 4 °C. Renal cortex is homogenized for 2 min in a medium containing 250 mmol/l mannitol, 10 mmol/l Tris, 16 mmol/l HEPES buffer (pH 7.5) using a Physcotron homogenizer. The homogenate is centrifuged at 2400 g for 10 min and the supernatant is centrifuged at 2800 g for 20 min. Subsequently, the supernatant is discarded and the loosely packed membrane-rich layer is flushed off from the bottom densely packed brown pellet. The membrane-rich layer is resuspended manually in 250 mmol/l mannitol containing 10 mmol/l Tris-HEPES (pH 7.5) using a Dounce homogenizer and $MgSO_4$ is added to a final concentration of 10 mmol/l. After standing for 20 min, the suspension is centrifuged at 2400 g for 20 min and the supernatant containing brush border membranes is recentrifuged two more times at 2400 g for 20 min. The final supernatant is centrifuged at 28 000 g for 20 min and the pellet is suspended in a small amount of medium containing 150 mmol/l mannitol, 50 mmol/l

potassium phosphate buffer (pH 7.5) and 2 mmol/l $MgSO_4$ to a final protein concentration of 4–8 mg/ml. The brush border membrane vesicle preparation is frozen and stored at – 80 ° C until use.

After preincubation of the brush border membrane vesicle preparation for 2 h, [2^{-14} C]urate uptake is initiated by adding 200 µl of incubation medium to 20 µl of the membrane suspension. The incubation medium has the following composition (mmol/l): 150 mannitol, 2 $MgSO_4$, 50 potassium phosphate buffer, pH 6.0 or 7.5, 0.02 [2^{-14} C]urate, and various concentrations of the inhibitor. At 10 s after the addition of the incubation medium, 200 µl portions of the suspension are pipetted onto the center of prewetted cellulose acetate filters kept under suction. The vesicles retaining on the filter are washed immediately with 5 ml of an ice-cold solution containing 150 mmol/l mannitol and 50 mmol/l potassium phosphate buffer, pH 6.0 or 7.5, which is used at the same pH as the incubation medium. Preincubations and incubations are performed at 23 ± 1 °C. Each experiment is performed in triplicate. Corrections are made for the radioactivity bound to the filters in the absence of membrane vesicles. The term of the OH^-gradient-dependent urate uptake is defined as the difference between the uptakes in the incubation medium at pH 6.0 and that at pH 7.5. The OH^-gradient-dependent urate uptake at 10 s is assumed to present an initial velocity.

EVALUATION

From a concentration-response curve relating log concentration of drug to the logit activity of the OH^- gradient-dependent urate uptake for 10 s, IC_{50} (concentration producing 50 % of inhibition) is determined by least-squares regression analysis.

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I.E.1.1.4
Patch Clamp Technique in Kidney Cells

PURPOSE AND RATIONALE

In the different parts of the kidney (proximal tubules, distal tubules, collecting ducts) fluid is reabsorbed and substances may be transported either from the tubule lumen to the blood side (reabsorption) or vice versa (secretion). Besides active transport and coupled transport systems, ion channels play an important role in the function of kidney cells. The various modes of the patch clamp technique (cell-attached, cell-excised, whole-cell mode) (Neher and Sakmann 1976; Hamill et al. 1981) allow the investigation of ion channels. In addition, the investigation of other electrogenic transport mechanisms, such as the sodium-coupled alanine transport can be studied.

PROCEDURE

The patch clamp technique can be applied to cultured kidney cells (Merot et al. 1988), to freshly isolated kidney cells (Hoyer and Gögelein 1991) or to cells of isolated perfused kidney tubules (Gögelein and Greger 1984). The latter method shall be described in more detail.

Segments of distal superficial proximal tubules of rabbit kidney are dissected and perfused from one end with a perfusion system (Burg et al. 1966; Greger and Hampel 1981). The non-cannulated end of the tubule is freely accessible to a patch pipette. Under optical control (differential interference contrast optics with 400 × magnification) the patch pipette can be moved through the open end into the tubule lumen and is brought in contact with the brush border membrane. After slight suction of the patch electrode, gigaseals form instantaneously and single potassium or sodium channels can be recorded in the cell-attached or inside-out cell-excised mode (Gögelein and Greger 1984, 1986).

In order to obtain exposed lateral cell membranes suitable to the application of the patch clamp method, pieces of the tubule are torn off by means of a glass pipette (diameter about 40 µm). In order to facilitate the tearing off, the tubules are incubated for about 5 min in 0.5 g/l collagenase (Sigma, C 2139) at room temperature. After tearing off part of the cannulated tubule, clean lateral cell membranes are exposed at the non-cannulated end. The patch pipette can be moved to the lateral cell membrane and gigaseals can be obtained. It was possible to investigate potassium channels (Gögelein and Greger 1987) and nonselective cation channels (Gögelein and Greger 1986) in these membranes.

As cells are still part of an epithelial layer and, therefore, are intracellularly coupled, the whole-cell technique is not appropriate in this preparation. On the other hand, cotransport systems can only be investigated by the whole-cell method because the transport

rate of a single event is much too small to be resolved in a similar manner as single ion channel events. Consequently, cells of rabbit proximal tubules are isolated as described in detail elsewhere (Hoyer and Gögelein 1991; Heidrich and Dew 1977). After euthanasia the kidneys are rapidly excised and placed in ice-cold solution [mmol/l]: 150 K-cyclamate, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$, pH 7.4. The following steps are performed on ice: After decapsulation, superficial cortical slices of about 0.5 mm thickness are dissected and minced with a scalpel. The tissue is homogenized in a Dounce homogenizer by three strokes with a loose-fitting pestle. The homogenate is then poured through graded sieves (250, 75 and 40 μm) to obtain a population of single cells. Since the predominant tubule section of the cortex of the rabbit kidney is the pars convoluta of the proximal tubule, it can be concluded that the majority of the isolated cells in the cell suspension are of proximal tubule origin. By light microscopy cells are identified by long microvilli distributed over the entire cell surface and can easily be discriminated from remaining erythrocytes, cell detritus and tubular fragments.

By application of the whole-cell mode of the patch clamp technique to freshly isolated cells of convoluted proximal tubules, the sodium-alanine cotransport system could be investigated in detail (Hoyer and Gögelein 1991).

EVALUATION

In isolated perfused renal tubules, concentration response curves of drugs which inhibit ion channels can be obtained with the patch clamp technique. In isolated cells of the proximal tubule, the whole-cell mode of the patch clamp technique enables the investigation of the sodium-alanine cotransport system. The apparent K_m values for sodium and L-alanine can be recorded.

MODIFICATIONS OF THE METHOD

Schlatter (1993) recorded membrane voltages of macula densa cells with the fast or slow whole-cell patch-clamp method. The effects of diuretics and the conductance properties of these cells were examined.

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I.E.1.1.5
Perfusion of Isolated Kidney Tubules

PURPOSE AND RATIONALE

The various tubule segments: proximal tubule (PT, S1-S3); descending thin limb of the loop of Henle (DTL); ascending thin limb of the loop of Henle (ATL); thick ascending limb of the loop of Henle (TAL); distal convoluted tubule (DCT); connecting tubule (CNT); cortical collecting duct (CCD); medullary collecting duct (MCD); papillary collecting duct (PCD) have different functional properties. The in vitro perfusion of isolated tubule segments (Burg et al. 1966) is the method of choice if one has to identify the site and the mechanism of action of a pharmacological agent which has been shown to act on kidney function in clearance and micropuncture studies.

PROCEDURE

After its invention by Burg et al. (1966) this technique has been used successfully in the kidney tubule segments of several species: man, rabbit, rat, mouse, hamster, snake, birds etc. The tubule segments are dissected from thin kidney slices (< 1 mm thickness). Usually dissection can be done using sharpened forceps or needles without the addition of proteases (collagenase). The segment is identified by its anatom-

ical location and by its appearance. A 20–50 × lens is used for dissection. Dark field illumination is helpful for the identification of the segment under study. PT, TAL, DCT, CNT, CCD, MCD can all be dissected quite easily. The dissection of DTL, ATL and PCD is much more difficult because these segments are damaged easily by the mechanical dissection. Dissection is usually performed at 4 °C in a Ringer type solution.

For smaller species, proximal tubule segments can also be isolated from the entire kidney using in situ collagenase perfusion techniques (Tyson et al. 1990). Following anesthesia, the kidneys may be perfused via the aorta (mice) or renal arteries (rats, rabbits) for five minutes to remove the residual blood; the kidneys and associated blood vessels are then removed and the perfusion continued for an additional 15–20 minutes with buffer containing 180 U/ml of Type I collagenase. The cortical tissue is then removed from the medulla and the tubules isolated as described above. The viability and function of tubules so isolated are comparable to those isolated without collagenase (Rodeheaver et al. 1990).

The dissected segment is transferred into the perfusion chamber by a transfer pipette. The perfusion chamber is mounted in the stage of an inverted microscope (20–400 ×). The chamber is usually kept at 37 °C, and the bath perfusate is also preheated to this temperature. The bath perfusate will depend on the tubule segment under study. In most instances it will contain HCO_3^- and will be bubbled with CO_2. The metabolic substrate will be acetate for PT and D-glucose for TAL, CCD etc. The actual perfusion is performed with two sets of concentric glass pipettes, one set at the perfusion end; and one at the collection end of the segment. These pipettes are manufactured with special glass forges. The most refined one has been designed by Hampel and Greger. The glass tube is rotated at approximately 1 rps, is moved in perpendicular direction by a remote control, and the heating filament is moved in xy direction also by a remote control. The shaping of the glass is observed continuously by a lens (5–50 ×). The pulling force is provided by weights fixed on the lower end of the glass tube. The pipettes are cut either by a diamond or by the pulling force of a small glass bead, fixed on the edge of a vertical platinum filament and melted sidewise on the pipette. When the heating current of the platinum filament is switched off the filament retracts and breaks the pipette at the desired site. Greger and Hampel (1981) have modified the original perfusion system of Burg and co-workers. Their device is optimized inasmuch as it guarantees concentric alignment of the various pipettes. The forward and backward movement is controlled by small electric motors. At the perfusion side they use 4 concentric pipettes. The outermost one contains sylgard and is driven over the perfused end of the tubule in order to seal this end. The tubule is held by a holding pipette with appropriate dimensions. The tubule is sucked into this pipette up to the constriction. Then the perfusion pipette with a tip diameter smaller than the inner diameter of the perfused segment is advanced into the segment held by the holding pipette. The perfusion pipette is put under hydrostatic pressure of a few to 100 cm to achieve a perfusion rate of 1–20 nl/min. Usually the collapsed tubule lumen opens when the perfusion pipette is advanced. The pipette is advanced in the lumen until it reaches an area of the segment where it appears intact by inspection (200–400×). Within its lumen the perfusion pipette contains yet another pipette, the fluid exchange pipette. With this pipette the composition of the perfusate can be replaced very rapidly (Greger and Schlatter 1983). The collection end of the tubule segment is sucked into a holding pipette. A sylgard pipette is advanced to seal the collection site. The holding pipette at the collection site will contain mineral oil in flux measurements. Then a collection pipette is advanced through the oil to quantitatively collect the perfusate delivered by the tubule.

The **measured parameters** can be as follows:

Flux measurements (Schafer et al. 1974). The collection rate (V_c, nl/min) can be measured by the constant bore collection pipette by timed collections. Radioactive tracers can be added to the lumen or bath fluid. For instance, radioactively labelled inulin can be added to the perfusate (In_p) and can be used to measure volume absorption DV = perfusion rate (V_i–V_c). Unidirectional fluxes, bath to lumen and lumen to bath, for any given substance can be quantified, and permeabilities (P_x) can be determined:

$$P_x = (V_i - V_c)L^{-1}[ln(x_p In_c x_c^{-1} In_p) + 1] ,$$

where L is the length of the segment; x_p and x_c are the concentrations of x in the perfusate and in the collected fluid; and In_c is the inulin concentration in the collected fluid. Net fluxes of x can be determined as the difference of the unidirectional fluxes or by the chemical determination of Dx (perfusate-collected fluid). This requires very sensitive methods. Electron probe analysis of Na^+, K^+, Ca^{2+}, Mg^{2+} etc. has been used to determine the net transport of these ions in various tubule segments (Wittner et al. 1988). Flux studies are usually performed at low luminal perfusion rates of a few nl/min. Substances under study can be added to the luminal and bath perfusate, and paired data can be obtained under control and experimental conditions

(Burg and Green 1973; Stoner et al. 1974; Burg 1980; Burg and Stoner 1976; Dillingham et al. 1993).

Transepithelial electrical measurements. The perfusion pipette can be connected to the high impedance input of an electrometer. The voltage is referenced to the grounded bath. The connections are usually made with agar bridges (80 g agar in 1l Ringer's solution), and appropriate corrections for liquid junction voltages must be applied. With identical solutions in the bath and in the lumen and with high luminal perfusion rates (> 10 nl/min), any transepithelial voltage (V_{te}) must be caused by active transport = active transport potential (Frömter 1984). Hence, the effectiveness of putative inhibitors of active transport can also be examined by the measurement of V_{te}. According to Ohm's law the determination of the flux of ions also requires the measurement of transepithelial resistance. Greger (1981) has introduced a method which utilizes a dual channel perfusion pipette, made of Q-shaped glass. One channel is used for perfusion and the other for current (I_{te}) injection. The current is defined by a resistor chosen such that the deflection in V_{te} generated by this pulse is in the order of 10–20 mV. Transepithelial resistance (R_{te}) can now be calculated from DV_{te} and I_{te}. The ratio of V_{te} and R_{te} is called equivalent short circuit current. It is directly proportional to active transport (Greger 1985). The measurement of V_{te} and R_{te} is much more efficient than flux studies for pharmacological screening, provided that the process under study produces a transepithelial voltage. Several substances can be examined in one single tubule in strictly paired fashion (Schlatter et al. 1983; Wangemann et al. 1986). The time resolution of the measurements is on the order of 1 s, whereas that of flux studies is several minutes at best.

Intracellular electrical measurements. Greger and Schlatter (1983) have developed a method for the use of impalement techniques in the isolated perfused tubule. Very fine tip microelectrodes ($\phi < 100$ nm) are used to impale the tubule cell across the basolateral membrane. The actual impalement is performed by a piezo stepper which accelerates the microelectrode to high speed, which makes it possible to penetrate the rigid basal membrane. The simultaneous measurement of V_{te}, R_{te}, and basolateral membrane voltage (V_{bl}) allows for a complete analysis of voltages and resistances (Greger 1985; Ullrich and Greger 1985). Ion selective microelectrodes can also be used in impalement studies, and the cytosolic ion activities for e.g. Na^+, K^+, Cl^- can also be determined (Greger 1985). These methods are all rather difficult to perform. They are of high relevance for the understanding of the function of a given tubule segment and for the detailed description of the mechanism of action of a drug, which, in preceding studies has been shown to act in a given tubule segment.

Patch clamp studies. The combination of in vitro perfusion of renal tubules and patch clamp analysis of ion channels in the luminal and basolateral membranes is described in Sect.C.1.1.4.

Fluorescent dyes in the isolated perfused tubule. Several fluorescent dyes for the monitoring of Na^+, K^+, Cl^-, Ca^{2+}, pH have become available during the past few years. These dyes can be used in the in vitro perfused tubule (Nitschke et al. 1991). The inverted microscope is equipped with an appropriate illumination and filter wheel for excitation. The emission is measured by photon counting or by a video camera. When compared with impalement methods, these techniques are probably easier for routine use.

EVALUATION

For each of the above protocols paired measurements of one or several given parameters of tubule transport are obtained under control conditions and in the presence of a substance under study. Also concentration response curves can be obtained in one single preparation (Schlatter et al. 1983; Wangemann et al. 1986; Wittner et al. 1987). Intracellular measurements are usually required to define the mechanism of action (Greger 1985). Especially the electrical and optical measurements have a very high reproducibility. For screening usually 3 preparations are sufficient. Approximately 10 preparations are required for concentration response curves.

MODIFICATIONS OF THE METHOD

All of the above assessments can also be performed in isolated nephron segments in an in vivo preparation. After appropriate anesthesia, the kidney is isolated and the segment of interest is identified on the surface; the segment of interest is then isolated from the glomerular filtrate by placing an oil and wax block between the segment of interest and the glomerulus. The proximal and distal tubules are readily identifiable and accessible on the kidney surface. With this technique it is also possible to perfuse the loop of Henle by placing the perfusion pipette into the last accessible loop of the proximal tubule and the collection pipette into the first accessible loop of the distal tubule (Ramsey and Knox 1996).

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I.E.1.1.6
Isolated Perfused Kidney

PURPOSE AND RATIONALE

The isolated kidney is a good tool for studying the proximal tubule, but is of limited value for distal tubule function. The kidney can be perfused in situ and/or isolated in vitro. The isolated kidney can be perfused by a pump using blood or plasma-like solutions. One specific problem of the blood-perfused dog kidney in vitro is its instability. After only 1 h of perfusion, glomerular filtration and renal blood flow decline markedly. It was reported that in situ-perfused isolated dog kidney seems to be more stable. In isolated perfused rat kidney plasma-like solutions are used for perfusion. This system, by inclusion of a dialyzing unit, provides optimal conditions for maintaining a constant electrolyte composition of the perfusate. However, function of distal tubule is also grossly impaired in this rat model. The isolated kidney does not acidify tubular fluid, and the concentrating ability is reduced.

PROCEDURE

Kidneys are obtained from anesthetized male rats with a body weight of 300 to 400 g. The donor animals are fasted overnight prior to surgery, but have free access to water. After the abdominal cavity is exposed by a ventricular incision, the right ureter is cannulated with PE-50 polyethylene tubing and heparin is injected into the vena cava (500 U/kg body weight). The venous cannula is introduced into the vena cava below the right renal vein. The right kidney is freed from the perirenal fat, not disrupting the renal capsule. The renal artery is cannulated via the superior mesenteric artery without interruption of flow. Thereafter, the kidney is continuously perfused with a perfusion solution fed from the gravity system situated 130 cm above the cannula. Ligatures around the renal artery and vena cava above the renal pedicle are tied. The kidney is then removed from the animal and placed in a Plexiglas chamber. A perfusion pressure of 80–90 mmHg in the renal artery is maintained by adjusting the speed of the perfusion pump. For more details see references.

EVALUATION

After the equilibration period, clearance periods of 20 min are used. Urine samples are collected and perfusate is obtained at midpoint of the clearance period for the evaluation of overall kidney function. For determination of glomerular filtration rate (GFR) and fluid transport, ^{3}H-labelled polyethylene glycol is added to a modified Krebs-Henseleit bicarbonate buffer. Electrolytes are determined in urine by standard flame photometry. Fractional excretions of water, electrolytes and test compounds are calculated.

MODIFICATIONS OF THE METHOD

Tarako et al. (1991) evaluated oxygen supply and energy state in the isolated perfused rat kidney. Metabolic activities of the isolated perfused rat kidney were described by Nishiitsutsuji-Uwo et al. (1967). Cox et al. (1990) used the isolated perfused rat kidney as a tool in the investigation of renal handling and effects of nonsteroidal anti-inflammatory drugs.

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I.E.1.1.7
Micropuncture Techniques in the Rat

PURPOSE AND RATIONALE

Micropuncture techniques have been applied to the direct investigation of the effect of diuretics on single nephron function. The observed changes in tubular fluid reabsorptive rates and electrolyte concentrations can be used to asses the mechanism of action. The rat is the model of choice since proximal and distal tubules as well as collecting ducts are accessible for micropuncture.

PROCEDURE

Clearance and free-flow micropuncture studies are performed in rats with a body weight of about 250 g. The animals are fasted for 16 h before the beginning of the experiment, but have free access to tap water. After anesthesia the animals are placed on a thermostatically heated table. Thereafter rats are tracheotomized and carotid artery and jugular vein are cannulated for blood pressure recording, blood sampling, and for infusion of compounds, respectively. The left kidney is carefully exposed by a flank incision, embedded in a small plastic vessel with cotton wool, and bathed with paraffin oil at 37 °C. The ureter is cannulated and rectal temperature monitored continuously. A bolus injection of 75 mCi inulin ^{3}H in 0.7 ml NaCl solution is given, followed by 0.85 % NaCl solution at a rate of 2.5 ml/min per 100 g body weight. The sustained infusion delivers 75 mCi inulin ^{3}H per hour. The control puncture of tubules is performed 45 min after beginning of the intravenous infusion. The direct collection of tubular fluid samples from proximal and distal tubules is carried out with glass capillaries of 8 to 10 mm external diameter using a micromanipulator and microscopic observation. Distal tubules are identified by intravenous injection of lissamine green. The control period is followed by the test period. After an equilibration period of 30 min with the compound to be tested, micropuncture is performed again and tubular fluid is collected. The ureteral urine is collected and blood sampling is performed in the middle of each clearance period.

EVALUATION

The following parameters may be determined: inulin clearance (GFR), single nephron GFR, fractional delivery of water, sodium and potassium in proximal and distal tubules and in urine. All data are expressed as mean values ± SEM. Comparison of the effects of compounds to be tested with controls is performed by one way analysis of variance and by Student'st-test for paired and unpaired data.

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I.E.1.1.8
Stop Flow Techniques

PURPOSE AND RATIONALE

This procedure is of considerable value in the localization of transport processes along the length of the nephron. During clamping of the ureter, glomerular filtration is grossly reduced. The contact time for the tubular fluid in the respective nephron segments increases, and the concentration of the constituents of tubular fluid should approximate the static-head situation. After release of the clamp, the rapid passage of the tubular fluid should modify the composition of the fluid only slightly. The first samples should correspond to the distal nephron segment, the latest to glomerular fluid. However, with introduction of the micropuncture technique, the stop-flow method appears less attractive.

PROCEDURE

This method can be performed in different animals during anesthesia and was originally described by Malvin et al. (1958). The ureter of an animal undergoing intense osmotic diuresis is clamped for several minutes allowing a relatively static column of urine to remain in contact with the various tubular segments for longer than the usual periods of time. Thus, the operation of each segment on the tubular fluid is exaggerated. Then the clamp is released, and the urine is sampled sequentially. Small serial samples are collected rapidly, the earliest sample representing fluid which had been in contact with the most distal nephron segment. Substances examined are administered along with inulin before the application of uretral occlusion. However, tubular segments downstream from the proximal segments may modify the tubular fluid during its egress.

EVALUATION

In each sample the concentration of a glomerular marker, such as inulin, and the concentration of the substance under study are measured. Fractional excretion of the substance and the glomerular marker are plotted versus the cumulative urinary volume.

MODIFICATIONS OF THE METHOD

Shinosaki and Yonetani (1989), Shinosaki et al. (1994) performed stop-flow studies on tubular transport of uric acid in rats treated with pyrazinoic acid, an inhibitor of tubular urate secretion.

Tanaka et al. (1990) used stop-flow experiments to test uricosuric and diuretic activities of new compounds in dogs.

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I.E.1.2
In vivo Techniques

I.E.1.2.1
Diuretic Activity in Rats (LIPSCHITZ Test)

PURPOSE AND RATIONALE

A method for testing diuretic activity in rats has been described by Lipschitz et al. (1943). The test is based on water and sodium excretion in test animals and compared to rats treated with a high dose of urea. The "Lipschitz-value" is the quotient between excretion by test animals and excretion by the urea control.

PROCEDURE

Male Wistar rats weighing 100–200 g are used. Three animals per group are placed in metabolic cages provided with a wire mesh bottom and a funnel to collect the urine. Stainless-steel sieves are placed in the funnel to retain feces and to allow the urine to pass. The rats are fed with standard diet and water ad libitum. Fifteen hours prior to the experiment food and water are withdrawn. Three animals are placed in one metabolic cage. For screening procedures two groups of three animals are used for one dose of the test compound. The test compound is applied orally at a dose of 50 mg/kg in 5.0 ml water/kg body weight. Two groups of 3 animals receive orally 1 g/kg urea. Additionally, 5 ml of 0.9 % NaCl solution per 100 g body weight are given by gavage. Urine excretion is recorded after 5 and after 24 h. The sodium content of the urine is determined by

flame photometry. Active compounds are tested again with lower doses.

EVALUATION

Urine volume excreted per 100 g body weight is calculated for each group. Results are expressed as the "Lipschitz-value", i.e., the ratio T /U, in which T is the response of the test compound, and U, that of urea treatment. Indices of 1.0 and more are regarded as a positive effect. With potent diuretics, Lipschitz values of 2.0 and more can be found. Calculating this index for the 24 h excretion period as well as for 5 h indicates the duration of the diuretic effect. Similar to urine volume, quotients can be calculated for sodium excretion. Dose-response curves can be established using various doses. Loop diuretics are characterized by a steep dose-response curve. Saluretic drugs, like hydrochlorothiazide, show Lipschitz values around 1.8, whereas loop diuretics (or high ceiling diuretics) like furosemide, bumetanide or piretanide reach values of 4.0 and more.

LIMITATIONS OF THE METHOD

The Lipschitz test has been proven to be a standard method and a very useful tool for screening of potential diuretics.

MODIFICATIONS OF THE METHOD

The method has been modified in various ways by several authors. Cummings et al. (1960) recommended a sequential procedure with criteria for acceptance or rejection of test drugs. Kau et al. (1984) recommended a method for screening diuretic agents in the rat using normal saline (4 % body weight) as hydrating fluid.

Homozygous Brattleboro rats exhibit symptoms of diabetes insipidus (Valtin et al. 1965). The condition is due to the failure of hypothalamic neurons to produce vasopressin, which is due to a single base point deletion in the vasopressin gene (Schmale and Richter 1984). The abnormal quinine drinking aversion in the Brattleboro rat with diabetes insipidus is reversed by a vasopressin agonist (Laycock et al. 1994). These animals can be used to study vasopressin agonism and antagonism and the aquaretic effects of synthetic drugs.

Klatt et al. (1975) described a method of collecting urine excreted by large animals. On the basis of urine funnels used in rats, an appropriate larger metabolism cage made out of transparent, rigid polyvinyl chloride was used. The cage was improved by a built-in sieve cone which assured good separation of urine and feces. A device to measure and record the time and amount of voided urine was attached. Urine was collected in a vessel with a hose connection from the bottom to a pressure sensor. An attached overflow tube could be occluded. The initial pressure of the sensor was fed into a linear recorder. Before the test, the recorder was calibrated with a sufficient amount of distilled water to adjust the number of division intervals for direct measurement of voided urine in milliliters. This allowed calculation of the time point of voiding from the chart speed.

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I.E.1.2.2
Saluretic Activity in Rats

PURPOSE AND RATIONALE

Excretion of electrolytes is as important as the excretion of water for treatment of peripheral edema

and ascites in congestive heart failure as well as for treatment of hypertension. Potassium loss has to be avoided. As a consequence, saluretic drugs and potassium-sparing diuretics were developed. The diuresis test in rats was modified in such a way that potassium and chloride as well as osmolality are determined in addition to water and sodium. Ratios between electrolytes can be calculated indicating carbonic anhydrase inhibition or a potassium sparing effect.

PROCEDURE

Male Wistar rats weighing 100–200 g fed with standard diet and water ad libitum are used. Fifteen hours prior to the test, food but not water is withdrawn. Test compounds are applied at a dose of 50 mg/kg orally. Three animals are placed in one metabolic cage provided with a wire mesh bottom and a funnel to collect the urine. Two groups of 3 animals are used for each dose of a test drug. Urine excretion is registered every hour up to 5 h. The 5 h urine is centrifuged to remove solid debris and analyzed by standard methods for sodium, potassium and chloride (Durst and Siggard-Andersen 1999; Scott et al. 1999). To evaluate compounds with prolonged effects the 24 h urine is collected and analyzed. Furosemide (25 mg/kg p.o.), hydrochlorothiazide (25 mg/kg p.o.), triamterene (50 mg/kg p.o.), or amiloride (50 mg/kg p.o.) are used as standards.

EVALUATION

- The sum of Na^+ and Cl^-excretion is calculated as parameter for saluretic activity.
- The ratio Na^+/K^+ is calculated for natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect. Ratios greater than 10.0 indicate a potassium-sparing effect.
- The ratio: (ion quotient) is calculated to estimate carbonic anhydrase inhibition.
- Carbonic anhydrase inhibition can be excluded at ratios between 1.0 and 0.8. With decreasing ratios slight to strong carbonic anhydrase inhibition can be assumed.

MODIFICATIONS OF THE METHOD

Adrenalectomized rats treated with DOCA or aldosterone can be utilized to test aldosterone antagonists. Spironolactone has no effect in the absence of a mineralocorticoid, but reverses in a dose-related manner the effect of DOCA on the Na^+/K^+ ratio in the urine (Kagawa et al. 1957; Bicking et al. 1965).

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I.E.1.2.3 Diuretic and Saluretic Activity in Dogs

PURPOSE AND RATIONALE

Dogs have been extensively used to study renal physiology and the action of diuretics. Renal physiology of the dog is claimed to be closer to man than that of rats. Oral absorbability of diuretic substances can appropriately be studied in dogs. Using catheters, interval collections of urine can be made with more reliability than in rats. Simultaneously, blood samples can be withdrawn to study pharmacokinetics.

PROCEDURE

Beagle dogs are placed in metabolic cages. At least 4 dogs are used as controls receiving water only, as standard controls (1 g/kg urea p.o. or 5 mg/kg furosemide p.o.) or the test drug group. Twenty-four hours prior to the experiment food but not water is withheld. On the morning of the experiment, the urinary bladder is emptied with a plastic catheter. The dogs receive 20 ml/kg body weight water by gavage, followed by hourly doses of 4 ml/kg body weight drinking water. The bladder is catheterized twice in an interval of 1 h and the urine collected for analysis of initial values. Then, the test compound or the standard is applied either orally or intravenously. Hourly catheterization is repeated over the next 6 h. Without further water dosage the animals are placed in metabolic cages overnight. Twenty-four hours after dosage of the test compound, the dogs are catheterized once more and this urine together with the urine collected over night in the metabolic cage registered.

The collected urine is centrifuged to remove solid debris and analyzed by standard methods for sodium, potassium and chloride (Durst and Siggard-Andersen, 1999, Scott et al. 1999). Osmolality is also measured with an osmometer (the freezing point depression type of instrument is recommended (Scott et al. 1999).

EVALUATION

Urine volume, electrolyte concentrations and osmolality are averaged for each group. The values are plotted against time to allow comparison with pretreatment values as well as with water controls and standards. The non-parametric U-test is used for statistical analysis.

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I.E.1.2.4
Evaluation of Renal Concentrating Ability

PURPOSE AND RATIONALE

Investigations of the clearance of solute-free water represent indirect methods for the evaluation of several aspects of renal function and provide information on the site of action of pharmacological agents within the nephron. The discovery of the countercurrent multiplier system as the mechanism responsible for the concentration and dilution of the urine has been the prerequisite for the identification of the site of action of diuretic drugs. A drug that acts solely in the proximal convoluted tubule, by causing the delivery of the increased amounts of filtrate to the loop of Henle and the distal convoluted tubule, would augment the clearance of electrolyte-free water (EC_{H_2O}) during water diuresis and the reabsorption of electrolyte-free water (ETC_{H_2O}) during water restriction. In contrast, drugs that inhibit sodium reabsorption in Henle's loop would impair both EC_{H_2O} and ETC_{H_2O}. On the other hand, drugs that act only in the distal tubule would reduce EC_{H_2O} but not ETC_{H_2O}.

PROCEDURE

These tests may be performed in any species from which urine and plasma can be readily collected, although low-level changes in concentrating ability may be more readily manifest in rats than in dogs (Sharratt and Frazer 1963; Osbourne et al. 1983). The general procedure involves initially placing the animals in a metabolism cage with unlimited access to water. After collection of a urine sample (16–24 hours is best), the water is withdrawn and the urine is collected for the next 12–16 hours. The urine specific gravity (or preferentially, urine osmolality) is measured and compared both with the value from the hydrated animal and the mean values from the water-deprived control group (Ragan and Weller 1999).

Assessment of renal urine diluting ability is more cumbersome but can be accomplished in rats by administration of an oral dose of water by gavage representing 5 % of the animal's body weight. The animals are then placed in metabolic cages and the urine is collected every 30 minutes for the next 2 hours (Sharratt and Frazer 1963). In dogs, water diuresis can be induced by oral administration of 50 ml of water per kg body weight and maintained by continuous infusion into jugular vein of 2.5 % glucose solution and 0.58 % NaCl solution at 0.5 ml/min per kg body weight. When water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter (Suki et al. 1965). The volume and specific gravity (or osmolality) of the collected urine is measured and the results expressed as the percent of the administered dose of water excreted during the time period. Time-matched plasma samples are collected for the determination of plasma osmolality (see formulas below).

MODIFICATIONS OF THE METHOD

In addition to gross assessment of renal dilution or concentrating ability (as described above), quantitative evaluation of either osmolar clearance, freewater clearance (C_{H_2O}), or solute-free water clearance (EC_{H_2O}) may add additional sensitivity to the evaluation of these functions.

Osmolar clearance can be calculated as follows:

$$C_{osm} = (U_{osm} \times V)/P_{osm} ,$$

where C_{osm} = osmolar clearance, U_{osm} = urine osmolality (in mosm/kg water), V = urine flow (measured in ml/min) and P_{osm} = plasma osmolality (measured in msom/kg water). C_{osm} is expressed in ml/min. C_{osm} less than V indicates excretion of a dilute urine (i.e., excess water is being excreted), but C_{osm} greater than V indicates excretion of concentrated urine (i.e., excretion of excess solute).

Freewater clearance (C_{H_2O}) provides an estimate of the amount of urine being excreted in excess that needed to clear solutes, and is calculated traditionally as follows:

$C_{H_2O} = V \text{ X } [(1 - U_{osm})/P_{osm}]$

(Wesson and Anslow 1952)

More accurate assessment of the ability of the kidney to appropriately regulate plasma tonicity and/or respond appropriately to antidiuretic hormone (ADH) can be made by calculation of electrolyte-free water clearance (EC_{H_2O}). This formula takes into account only water needed to excrete excess "effective" osmolytes (in general, monovalent electrolytes and their associated anions) and is calculated as follows:

$$EC_{H_2O} = V \text{ X}\{1 - \left(\frac{[Na^+ + K^+]_{urine}}{[Na^+ + K^+]_{plasma}}\right).$$

(Shoker 1994)

LIMITATIONS OF THE METHOD

In general the sensitivity of either urine concentration or dilution tests to detect the effects of toxicants on renal function is quite low, regardless of species (Ragan and Weller 1999). Furthermore, these tests (especially the urine dilution test) may be significantly altered by extrarenal effects (e.g., vomiting, diarrhea, delayed GI absorption, altered adrenal cortical function). Calculation of osmolar, freewater and electrolyte-free water clearances require accurate assessment of urine flow rates, which will require accurate and complete collection of all urine produced during the time interval, not always easily accomplished in small animal species. Catheterized models should be utilized in this circumstance. In addition, calculated electrolyte-free water clearances may be influenced by the presence of excess effective osmolytes in either urine or plasma (e.g., in circumstances of hyperglycemia or metabolic acidosis/alkalosis) and the formula must be corrected to account for these if they are known to be present (Shoker 1994; Nguyen and Kurtz 2005).

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I.E.1.2.5 Clearance Methods

PURPOSE AND RATIONALE

The renal clearance of a compound is the volume of plasma from which that compound is completed removed by the kidneys per unit time (Pitts 1968). The material removed from the plasma is generally excreted in the urine.

Clearance methods can be used for a variety of purposes:

- Determination the effects of toxicants on glomerular filtration rate (GFR) and renal blood flow (RBF)
- Determination of the mechanism(s) involved in the renal excretion of a toxicant.

PROCEDURE

Clearance procedures can be conducted in all laboratory animal species; anesthetized and conscious animal models may be used. Measurement of arterial pressure is advisable, especially in anesthetized preparations, to insure that renal perfusion pressure remains within the autoregulatory range (usually 80–120 mmHg). Vascular access ports can be helpful to provide continuous arterial access for pressure measurements (Mann et al. 1987).

Carefully timed and complete collection of urine is critical to clearance studies. Urine can be collected directly from the ureters (anesthetized preparations) or via repeated catheterization of the urinary bladder in large animals (conscious preparations). Cannulated

rats can also be used to collect accurate and complete timed urine samples (Mandavilli et al. 1991; Horst et al. 1988). Care must be taken to ensure complete urine collection in catheterized/cannulated models by flushing the bladder with saline (adding the wash to the urine volume) and instilling air at the end of the collection to completely empty the bladder.

Tracers are administered intravenously to achieve near steady-state concentrations; a priming dose loads the plasma and extracellular compartments, and subsequent infusion replaces renal losses. Once steady state plasma tracer levels are approached, a series of timed urine collections (clearance periods) are performed, with blood samples collected at the either the midpoint or beginning and end of the clearance periods. The urine and blood (plasma or serum) samples are analyzed for tracer(s) and the test compound.

Tracers for the determination of GFR must be freely filtered and then neither secreted nor reabsorbed. This allows the assumption that the amount of plasma cleared of the tracer per unit time represnts that which has been filtered through the glomeruli (i.e., GFR). The fructose polysaccharide, inulin (mw ~ 5200) is the most commonly used tracer in all species and serves as the 'gold standard' to which others are compared (Finco 1983; Ragan and Weller 1999). Other indicators include isotopes of vitamin B_{12}, sodium iodothalamate, iohexol and radiolabelled metal chelates of ethylenediamintetracetate (EDTA) and diethylaminotriaminepentaacetate (DPTA) (Sarkar et al. 1988; 1991; Gaspari et al. 1997; Ragan and Weller 1999).

Tracers for the assessment of renal plasma flow must be completely cleared (combination of filtration and efficient tubular secretion to the urine) on first pass through the kidney. This allows the assumption that the volume of plasma cleared per unit time represents that which was either filtered by the glomeruli or bypassed the glomeruli and perfused the tubules. p-aminohippurate (PAH) is used for the assessment of RBF, because it is both freely filtered by the glomeruli and actively secreted by the organic acid transport pathway of the proximal tubule. First-pass PAH extraction by the kidneys varies from about 70 % to 90 % in rats, dogs, and humans (Brenner et al. 1976), but for the purpose of estimating RBF it is assumed to be 100 %. Using this assumption, RPF is always slightly underestimated. Tetraethylammonium bromide (TEA), a substrate for the renal cation transporter, may also be used, and is subject to the same limitations (Ragan and Weller 1999).

Inulin is measured colorimetrically, either by acid hydrolysis to generate a green product, or by a series of enzymatic reactions based on inulinase with subsequent reduction of NADH. HPLC methods are used for the remaining exogenous GFR tracers. PAH and TEA are measured colorimetrically (Newman and Price 1999).

EVALUATION

Renal clearance (Cl) of any compound (X) can be determined by comparing the urinary excretion rate of compound X to the plasma concentration of compound X.

The urinary excretion rate is calculated as:

$$\text{Urinary excretion rate (mg/min)} = U_x(\text{mg/ml}) \times V(\text{ml/min}) ,$$

where U_x represents the concentration of substance X in urine (in mg/ml) and V represents the volume of urine collected per unit time (in ml/min). Thus, the clearance equation may be constructed:

$$Cl_x(\text{ml/minute}) = \frac{U_x(\text{mg/ml}) \times V(\text{ml/min})}{P_x(\text{mg/ml})} ,$$

where P_x is the concentration of compound X in plasma (in mg/ml).

GFR is estimated by calculating the clearance of the freely-filtered tracer or endogenous substance. RPF estimated using PAH clearance is often designated effective renal plasma flow (ERPF). Renal plasma flow is converted to renal blood flow (RBF) by dividing ERPF by the plasma fraction of whole blood, as estimated from the hematocrit (Hct):

$$\text{RBF} = \text{ERPF}/(1 - \text{Hct}).$$

The clearances of other compounds can be compared with inulin clearances to determine how the kidney functions in the elimination of the test compound. A clearance ratio is constructed by dividing the renal clearance of the test compound (X) by the renal clearance of inulin:

$$\text{Clearance Ratio} = CL_x, (\text{ml/min})/Cl_{inulin}, (\text{ml/min}).$$

A clearance ratio < 1.0 indicates reabsorption of the test substance following filtration, whereas active secretion will result in a clearance ratio of > 1.0.

MODIFICATIONS OF THE METHOD

Endogenous compounds can administered exogenously in place of tracer substances. This has been done for the dog using creatinine (Sapirstein et al.

1955; Finco et al. 2001) and for the rat using cystatin C (Tenstad et al. 1996). Either urine or plasma clearance methods can be used for the former. For the latter, plasma clearance was used to estimate GFR due to the fact that cystatin C is reabsorbed and degraded by the proximal tubule and does not appear in the urine.

Endogenous creatinine clearance may also be used to estimate GFR. A single timed urine collection and matched plasma sample are used. Clearance is calculated as noted above.

GFR and RPF can be measured without collection of urine by measuring the disappearance of the appropriate tracers from the blood over time (plasma clearance). In this technique, precise amounts of tracers are injected intravenously as a bolus and subsequent serial samples at precise intervals are collected. The disappearance of the tracer from the blood used to calculate the renal clearance (Bailey et al. 1970; Ronnhedh et al. 1996).

LIMITATIONS OF THE METHOD

First-pass extraction of PAH is highly variable both between species and between individuals within a species, which adds to the inherent inaccuracy of the estimate of RBF by this method. Furthermore, the test compound may interfere with the extraction of either PAH or TEA by competing for transport by the organic anion or cation transporters (Newman and Price 1999; Ragan and Weller 1999).

The limitations of the Jaffe method for creatinine determination are discussed under "Assessment of Renal Injury by Serum Chemistry". Exogenous creatinine clearance compensates for the insensitivity of the method as well as the interference by endogenous chromagens by artificially increasing the plasma creatinine concentration (Finco 1997).

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I.E.1.2.6 Fractional Excretion Methods

PURPOSE AND RATIONALE

Since one of the kidney's primary functions is maintaining electrolyte and mineral homeostasis in the face of fluctuating dietary intake and body needs, examination of plasma and urine electrolyte levels will provide some insight into renal function. Because of the large functional mass of the kidney, decrements in plasma electrolyte levels are usually not detected until the amount of renal functional diminution is significant. In contrast, urine electrolyte levels examined with knowledge of plasma levels and dietary intake can serve as an extremely sensitive index to the effect of drugs or chemicals on the functional state of the kidney.

In animal studies the diet can be carefully controlled and thus the intake (and hence the plasma electrolyte levels) can be assumed to be fairly constant. Provided there are no sources of significant electrolyte loss resulting from the experimental manipulations (e.g.,

vomiting, diarrhea, salivation), urine electrolyte levels will reflect the effects of the compound on either GFR (determines the filtered load) or tubular secretion or reabsorption (determines the final urine electrolyte composition).

The most useful way to utilize urine electrolyte information is to calculate the fractional excretion (FE), which is the proportion of the filtered load that is excreted from the plasma. If both tubular function and plasma electrolyte values are normal, increases in electrolyte FE values clearly reflect a decrement in GFR. With tubular malfunction, the direction of the change in FE values depends on the net direction of electrolyte transport (i.e., FE will increase for electrolytes that are primarily reabsorbed and will decrease for secreted electrolytes) (Finco 1997; Stockham and Scott 2002).

PROCEDURE

FE assessments can be performed in any species, as it requires only carefully timed complete urine collections and a concurrent assessment of GFR. FE will be unitless if the urine collection period is expressed in minutes, thus (Finco 1997):

$$\mathrm{FE} = \frac{\left(\begin{array}{c}\text{urine electrolyte concentration (mmol/L)}\\ \times\text{urine volume(mL/collection period)}\end{array}\right)}{\left(\begin{array}{c}\text{GFR (mL/min)}\\ \times\text{plasma electrolyte concentration (mmol/L)}\end{array}\right)}.$$

To eliminate the need for both complete timed urine collections (difficult to do in most animals) and concurrent assessment of GFR, FE values are usually calculated based on point-in-time urine collections by using creatinine excretion during the same time period as an estimator of GFR (Finco 1997).

Animals are placed in appropriately-sized metabolism cages for an appropriate period of time to allow collection of an adequate volume of urine (for large animals only a few hours may be needed; for rodents, 12–24 hours might be required). To preserve the quality of the urine specimens, the collection vial must have a small neck (to prevent evaporation of water) and it should be surrounded by wet ice or frozen cold packs to ensure the urine is maintained at 4 °C for the duration of the collection period (Emeigh Hart and Kinter 2005). At the end of the collection period a blood sample is obtained under appropriate anesthesia (note: the use of CO_2 will falsely elevate plasma potassium levels and render the method inaccurate) for determination of electrolyte and creatinine levels.

Plasma and urine electrolytes and creatinine are determined by standard methods (Durst and Siggard-Andersen 1999; Newman and Price 1999; Scott et al. 1999). FE is calculated from the results as outlined below (Stockham and Scott 2002):

$$\mathrm{FE} = \frac{\left(\begin{array}{c}\text{urine electrolyte concentration}\\ \times\text{plasma creatinine concentration}\end{array}\right)}{\left(\begin{array}{c}\text{urine creatinine concentration}\\ \times\text{plasma electrolyte concentration}\end{array}\right)}.$$

LIMITATIONS OF THE METHOD

The inherent inaccuracy in the use of creatinine clearance as an estimator of GFR has been discussed previously. If the Jaffe method for creatinine is used, the investigator must be aware of potential interference due to endogenous chromogens (the error is magnified in species where these chromogens are present in higher concentration in the plasma than in the urine; dog, mouse) (Finco 1997; Dunn et al. 2004) or possibly the test compound (Sonntag and Scholer 2001). Additionally, plasma electrolyte levels may fluctuate as the result of eating or due to diurnal rhythms (Finco 1997). The impact of inaccuracies can be minimized by consistent timing of urine collection, fasting of animals before and during urine collection and the inclusion of a concurrent untreated (vehicle control) group in all studies.

MODIFICATIONS OF THE METHOD

Although FE of sodium is most commonly used to assess tubular function, FE of magnesium has been shown to be the most sensitive index in detecting low-level tubular injury in humans (Futrakul et al. 1999; Kang et al. 2000; Oladipo et al. 2003). Increases in FE of magnesium and/or calcium have also been shown to serve as sensitive indices of effects on renal function unrelated to overt renal injury (Lam and Adelstein 1986; Barton et al. 1987; Tuso and Nortman 1992; Eliott et al. 2000).

FE of urea has been recently shown to be more useful than the FE of sodium in distinguishing between prerenal and renal azotemia in humans (Carvounis et al. 2002) and thus may also be useful in making this distinction in animal models. Changes in the FE of urea also reflect changes in urine flow rates (in general, these values move parallel to each other) and can be used as an estimate of this parameter (Finco 1997). The methods used to detect urea in urine are the same as those used in serum (Newmann and Price 1999).

FE of anions (ammonium, bicarbonate) can be used to determine the potential mechanism underlying sys-

temic acid base imbalances (Rothstein et al. 1990; Kim et al. 2001).

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I.E.1.2.7
Diuretic and Uricosuric Activity in Mice

PURPOSE AND RATIONALE

Renal excretion of uric acid consists of three components: complete filterability of uric acid in the glomerulus, subsequent tubular reabsorption, and tubular secretion (Gutman and Yü 1961). Pronounced species differences have been described in uric acid metabolism including man. Mice were recommended for primary screening of uricosuric drugs.

PROCEDURE

Male NMRI mice weighing 25–30 g are used. On the evening prior to the experiment, food but not water is withheld. In the morning, the mice are orally loaded with 50 ml/kg 0.9 % NaCl-solution. Together with the sodium load the test compound is applied by gavage in 2 % starch suspension. Controls receive saline and starch suspension only. Groups of 5 mice are placed into metabolism cages. Urine is collected over 4 h. In the urine sodium and potassium are determined by flame photometry, chloride by argentometrically with potentiometrical end point titration (Chloride titrator, Aminco), uric acid by the Uriquant-method, creatinine by the Jaffé-reaction, as well as pH and osmolality.

EVALUATION

Urine excretion is calculated in ml/kg. Uric acid-, creatinine- and ion-excretions are calculated in mmol/kg and expressed as percent changes versus controls. The changes are evaluated statistically using Student's t-test.

LIMITATIONS OF THE METHOD

Some saluretic-diuretic agents, like ethacrynic acid, are inactive in the rat, when given orally. Moreover, uricosuric activity in mice is less reliable than that in primates.

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I.E.1.2.8
Inhibition of Allantoxanamide-Induced Hyperuricemia in Rats

PURPOSE AND RATIONALE

Most species used for pharmacological experiments have rather low blood levels of uric acid. Experimental hyperuricemia can by induced by inhibition of the enzyme uricase. In most species uricase metabolizes uric acid to allantoin. Allantoxanamide blocks uricase and increases endogenously synthesized uric acid. This increase is blocked by compounds like allopurinol.

PROCEDURE

Non fasted male Sprague Dawley rats weighing 230–280 g are treated by intraperitoneal injection of 250 mg/kg allantoxanamide suspended in 5 ml/kg sesame oil. The test compound is applied orally in a dose of 50 mg/kg in 40 ml/kg water. Likewise, the standard compound allopurinol is given in a dose of 50 mg/kg. Eight rats are used for the each dose of test drugs and standard. The animals are placed individually into metabolism cages with free access to food and water. Urine is collected during the periods of 1 to 6 and 7 to 24 h. Blood is withdrawn by retroorbital puncture prior and 2, 6 and 24 h after compound administration. Uric acid is determined with the Uric-aquant°-method in plasma [mmol/l] and urine [mmol/l].

EVALUATION

Mean values of uric acid concentrations in plasma at the different time intervals and mean values of uric acid excretion after 6 and 24 h of the test group are compared with the control group (allantoxanamide treated only) using Student's t-test.

MODIFICATION OF THE METHOD

Potassium oxonate may be used as a uricase inhibitor in rats as an alternative to allantoxamide, but its effectiveness requires a special diet. Rats are offered a diet containing 5 % fructose, 3 % uric acid, 2 % potassium oxonate (2,4-dihydroxy-1,3-triazine-6-carbonic acid) and 0.001 % artificial sweetener. Drinking water consists of a 0.5 % solution of potassium oxonate. Allopurinol is used as a positive control for the test compound. Animals are assessd following three days on this treatment regimen. Clearance techniques in oxonate-treated rats may also be used (Yonetani et al. 1987; Shinosaki et al. 1991; Dan et al. 1994). Sugino and Shimada (1995) tested uricosuric effects in oxonate-loaded rats, in the pyrazinoic acid suppression test and in the phenolsulfonphthalein test.

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I.E.1.2.9
Phenol Red Excretion in Rats

PURPOSE AND RATIONALE

Phenol red (= phenolsulfonphthalein) excretion is an indirect test for uricosuric activity. After intravenous injection phenol red is mainly eliminated by active secretion in the proximal tubulus of the kidney. Treatment with uricosuric agents decreases the secretory activity of tubulus cells resulting in a delayed excretion of phenol red. Plasma values of phenol red are increased in treated animals as compared to controls.

PROCEDURE

Male Wistar rats weighing 120–150 g are treated orally with the test compound or the standard 30 min prior to intravenous injection via the tail vein with 2.5 ml/kg of a 3 % aqueous solution of phenolsulfonphthalein. For intravenous application, 5.0 ml/kg of the test drug solution are injected immediately after the phenolsulfonphthalein injection followed by flushing with 2.5 ml/kg saline. By retro-orbital puncture blood samples are withdrawn after 30, 60 and 180 min. Blood (0.2 ml) is diluted with 2 ml 0.9 % NaCl-solution and centrifuged. To 1 ml of the supernatant 1 ml of 1 % sodium carbonate solution and 8 ml of saline are added. Using a spectrophotometer (Eppendorf, Hamburg) extinction at 546 nm is determined.

EVALUATION

Extinction values are calculated for total blood. At each time interval the values in treated rats are compared statistically with those of controls.

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I.E.1.2.10
Uricosuric Activity in Relevant Animal Models

PURPOSE AND RATIONALE

Most species have low plasma levels and low renal excretion of uric acid and thus are poor models for the human. There are two exceptions, however: the Dalmatian dog and the Cebus monkey.

The Dalmatian dog has excessive uric acid excretion and relatively high plasma levels. This is due to a genetically determined defect in tubular reabsorption of filtered urate (Friedman and Byers 1948; Kessler et al. 1959) and defective hepatic uricase activity (Yü et al. 1971). The Dalmatian dog can therefore being used for studies of uricosuric agents.

The Cebus monkey is described not to possess uricase (Simkin 1971) and to have different metabolic conditions for uric acid than other experimental animals. This species is chosen for special studies of anti-uricopathic drugs since it resembles more closely human uric acid metabolism.

PROCEDURE

Fasted male Dalmatian dogs with a body weight of about 20 kg are used. The animals are placed individually in metabolic cages. The urinary bladder is emptied by catheterization. Twenty ml/kg drinking water is administered by gavage. Urine and plasma samples are obtained at 2 h and an additional 8 ml/kg drinking water is administered by gavage. The urine and plasma values obtained after the first 2 h serve as control. Then, the test compound is administered either i.v. or orally. Blood and urine samples are collected every 2 h for up to 8 h following treatment. No water is given after the last sampling. The dogs stay over night in the metabolic cages. Twenty-four hours after beginning of the experiment, venous puncture and catheterization are performed once more.

Fasted Cebus monkeys (*Cebus albifrons*) of either sex weighing 3.0 to 5.0 kg are used. On the morning of the experiment, the animals receive 20 ml/kg drinking water by gavage, followed by oral administration of the test compound. Allopurinol and probenecid are used as control compounds. Control animals receive water only. The animals are placed in individual metabolism cages and the spontaneously voided urine is collected after 2, 6, and 24 h. After 2 and 6 h, an additional 4 ml/kg water is given by gavage. From a cubital vein blood is withdrawn prior to the experiment and 2, 6 and 24 h after application.

Urine and serum samples are analyzed for uric acid (Uricaquant-method), creatinine (Jaffé reaction), sodium and potassium (flame photometry), calcium and magnesium (atom absorption method), and chloride (argentometry) as well as for osmolality.

MODIFICATION OF THE METHOD

Onuma et al. (1988) used Cebus monkeys for evaluation of uricosuric effects of an aryloxyacetic derivative. Yonetani et al. (1987) performed clearance experiments with uricosuric drugs in anesthetized chimpanzees. Dan et al. (1989) tested the activity of AA-193, an uricosuric agent in rats, mice and Cebus monkeys.

EVALUATION

The values after administration of the drug are compared with predrug values, and are compared to the results with the control compounds.

LIMITATIONS OF THE METHOD

Dalmatian dogs bred by commercial breeders are not always homozygous. Therefore, not every dog is suitable for experiments on uric acid excretion. The use of the Cebus monkey as animal model has been proven to be

the most valuable method to test putative hypouricemic compounds (Hropot, unpublished data).

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I.E.2
Assessment of Renal Injury

I.E.2.1
Assessment of Renal Injury by Serum Chemistry

PURPOSE AND RATIONALE

Estimation of the glomerular filtration rate (GFR) is considered a sensitive index of functional nephron mass (Newman and Price 1999). Point measurements of the plasma levels of several endogenous small molecules (urea, creatinine, 2-(α-mannopyranosyl)-L-tryptophan) or small (less than 66 kDa) proteins (cystatin C (γ-trace), prostaglandin D synthase (β-trace protein), α_1-microglobulin, β_2-microglobulin, and retinol binding protein) have been used to assess GFR in many species.

PROCEDURE

Serum or plasma samples are collected from the test animals (if plasma is used, blood should be collected in heparinized tubes). Assay methods vary, and are outlined below.

Urea is most commonly assayed by combined urease methods, in which the urea is first converted to two ammonium ions. The ammonium generated is then measured by either enzymatic or chemical methods. Urea nitrogen values determined by this method (mg/ml) are converted to urea values by the use of appropriate factors (2.14 for urea in mg/ml, 0.357 for urea in mmol/L) (Emeigh Hart and Kinter 2005).

Creatinine is most commonly measured by the Jaffe reaction of creatinine with picrate to generate an orange chromogen. Several enzymatic assays (based on reactions with creatinase or creatinine deaminase) have also been developed. These are equal in sensitivity to the Jaffe method but are less likely to be subject to interference by endogenous or exogenous chromogens (Finco et al. 1995; Finco 1997; Newman and Price 1999). A recently-developed HPLC assay has been shown to be much more sensitive than the Jaffe method for mouse plasma (Dunn et al. 2004).

2-(α-mannopyranosyl)-L-tryptophan (MPT) is measured by HPLC (Takahira et al. 2001). Cystatin C assays are all antibody based (nephelometric, agglutination or sandwich ELISA) (Pergande and Jung 1993; Finney et al. 1997; Jensen et al. 2001), and have been used successfully in dogs, rats, mice and cats (Hakansson et al. 1996; Boekenkamp et al. 2001; Braun et al. 2002; Martin et al. 2002). The other small molecular weight proteins are detected by immunoassays as well.

EVALUATION

Analyte levels are compared both to the concomitant control group (using appropriate statistical methods of the group size is large enough) and additionally to laboratory-specific reference intervals for the species, strain, age and sex in question.

Algorithms can be used to convert plasma creatinine to a reasonably accurate estimate of GFR in humans (Cockroft and Gault 1976) and dogs (Finco et al. 1995).

Both urea and creatinine will be elevated with renal injury, and the elevations in both are usually proportional. Calculation of the urea:creatinine ratio may implicate extrarenal causes for the analyte elevations (see “Limitations of the Method”, below). Extremely high urea:creatinine when both are elevated (where urea is elevated markedly out of proportion to creatinine) indicates decreased renal blood flow, urinary tract obstruction or extravasation of urine into the peritoneal cavity. Elevation in the urea:creatinine ratio as a consequence of pure urea elevation implicates gastrointestinal hemorrhage, high protein diet, increased protein catabolism, or loss of muscle mass. A decreased ratio indicates primary liver dysfunction (due

to decreased urea synthesis), decreased protein intake, extremely muscular individuals or tissue anabolism (Baum et al. 1975; Newman and Price 1999).

LIMITATIONS OF THE METHOD

Both prerenal factors (dehydration, blood loss, altered vasomotor tone, age-related decreases in renal blood flow in rats) and postrenal factors (obstruction or extravasation of urine to the peritoneal cavity) may cause elevations of the commonly measured analytes that do not reflect primary kidney injury. Plasma analytes also cannot be used to determine the location of renal injury (glomerular versus tubular, or tubular segment affected) (Baum et al. 1975; Corman and Michel 1987; Finco 1997; Newman and Price 1999).

Urea and creatinine elevations in plasma are in general not sensitive enough to detect low-level alterations (less than 75 % loss) in functional nephron mass, due to the contribution of renal secretion and/or reabsorption to their overall excretion, (which can compensate for their decreased filtration), to wide variations in baseline levels of some analytes, and to inherent imprecision in the assays used (Finn and Porter 1998; Price 2002; Starr et al. 2002; Shemesh et al. 1985). In particular, urea will underestimate GFR (due to extensive tubular reabsorption with decreased GFR) (Baum et al. 1975; Kaplan and Kohn 1992; Newman and Price 1999). Creatinine tends to overestimate GFR because it is secreted by the tubule in many species and secretion increases with reduced GFR (Shemesh et al. 1985; Andreev et al. 1999; Newman and Price 1999; Starr et al. 2002). In addition, creatinine synthesis is regulated by feedback inhibition which limits the degree of elevation than can occur in plasma with renal injury (Watson et al. 2002).

The Jaffe reaction for creatinine is subject to interference by numerous endogenous substrates and drugs or compounds (Schwendenwein and Gabler 2001; Sonntag and Scholer 2001; Dunn et al. 2004). This effect can be minimized by using appropriate substrate extraction or by the use of kinetic assessments. The urease assay is specific for urea, but increased circulating ammonia (such as occurs in aged plasma samples, metabolic disorders and portocaval shunting) will react with the subsequent reaction and result in falsely elevated plasma levels (Newman and Price 1999).

Baseline levels of urea and creatinine can be highly variable. Plasma urea reflects hepatic synthesis rate and will be elevated with increased protein catabolism (increased dietary protein intake, gastrointestinal hemorrhage, fever, severe burns, corticosteroid administration, sustained exercise or muscle wasting), and decreased with low or poor quality protein diets, modest food restriction in rodents or hepatic insufficiency (Pickering and Pickering 1984; Finco 1997; Hamberg 1997; Tauson and Wamberg 1998; Newman and Price 1999). Baseline creatinine reflects muscle catabolism and will be elevated in individuals with higher muscle mass or following sustained exercise or acute muscle damage; it will be lower in individuals with loss of muscle mass (Finco 1997; Newman and Price 1999). Drugs which compete with creatinine for renal excretion may falsely elevate plasma levels in the absence of renal injury (Andreev et al. 1999). There are also significant diurnal variations in some species (Loeb and Quimby 1999).

2-(α-mannopyranosyl)-L-tryptophan (MPT) appears to be less likely to be affected by muscle mass than creatinine. Point-in-time measurements of this tryptophan glycoconjugate correlated extremely well with the inulin clearance, suggesting it may be a superior indicator of GFR. However, the renal handling of MPT has not been examined to determine if plasma levels may be influenced by either reabsorption or secretion (Horiuchi et al. 1994; Gutsche et al. 1999; Takahira et al. 2001).

Cystatin C does not appear to be a sensitive index of GFR in the cat (Martin et al. 2002).

The other small protein markers of GFR are detected by immunoassays with reagents specific for the human proteins; the cross-reactivity of these reagents with other species and the usefulness of these markers in animal models have not been well established (Loeb 1998).

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I.E.2.2
Assessment of Renal Injury by Urinalysis

PURPOSE AND RATIONALE

Urinalysis provides a unique opportunity to selectively and non-invasively "sample" a single target organ by examination of its endproduct. The levels of proteins and small molecules normally filtered, excluded from filtration, secreted or reabsorbed by the tubules can be used as indicators of the functional status of certain nephron segments, while injury can be assessed by examination of cellular enzymes which are preferentially leaked into the urine (Loeb 1998), or proteins which are either upregulated or leaked in circumstances of cellular injury and subsequently appear in the urine.

PROCEDURE

Urine samples are collected from the test species by standard methods (metabolic cage, catheterization, cystocentesis or "free catch" in the case of dogs). Samples must be collected in clean containers and must be kept free of contamination from food, drinking water, feces, blood and bacteria. To preserve the quality of the specimens during prolonged collection times, the opening of the collection vial needs to be small to prevent evaporation and the vial should be surrounded either with wet ice or frozen cold packs to chill the sample promptly once it is deposited (Loeb 1998; Loeb and Quimby 1999). For best results, samples should be analyzed promptly (ideally within one hour) after collection, but where analysis must be delayed, the sample must be protected from chemical degradation and evaporation, best accomplished by using collection containers that are appropriately sized for the species in question, tightly sealing the containers when possible, and keeping the specimen cold (4 °C)

until analysis. If chilled or frozen, samples must be allowed to slowly equilibrate to room temperature before analysis. Samples for which urine sediment evaluation is to be performed must not be frozen.

The volume and specific gravity (by refractometry) or osmolality are measured (see previously under "Diuretic and Saluretic Activity in Dogs") and a visual assessment for color and clarity is done. Additional analysis should include pH determination and quantitative or semiquantitative assessment of urine glucose and protein (Weingand et al. 1996). Quantitative assessment of urine creatinine should be performed in any circumstance where quantitative assessment of another analyte is done. Additional components (ketones, bilirubin, urobilinogen, hemoglobin, nitrite and leukocyte esterases) can be determined semiquantitatively using commercially available "dipsticks" (Multistix, Bayer Diagnostics Corporation) that can be evaluated either manually or with automated equipment (Clinitek, Bayer Diagnostics Corporation) (Newman and Price 1999). In animal studies, quantitative assessment of urine protein is recommended (see below under "Limitations of the Method"). The Folin phenol (Lowry), Coomassie brilliant blue and Ponceau S methods have been recommended as being particularly precise for urine samples (Peterson et al. 1969; Dilena et al. 1983; Finco 1997; Newman and Price 1999). Five ml of the urine is then centrifuged at 700 g for five minutes and the sediment is examined microscopically for the presence of cells, casts, crystals and miscellaneous components.

EVALUATION

The urine creatinine concentration should be used to normalize the quantity of any analyte of interest, as this will correct for incomplete urine collection or urine dilution that may have resulted from drinking water spillage within the metabolism cages (Haas et al. 1997). The quantity of creatinine in a spot urine sample serves as an accurate index of the 24 hour urine output in most species.

Glucosuria in the face of normal plasma glucose may indicate a functional deficit in the proximal tubule that may precede the appearance of actual tubular necrosis or injury (Stonard et al. 1987; Finco 1997; Loeb and Quimby 1999; Newman and Price 1999; Aleo et al. 2002). Glucosuria is normal in gerbils.

Excretion of markedly elevated levels of protein is indicative of glomerular disease, whereas low-level proteinuria indicates tubular damage or very early/lowgrade glomerular injury (Peterson et al. 1969; Finco 1997). Further determination of the severity and site of nephron injury requires qualitative identification and quantitation of the individual proteins present in the urine.

The presence of greater than 1–2 erythrocytes or neutrophils per high-power field confirms a positive result of dipstick analysis for blood or leukocyte esterase. Increased number of hyaline casts (small numbers are normal in most species), or the presence of granules (cells or cellular debris) within casts may be indicative of tubular injury. The presence of renal tubular epithelial cells in urine sediment strongly indicates tubular injury (Stonard 1990; Hofmann et al. 1994; Finco 1997; Finn and Porter 1998; Newman and Price 1999).

Small numbers of lipid droplets (representing neutral lipid, lipoprotein or phospholipid) are considered normal in dogs, cats, mice and humans (Gross et al. 1991; Streather et al. 1993; Finco 1997). Increased excretion of lipid droplets (which may cause increased urine turbidity) may indicate glomerular injury (de Mendoza et al. 1976; Gherardi and Calandra 1982). Increased urinary phospholipid excretion (confirmed by thin layer chromatography) may indicate early papillary injury/renal papillary necrosis in rodents (Thanh et al. 2001).

LIMITATIONS OF THE METHOD

The glucose oxidase method (used in the dipsticks and by many automated analyzers) can show a false positive result in some species (e.g, dog, mouse) with high urinary ascorbate levels or in urine contaminated with hypochlorite (bleach) used as a disinfectant (Finco 1997; Loeb and Quimby 1999).

The dipstick reagent for blood detects hemoglobin but cannot distinguish between free hemoglobin (possibly due to intravenous hemolysis) or intact erythrocytes and also cross-reacts with myoglobin (present in urine as the consequence of muscle injury). Addition of ammonium sulfate to the urine (which precipitates hemoglobin but not myoglobin) can help differentiate the two pigments (Graff 1983).

The dipstick test uses the bromphenol blue method for protein that is most sensitive for albumin and is optimized for protein levels/urine pH common in humans (Newman and Price 1999) False positives are thus common in animals because of their higher urine pH and background urine protein (Finco 1997; Loeb and Quimby 1999). A positive result with a urine dipstick test must therefore be followed by a more detailed quantitative and qualitative assessment of the increase in protein excretion in order to determine the site and nature of the renal injury present.

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I.E.2.3
Assessment of Renal Injury by Urine Proteins

PURPOSE AND RATIONALE

The combination of qualitative and quantitative assessment of the normally-excreted urine protein (see previous section "Assessment of Renal Injury by Urinalysis") can serve as a reliable indicator of the nephron segment(s) affected by the injury. Proteins may also appear in the urine as the consequence of their release from damaged nephrons. Many of these are unique to the kidney; those that are not are either too large to pass an intact glomerular filter or have not been shown to circulate in plasma. Furthermore, several of these are both upregulated and shed to the urine, thus increasing their sensitivity as injury biomarkers.

PROCEDURE

Twenty-four hour urine collection is preferred for best accuracy, although point-in-time samples can be used. Urine samples are collected from the species in question by appropriate methods (see section "Assessment of Renal Injury by Urinalysis"). Total protein and creatinine are determined by appropriate methods (see under "Assessment of Renal Injury by Serum Chemistry" and "Assessment of Renal Injury by Urinalysis") and a urine protein:creatinine ratio is calculated.

Albumin can be measured quantitatively by the bromcresol green method in most species (Evans and Duncan 2003). Other proteins (described below) are measured by immunometric methods. Newer methods based on proteomics technology (concentration of proteins by acetone precipitation or ultracentrifugation, separation by 2-d gel electrophoresis or chromatographic techniques with subsequent identification and quantitation by mass spectrometry) have been used experimentally (Bandara and Kennedy 2002; Chapman 2002; Thongboonkerd et al. 2002a, b).

EVALUATION

Protein:creatinine ratios of >1.0 are considered indicative of significant renal proteinuria in most species (Gregory 2003). Excretion of markedly elevated levels of protein (protein:creatinine ratio > 5.0) is indicative of glomerular disease, whereas low-level proteinuria indicates tubular damage or very early/lowgrade glomerular injury (Peterson et al. 1969; Finco 1997; Gregory 2003).

Increased albumin usually indicates glomerular injury, although it is not a specific marker for any one nephron site (Guder and Hofmann 1992; Price et al. 1996; Finn and Porter 1998). High levels of albumin in urine are invariably the result of glomerular malfunction. Low level increases in urine albumin, especially in circumstances where total urine protein excretion is not elevated ("microalbuminuria") can result either from increased glomerular filtration or decreased tubular reabsorption, and albumin values

in this range must be interpreted in comparison to the excretion of other protein biomarkers. Concomitant elevation of albumin and one or more low molecular weight proteins indicates tubular malfunction, while albumin elevation alone or concurrently with a high molecular weight protein indicates primary glomerular injury (Peterson et al. 1969; Finn and Porter 1998; Guder et al. 1998; Umbreit and Wiedemann 2000). Calculation of the relative clearance of albumin and either immunoglobulin or transferrin is a useful indicator of the degree of glomerular injury (the higher the ratio, the more severe the damage) (Tencer et al. 1998; 2000).

Elevation of any of the filtered low molecular weight proteins in urine is an indication of a primary defect in tubular uptake, either as a consequence of decreased nephron mass or competition for the endocytic pathway by a competing substrate (Finn and Porter 1998; Aleo et al. 2002; 2003). The most commonly used of these are α-1 and β-2 microglobulins; in particular, α-1-microglobulin is present in fairly high abundance in normal urine, shows robust elevation with tubular disease and is slightly more stable to degradation than β-2-microglobulins (Donaldson et al. 1989; Price et al. 1996; 1997; Price 2000; 2002). Other low molecular weight proteins which are filtered and thus may be used as indicators of proximal tubule function include retinol binding protein and cystatin C (Aleo et al. 2002; 2003; Donaldson et al. 1990; Herget-Rosenthal et al. 2001; Uchida and Gotoh 2002).

Increases in the urinary levels of proteins that are either shed or secreted into the urine from the site of injury may be more sensitive than alterations in filtered proteins in detecting renal injury. These include fibronectin, collagen IV and liver fatty acid binding protein (LFBP) as markers of glomerular injury, α-Glutathione-S-transferase, clusterin, kidney injury molecule-1 (KIM-1), and cysteine-rich protein 61 (CYR61) for the proximal tubule, π- or μ-glutathione-S-transferase for the distal tubule and Pap X 5C10 antigen (Pap A1) for the medullary collecting ducts (for reviews see Emeigh Hart 2005; Emeigh Hart and Kinter 2005).

LIMITATIONS OF THE METHOD

The majority of assay methods for excreted low molecular weight proteins are based in immunometric methods (gel immunodiffusion, nephelometry or ELISA) and the antibodies do not cross-react with homologous proteins in animal urine (the exception is β_2-microglobulin, for which a rat-specific latex agglutination assay exists) (Poulik et al. 1981; MacNeil 1991; Viau et al. 1986; Loeb 1998; Twyman et al. 2000). Measurement of urinary retinol has been shown to serve as an acceptable surrogate for direct measurement of retinol binding protein in the rat (M. Aleo, personal communication).

Dipstick tests designed to assess microalbuminuria in humans (Clinitek Microalbumin Test, Bayer Diagnostics Corporation) are not accurate for use in animals (Pressler et al. 2002). Dipsticks for point-in-time assessment of microalbuminuria in dogs and cats are commercially available (Heska Corporation).

Although there are published antibody-based methods for many of the kidney-specific proteins listed above, antibodies are not commercially available currently for many of them (specifically, KIM-1, CYR-61 and Pap A1). Where the antibodies are readily available, cross-reactivity across species has not always been established (these have in general been used only in human and/or rat). Heart and kidney specific isoforms of fatty acid binding protein exist that may cross-react in some species with the LFBP antibodies; additionally, α-2μ-globulin, a normal component of male rat urine, is homologous to K-FABP and may cross-react with the antibodies to LFBP (for reviews see Emeigh Hart, 2005, Emeigh Hart and Kinter 2005).

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I.E.2.4 Assessment of Renal Injury by Urine Enzymes

PURPOSE AND RATIONALE

Urinary enzyme activity provides a means to determine the presence and location of renal tubular injury as opposed to an index to the functional status of the nephron. Urine enzymes provide several advantages over urine protein for the assessment of tubular injury (Stonard et al. 1987; Vanderlinde et al. 1981; Price 1982; Plummer et al. 1986; Clemo 1998; Dubach et al. 1988; Westhuyzen et al. 2003):

- Increased sensitivity – enzyme levels in urine are frequently elevated in advance of overt evidence of renal malfunction and can be used to predict its onset
- Dose-response – the amount of enzyme activity in the urine accurately reflects the degree of tubular injury present
- Ease of analysis – most use chromatographic assays, which have been well validated for the same enzymes present in plasma and can be performed on automated equipment
- Utility in a variety of species – activity as opposed to antigen mass is measured, so cross-reactivity of antibodies or probes across species is not a consideration
- Repeatability – enzyme measurements over time can be used to determine the reversibility or progression of renal lesions
- Ability to localize the injury to a specific nephron site/subcellular location – serves as an index to the severity of the underlying injury. As a general rule, brush border enzymes indicate less severe damage than cytosolic, mitochondrial or microsomal enzymes.

For best results in determining the presence and site of nephron damage, a battery of enzymes as opposed to a single biomarker should be examined, especially since the most sensitive assay is highly variable, depending on the toxicant and species. Additionally, since many of the enzymes are not completely specific to a selected nephron segment, use of a battery of tests allows more precise localization of the site of injury (Price 1982).

PROCEDURE

Urine samples are collected and maintained as described in section "Assessment of Renal Injury by Urinalysis". There are some special considerations in sample collection and handling that are critical to accurate assessment of urine enzyme activity (Vanderlinde, 1981; Price, 1982; Plummer et al. 1986; Mueller et al. 1986, 1989, Loeb et al. 1997, Clemo 1998, Jung and Grutzmann 1988, Loeb 1998):

- Contamination of collected specimens must be carefully avoided, as enzymes present in feces, food or bacteria can contribute significantly to the activity present in the urine
- Enzyme activity can also result from increased numbers of erythrocytes, leukocytes or epithelial cells in the urine. Centrifugation of urine to remove contaminating cells will help to mitigate this source of error, and examination of the resulting sediment will allow the investigator to discard the results from heavily contaminated samples
- Normal urine frequently contains a variety of low-molecular weight substances (urea is a notable example) that can inhibit the enzymes of interest The toxicant may also act as an inhibitor. These substances usually can be removed by dialysis, dilution, Sephadex filtration, ultrafiltration or gel filtration
- The effects of dilution and pH on enzyme activity must also be considered, as these factors are highly variable in urine samples. Most enzymes are very labile to acid pH and the activity of many can be decreased substantially in concentrated urine
- Enzymes are generally less stable in urine than they are in high protein matrices such as serum or plasma (especially true for γ-glutamyl transpeptidase). Assays must be performed within two hours of sample collection or a stabilizing substrate needs to be added to the sample, preferably during collection. Albumin, ethylene glycol, glycerol, or erythritol will satisfactorily preserve the activity of most commonly used enzymes when the samples are stored at – 20 ° C.

Either 24 hour urine collection or timed urine samples collected at the same time each day is recommended, with the activity expressed per unit of time (Price 1982, Plummer et al. 1986). If the assessment is to be repeated with time, the samples should be collected over the same time period on each day because there is pronounced diurnal variation in excretion rate of some enzymes (Maruhn et al. 1977, Price 1982, Gossett et al. 1987). For spot urine samples or those where accurate timed collection is not possible, normalization of activity per unit of creatinine can be done and this has been shown to be reasonably well correlated to 24 hour enzyme activity (Vanderlinde 1981, Grauer et al. 1995). Diet and age-matched controls must be included if enzyme activity is to be normalized to creatinine, to control for the effects of these variables on creatinine excretion (Plummer et al. 1986, Casadevall et al. 1995).

Most of these assays can be performed using the same automated equipment that is used for plasma, but the assays will need to be validated separately for urine to ensure that the matrix (urine *versus* plasma) does not interfere with the method and that the enzyme levels present in urine, either endogenously or following injury, are within the limits of linearity. Immunoassays (ELISA) are commercially available for α, π or μ - glutathione-S-transferase in the rat and human (Biotrin International, Dublin).

EVALUATION

Enzyme activity is expressed either per unit of time or per unit of creatinine. Normalization of enzyme activity per unit of volume or osmolality is not recommended because of the high degree of variability of these parameters (Price 1982; Plummer et al. 1986).

N-acetyl-β-D-glucosaminidase (NAG), lysozyme (muraminidase), β-galactosidase, β-glucuronidase, β-glucosidase, alanine aminopeptidase (AAP), leucine aminopeptidase (LAP), leucine aminopeptidase, γ-glutamyl transpeptidase (GGT), intestinal alkaline phosphatase (IALP), and α-glutathione-S-transferase (α-GST, ligandin) are localized to the proximal tubule and are considered to indicate injury to this segment. LAP, GGT and IALP are brush border enzymes and thus appear earlier and/or denote less severe injury whereas the others are either cytosolic (α-GST), lysosomal or microsomal and appear later and with more severe injury. NAG is the most commonly used of these, as it has been validated for the greatest number of species and has less diurnal variation in excretion than many of the others (thus is more readily amenable

to use with spot urine collection) (reviewed in Emeigh Hart 2005; Emeigh Hart and Kinter 2005).

π (human) or μ (rat) -GST has been used as a marker for the distal tubule or collecting duct. Its activity is identical to that of α-GST and the two isoenzymes cannot be distinguished using standard assay methods (since α-GST accounts for the majority of the urinary activity even with distal tubular injury) and an immunoassay must be used to evaluate this enzyme (Sundberg et al. 1994; Van Kreel et al. 2002). For best results, a stabilizer (available from the test kit manufacturer) should be added to urine during collection (Shaw, personal communication).

Other enzymes (lactate dehydrogenase, LDH, and aspartate aminotransferase, AST) also indicate tubular injury when present in the urine but they cannot be localized to a specific nephron segment. Despite this, LDH is commonly used as a marker for distal tubular injury (Price 1982; Guder and Ross 1984; Clemo 1998).

LIMITATIONS OF THE METHOD

General Limitations of Urine Enzyme Analysis

The correct sample handling methodologies have not been well validated for all enzymes in all species and it may require some effort on the part of the investigator to determine the need and optimal method for sample preparation.

The inherent limitations of the Jaffe method for determination of creatinine have been discussed in section "Assessment of Renal Injury by Serum Chemistry". Factors which result in reduced excretion of creatinine without acute tubular injury (e.g., chronic renal disease in aged animals with pronounced loss of nephron mass, prerenal reduction of GFR) will also result in reduced urine creatinine and falsely elevated enzyme activity when normalized to creatinine (Price 1982, Plummer et al. 1986, Casadevall et al. 1995).

Limitations of Specific Enzymes

NAG is not specific for the proximal tubule, but also increases with papillary injury and glomerular disorders. Concomitant evaluation of one of the proximal tubule specific brush border enzymes (LAP, GGT or IALP) allows correct interpretation of the NAG increase. There are also significant increases in basal excretion as a function of age in rats and gender in dogs (higher in males), so age and sex-matched controls must be used (reviewed in Emeigh Hart and Kinter 2005).

GGT is notoriously unstable in urine and must be assayed very rapidly, even in the presence of urine stabilizers (Loeb 1998). There is high diurnal variation in baseline excretion rate in dogs that cannot be corrected in spot urine samples by creatinine normalization, so timed urine collection must be used with this enzyme in this species (Gossett et al. 1987).

LAP is also seen in the urine with very early glomerular disease (Bedir et al. 1996). The assay substrate is also a substrate for leukocyte esterase and thus increased neutrophils in the urine (pyuria) will cause a false positive result (Vlaskow et al. 2000).

Due to size limitations, most enzymes present in plasma are not filtered into the urine but there are some exceptions (e.g., lysozyme); these will be increased in the urine if tubular function (and uptake of filtered protein) is decreased. In addition, if glomerular injury accompanies tubular injury leakage of larger molecular weight proteins may occur and plasma source enzymes that ordinarily would not be filtered may appear in urine. AST, LDH and IALP may thus appear in urine.

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I.E.3 Experimental Models of Renal Failure

I.E.3.1 Chronic Renal Failure in the Rat

PURPOSE AND RATIONALE

Chronic renal failure is a frequent pathological condition in man. An animal model as described by Acott et al. (1987) is of value to test new diuretics under these conditions.

PROCEDURE

Sprague-Dawley rats weighing 150–200 g are anesthetized. Through a 6-cm midline incision in the abdominal wall the small bowel and cecum are lifted and placed on saline-soaked gauze sponges. The exposed right kidney is dissected from the retroperitoneal area and the vascular and ureteric pedicles are ligated with 2–0 silk sutures, transected, and the kidney is removed. The renal artery of the left kidney is dissected into the hilum to expose the three main segmental renal arteries. The kidney is not dissected out of the peritoneum. The anterior caudal branch of the artery is then temporarily ligated to establish the volume of renal tissue supplied. The area of ischemia becomes demarcated within 10–15 s. If this approximates 1/4 to 1/3 of the kidney, a permanent ligature is placed. The viscera are then carefully replaced in the abdomen and peritoneum and linea alba are closed with a continuous suture. The skin is closed with stainless-steel clips.

Blood for serum creatinine is collected at various time intervals up to 12 months. In association with this, 24-hour urines are collected for measurement of creatinine, protein, and specific gravity.

EVALUATION

Serum creatinine increases up to 500 mM/L after 12 months, whereas creatinine clearance decreases. Significantly increased urine volumes are accompanied by decreased urine specific gravity indicating a decreased concentrating ability. Proteinuria is significantly increased. Terminal uremia occurs after 14–15 months.

CRITICAL ASSESSMENT OF THE METHOD

The method may be used for special pharmacological studies as well as for evaluation of renal toxicity of new chemicals.

MODIFICATIONS OF THE METHOD

Sancho et al. (1989) used a similar procedure in rats ligating two of the three terminal branches of the left renal artery, followed by right nephrectomy.

Freeman (1971) induced azotemia combined with hypothermia in rats by ligation of the urinary bladder at the base.

Williams et al. (1997) described renal ischemia-reperfusion injury in rats. The animals were anesthetized and subjected to 45min of bilateral renal occlusion using atraumatic vascular clamps before renal perfusion was reestablished. After various time interval (up to 1week) blood urea nitrogen, creatinine and myeloperoxidase activity in the kidney were determined. The protective effects of an intracellular adhesion molecule monoclonal antibody were tested.

Ishidoya et al. (1995), Klahr and Morrissey (1997) induced interstitial renal fibrosis by unilateral ureteral obstruction in Sprague Dawley rats and tested the effect of ACE inhibitors and angiotensinII receptor antagonists.

Hartenbower and Coburn (1972) described a method for producing chronic renal insufficiency in the *chick*.

By urethral ligation, the function of one kidney was completely eliminated, and the functional mass of the other was reduced by two-thirds. The method resulted in elevation of plasma concentration of uric acid, the major product of protein catabolism in avian plasma, to levels 2–4 times normal for periods as long as 3 weeks.

Two to 3 weeks old White Leghorn cockerels are anesthetized and the abdominal feathers are clipped. The chick is placed supine on a small operating board with hips flexed and legs extended over the head. An incision is made along the left side of the abdomen extending into the peritoneal cavity. Self-retaining retractors are used to maintain exposure. The right ureter is identified and ligated just proximal to its junction with the cloaca. The left ureter and renal vein are ligated with a single suture near the middle of the left kidney.

The degree of azotemia is assessed by measuring urea and/or creatinine levels in blood samples obtained at intervals of 2–6 days after surgery. Plasma analytes are compared between operated and sham-operated animals. Histological examination is performed after sacrifice of the animals.

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I.E.3.2 Chronic Renal Failure after Subtotal (Five-Sixths) Nephrectomy in Rats

PURPOSE AND RATIONALE

Subtotal (five-sixths) nephrectomy in rats has been used by many authors as model for chronic renal failure. Levine et al. (1997) used this model to evaluate the hypothesis that under these conditions endogenous angiotensin II modulates in vivo bicarbonate reabsorption (J_{tCO2}) in distal tubules via H^+-adenosinetriphosphatase and Na^+/H^+-exchange. Bicarbonate reabsorption (J_{tCO2}) in distal tubules is significantly increased in five-sixths nephrectomized rats.

PROCEDURE

Surgical Procedure

Male Sprague-Dawley rats weighing 230–280 g are anesthetized. Prior to the first incision, the animal is given 20 ml/kg normal saline subcutaneously. On a heated table, a midline laparatomy is performed 2 cm below the xiphoid bone cartilage and the abdomen is opened. The right kidney is isolated and brought out of the abdomen by grasping the fat at the lower pole of the kidney. This fat is blunt dissected (sparing the adrenal gland), and three silk 4–0 ligatures are passed under the ureter, renal artery, and renal vein, tying one distally and two proximally. The ureter, renal artery and renal vein are then cut between the ligatures, the proximal ties are cut short, and the distal tie is pulled out, removing the kidney. After the area is checked for bleeding, the left kidney is the similarly isolated and brought out of the abdomen. The fat around the kidney is blunt dissected, avoiding excessive handling of the kidney or damage to the ureter, and the left and right renal poles are removed (two-thirds nephrectomy). The left remnant kidney is then returned to the abdomen and moistened with 5 ml of normal saline. The abdomen is closed with sutures and the skin with autoclips. Sham rats undergo the same procedure, except that the kidneys are only touched by the instruments. All rats are allowed 13–16 days recovery prior to microperfusion.

Microperfusion Experiments

The rats are anesthetized with 100 mg/kg thiobutabarbital sodium and placed on a heated operating table. After tracheostomy, using a PE-240 tubing, the left carotid artery is cannulated for continuous blood pressure measurement and collection of blood for acid-base and electrolyte analyses while the left jugular vein is cannulated with three lines for infusion of fluid, pentobarbital sodium anesthetic and 10 % Lissamine green (Levine et al. 1996). The left kidney is exposed by flank incision, carefully dissected from the adrenal gland, and immobilized in a stainless steel cup covered with mineral oil. The ureter is catheterized with PE-50 tubing to ensure proper urine flow.

To replace surgical fluid losses, the rats are infused at 1 % body wt/h for 30 min via the jugular vein with

donor plasma from control rats. The animals are then maintained on 0.9 % saline at 1 % body wt/h for the remainder of the experiment.

Two-loop perfusable surface distal tubules are identified by injecting a bolus of 1 % Lissamine green into surface proximal loops and observing its passage through the nephron. The distal tubules are perfused at 15nl/min with a hypotonic solution containing (in mM) 28 HCO_3^-, 26 Cl^-, 56 Na^+, 2 K^+, 1.8 Ca^{2+}, 22 urea, and 4 gluconate, as well as 0.05 % FD and C green dye no. 3 (Keystone, Chicago, IL) and 0.1 % albumin. The perfused bicarbonate load, higher than in free flow, is chosen to more easily reveal the effect of inhibitors. Sample collections are quantitative and timed. A 10-min preperfusion period precedes all collections.

Groups of five-sixths nephrectomized rats are treated with various agents, e.g., angiotensinII, angiotensin1 receptor antagonists, or Na^+/H^+ antiporter inhibitors.

Analyses

Whole blood and urine pH and P_{CO2} are measured quantitatively by an electrode blood-gas system and HCO_3 concentrations are calculated. Plasma and urine Na^+ and K^+ concentrations are measured by flame photometry and Cl^- concentrations by electrotitration. Plasma total protein concentrations and urine specific gravity are measured by refractometry, and hematocrits are determined by a microcapillary reader. Urine osmolalities are determined by freezing-point osmometry. Plasma creatinine concentrations are determined by the Jaffé method without deproteinization.

Perfusate and sample total CO_2 concentrations are measured by microcalorimetry (Levine et al. 1996). A standard curve is run before sample analysis, and standard samples bracket the determination of sample and perfusate CO_2 determination.

EVALUATION

The perfusion rate (R_P) is calculated as the product of the measured collection rate (R_C) and the ratio of inulin concentration in collected tubular fluid and perfusate. Water absorption (J_V) is calculated as the difference between the calculated perfusion rate and the measured collection rate (R_P minus R_C). Jt_{CO2} is calculated as

$$J_{tCO2} = [(R_P C_P)-(R_C C_C)/L,$$

whereby C_P and C_C are the measured CO_2 concentrations in perfusate and collected fluid, respectively, and L is the tubular length in millimeters, measured by dissection after latex injection.

Data are expressed as the means ± SE. Statistical significance is assessed by two-tailed unpaired Student's t-test or one way analysis of variance (ANOVA) followed by either Dunnett's test for multiple comparisons vs. control or the Newman-Keuls test for all pair-wise comparisons. Tests indicating a value of $P < 0.05$ indicate a statistically significant difference between groups.

MODIFICATIONS OF THE METHOD

Function of the remnant kidney after subtotal (5/6) nephrectomy in **rats** has been used for many purposes:

- To test the potential benefit of calcium antagonists (Tolins and Raij 1990; Jarusiripipat et al. 1992; Van den Branden et al. 1997) or antioxidants (Vaziri et al. 1998)
- To verify the effect of ACE-inhibitors (Pelayo et al. 1990; Kakinuma et al. 1992; Ashab et al. 1995; Liu et al. 1996; Ali et al. 1998; Cohen et al. 1998; MacLaughlin et al. 1998), angiotensin antagonists (Kohzuki et al. 1994; 1995; Brooks et al. 1995; Barreto-Chaves and Mello-Aires 1996; Noda et al. 1997; Lariviere et al. 1998; Roczniak et al. 1999) and endothelin receptor antagonists (Nabokov et al. 1996; Potter et al. 1997; Wolf et al. 1999; Brochu et al. 1999; Shimizu et al. 1999), or Na^+/H^+ antiporter inhibitors (Fernandez et al. 1994)
- To study the influence of hormones, such as parathyroid hormone (Fukagawa et al. 1991; Urena et al. 1994; Yi et al. 1995; Schaefer et al. 1996), growth hormone (Santos et al. 1992; Garcia de Boto et al. 1996), insulin-like growth factorI (Hazel et al. 1994; Mak and Pak 1996; Tonshoff et al. 1997), vasopressin (Bardoux et al. 1999), atrial natriuretic factor (Wong and Wong 1991; 1992); Luk et al. 1995) or erythropoietin (Poux et al. 1995; Zhou et al. 1997), during development of chronic renal failure.

Kimura et al. (1999) reported a model of progressive chronic renal failure in *rats*, produced by a single injection of microspheres (20 to 30 μM in diameter) into the left renal artery after right nephrectomy.

Cowley et al. (1996) described the **Han:SRPD rat** strain which develops autosomal dominant polycystic kidney disease with chronic renal failure that resembles human autosomal dominant polycystic kidney disease.

Chronic renal failure can be induced by feeding a lithium containing diet (40–50 mmol/kg) to newborn *rats* until an age of 55–65 weeks (Christensen et al. 1992; 1997; Nyengaard et al. 1994).

Stockelman et al. (1998) described chronic renal failure in a **mouse** model of human adenine phosphoribo-syltransferase deficiency. Hamilton and Cotes (1994) used a partial nephrectomy model in *mice* with two-thirds of the total renal mass excised to evaluate erythropoiesis and erythropoietin production from extrarenal sources such as the submandibulary salivary gland. Koumegawa et al. (1991) suggested the DBA/2FG-pcy *mouse*, which develops numerous cysts in kidney cortex and medulla, a progressive anemia and an elevation of blood urea nitrogen, as a useful spontaneous model of progressive renal failure.

Brown et al. (1990) studied the metabolism of erythropoietin in normal and uremic **rabbits** with 5/6 nephrectomy. Bonilla-Felix used *rabbits* after 75 % nephrectomy to study the response of cortical collecting ducts from remnant kidneys to arginine vasopressin.

Fine et al. (1990), Vaneerdeweg et al. (1992) described surgical techniques for kidney resection to produce chronic renal failure in **dogs**.

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I.E.3.3 Experimental (Immune-Mediated) Glomerulonephritis

PURPOSE AND RATIONALE

Various experimental procedures were described as models for glomerulonephritis in human beings. Most of them were developed in rats and rabbits. They involve the reactions of antibodies against renal components, such as Masugi nephritis (Masugi and Sato 1934), Heymann nephritis (Heymann 1959), nephrotoxic serum nephritis (Unanue and Dixon 1967), crescentic type anti-glomerular membrane nephritis (Nagoe et al. 1994, 1998), anti-Thy1 nephritis (Chen et al. 1999).

Moreover, MRL Mpf lpr/lpr (MRL/lpr)-mice were described which spontaneously develop a severe disease with many symptoms very similar to human systemic lupus erythematodes, i.e. hypergammaglobulinemia, and glomerulonephritis (Theofilopoulos and Dixon 1981).

Nephrotoxic serum nephritis is produced in animals by administration of heterologous antibody against

glomerular basement membrane. This is regarded as an experimental model of human glomerular immune injury resulting in glomerulonephritis (Unanue and Dixon 1967). The glomerular lesions induced by nephrotoxic serum nephritis vary with species. The Wistar-Kyoto rat is susceptible to the induction of a crescentic glomerulonephritis following small doses of nephrotoxic serum (Kushiro et al. 1998; Suzuki et al. 1998).

PROCEDURE

Preparation of Nephrotoxic Serum

Normal Wistar rat kidneys are fully perfused with physiologic saline through a catheter placed in the aorta. Renal cortical tissue is removed, homogenized and diluted with physiological saline at about 20 % suspension. Two ml of renal cortical homogenate are emulsified with an equal volume of Freund's complete adjuvant. This emulsion is injected subcutaneously into rabbits twice a month for two months. Seven days after the last injection, the rabbits are bled from the carotid artery under anesthesia. The sera are decomplemented for 30 min at 56 °C and absorbed with freshly harvested rat erythrocytes.

Experimental Protocol

Male Wistar-Kyoto rats weighing 150 g receive either continuous administration of the test drug by an osmotic pump (ALZA Co., Palo Alto, USA) or saline. Twenty-four hours later, the rats are injected with 1 ml of nephrotoxic serum. At 9, 12, and 14 days, urine samples are collected and urinary protein levels are measured using the Lowry method. At 14 days the rats are sacrificed under ether anesthesia, and both kidneys are removed. Portions of these tissues are processed for light microscopy, immunofluorescence staining and immunoperoxidase staining.

For light microscopy, tissues are fixed and embedded in paraffin. Sections are stained with hematoxylin and eosin, and periodic acid Schiff's reagent. Twenty glomeruli are examined per rat and number of glomeruli with injury (crescent formation) are determined.

Indirect immunofluorescence studies are performed on 3 μm-thick cryostat sections which are air dried and incubated with anti-rat intercellular adhesion molecule-1 (ICAM-1) antibody (Tamatani and Miyasaka 1990) for 60 min at room temperature. After washing the antibody binding is visualized by incubating the sections for 30 min with fluorescein isothiocyanate-labeled goat anti-mouse IgG.

Direct immunofluorescence studies are performed on 4mm thick cryostat sections, which are incubated with fluorescein isothiocyanate-labeled goat anti-rat IgG, goat anti-rat C3, goat anti-rat fibrinogen and goat anti-rabbit IgG. (Note: these procedures may also be performed on the paraffin sections using standard immunohistochemistry techniques).

The staining intensity of 20 glomeruli per rat is semi-quantitatively assessed into 4 grades.

The distribution of leukocytes is examined using an immunoperoxidase ABC kit (Vector Lab, Burlingame, USA). Nonspecific protein binding is blocked by incubating the cryostat sections with 10 % bovine serum in Tris-buffered saline for 20 min. Nonspecific staining is blocked by 15 min incubation with avidin and then biotin using the avidin-biotin blocking kit (Vector Lab). Endogenous peroxidase activity is inhibited by incubating the sections in methanol containing 0.3 % H_2O_2 for 20 min. Sections are first incubated for 60 min with primary antibodies at room temperature, incubating monoclonal antibodies against rat monocytes/macrophages (ED-1), rat CD4 and rat CD8. Then the sections are incubated with biotinylated donkey anti-mouse IgG for 30 min at room temperature. Biotinylated horseradish peroxidase is applied for 30 min at room temperature. Peroxidase activity is developed in 3,3-diaminobenzidine and hydrogen peroxide. The sections are then counterstained with Mayer's hematoxylin. The number of ED-1 positive cells, CD4 positive cells and CD8 positive cells per glomerular cross-section is counted in 20 glomeruli per rat.

EVALUATION

All quantitative, continuously distributed data are expressed as mean ± SEM. Significance of differences between groups is determined using Wilcoxon's test. Statistical evaluation of severity grades to determine differences between treatment groups is inappropriate.

MODIFICATIONS OF THE METHOD

Masugi and Sato (1934), Krakower and Greenspon (1951), Heyman et al. (1959, 1965), Eddington et al. (1968) already described experimental allergic glomerulonephritis in rats.

Ito et al. (1983), Nagao et al. (1994, 1998) induced crescentic type antiglomerular basement membrane nephritis in male Sprague Dawley rats by injecting 6.5 mg rabbit gamma-globulin in 0.25 ml Freund's complete adjuvant into the hind foot pads, following the injection of 0.6 ml of rabbit anti-rat glomerular basement membrane serum into the tail vein.

Couser et al. (1978) studied the development of immune deposits on the subepithelial surface of the glomerular capillary wall in isolated rat kidneys perfused at controlled perfusion pressure, pH, temperature, and flow rates with recirculating oxygenated perfusate containing bovine serum albumin in buffer and sheep antibody to rat proximal tubular epithelial cell brush border antigen.

Hayashi et al. (1996) tested the effects of a flavonoid in original-type anti-glomerular basement membrane antibody associated glomerulonephritis in male Sprague Dawley rats on upregulation of intracellular adhesion molecule expression and on increase in leukocyte function-associated antigen positive cells in nephritic glomeruli.

Nagamatsu et al. (1999) found beneficial effects of an angiotensin II type I receptor antagonist in anti-glomerular basement membrane antibody-associated nephritis in rats.

Sanaka et al. (1997) evaluated the effects of a free radical scavenger on the progression of nephrotoxic serum nephritis in male Sprague Dawley rats. The rat glomerular basement membrane was prepared according to the method of Krakower and Greenspon (1951) who localized the nephrotoxic antigen within the isolated renal glomerulus.

Kawasaki et al. (1992) induced crescentic glomerulonephritis with a small dose of nephrotoxic serum in WKY rats, which was characterized by the early infiltration of CD8 positive cells in glomeruli. In vivo depletion of CD8 positive cells completely prevented proteinuria and crescent formation.

Okuda et al. (1990) provided evidence of an elevated expression of transforming growth factor-β, proteoglycans and fibronectin in glomerulonephritis induced in rats by injection of anti-thymocyte serum.

Hamada and Nagase (1996), Chen et al. (1999) induced anti-Thy1 nephritis with the antibody to the Thy-1 antigen which is present in the mesangial cells of the glomeruli. The early pathobiological cellular events are characterized by invasion of platelets, polymorphonuclear leukocytes, and monocytes into the glomerulus which occurs within hours after induction of nephritis. Complement-dependent mesangiolysis then ensues between day 1 and 3.

Passive Heymann nephritis (Heymann 1959) was used a model by Hara et al. (1991), Nagao et al. (1996), Heise et al. (1998). This disease is induced in rats by heterologous antibody to crude renal border antigen Fx1A. The model is characterized by granular deposition of heterologous and homologous antibody and complement along the glomerular capillary wall and as a counterpart, extensive electron-dense subepithelial deposits are seen at the ultrastructural level. Massive proteinuria develops after a latent period of 2 to 4 days in the absence of glomerular hypercellularity.

Kawasaki et al. (1995) studied the therapeutic effect of combined treatment with monoclonal antibodies against intercellular adhesion molecule1 (ICAM-1) and lymphocyte-function-associated antigen1 (LFA-1) in **Masugi nephritis** of Wistar-Kyoto rats.

Thaiss et al. (1989) evaluated the effect of the immunosuppressant cyclosporin A on an active model of in situ immune complex glomerulonephritis. Wistar rats were preimmunized with human IgG and 2weeks after the last antigen injection, the left kidney was perfused with cationized human IgG in order to induce unilateral in situ immune complex glomerulonephritis.

Okubo et al. (1990) studied the immunosuppressive effects of FK506 on active Heymann's nephritis and the autologous phase of Masugi nephritis.

Rennke et al. (1994) developed a model system of acute nephritis in the rat whereby a chemically reactive form of the hapten azobenzenearsonate is introduced directly in to the left kidney of pre-immunized Brown Norway rats.

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I.E.3.4 Toxicant-Induced Renal Injury

PURPOSE AND RATIONALE

Some compounds used in antineoplastic therapy induce acute renal failure in animals. Examples include the antibiotic adriamycin (doxorubicin) (Milner et al. 1991; 1994; Chagnac et al. 1994; Wapstra et al. 1996; De Boer et al. 1999; Mutti et al. 1999), daunomycin (Kimura et al. 1993) or puromycin aminonucleoside (Yayama et al. 1993; Guoji et al. 1994; Magil 1996; Ebihara et al. 1997; Nosaka et al. 1997; Park et al. 1998; Asami et al. 1999; Pedraza-Chaverri et al. 1999) or cisplatin (Abdel-Gayoum et al. 1999), which cause injury to glomerular epithelial cells, resulting in profound proteinuria and resulting tubular injury. Other compounds (e.g., cisplatin (Wapstra et al. 1996) selectively damage the renal proximal tubules. These models allow the investigator to test the protective effects of drugs on the genesis of renal disease or in established injury, i.e., mimic the clinical situation.

PROCEDURE

The species, dose and time needed to induce injury and the appropriate endpoints will vary with the toxicant selected. The following is an example of such a model:

Procedure:

Male Wistar rats with an initial weight of about 300 g receive a single intravenous dose of 2 mg/kg adriamycin. Twice a week during a 12-weeks period, the animals are weighed, 24-h urine is collected and blood pressure is measured by the tail-cuff method.

During the first 5 weeks, all animals are kept on a low sodium diet with tap water ad libitum. After stabilization of proteinuria (5 weeks), animals are divided into two groups receiving either low sodium or normal sodium diet. After a week of stabilization on these diets, animals may be treated with test componds.

The regimen is continued until the end of the study (week 12), at which time all animals are sacrificed and blood samples and kidney tissue are obtained.

During each blood pressure measurement session, five measurements are recorded for each animal. The blood pressure is taken as the mean of the last 3 recordings. Urinary protein is determined by the Pyrogallol Red-molybdate method (RA-1000 Technicon). Urinary sodium, creatinine and urea and serum electrolytes, creatinine, albumin, cholesterol and triacylglycerols are measured by a standard autoanalyser technique. Kidney samples are fixed in formalin and embedded in paraffin. Sections are stained with the periodic acid/Schiff technique. Focal glomerular sclerosis is scored semiquantitatively by light microscopy.

EVALUATION

Quantitative continuous data may be evaluated by standard statistical methods. It is inappropriate to use parametric statistical methods on semiquantitative data (i.e., renal injury light miscroscopic assessment scores), although appropriate non-parametric methods (e.g., Duncan's rank-sum procedure) may be used.

MODIFICATIONS OF THE METHOD

Animal models with spontaneous disease exist and can be used in place of the toxicant-induced models. In addition, an investigator may take advantage of either genetically-modified animals or exploit strain differences in sensitivity to xenobiotics to examine mechanisms of renal injury.

Fawn-Hooded rats develop systemic hypertension and spontaneous age-dependent glomerulosclerosis with proteinuria (Mackenzie et al. 1997). Spontaneous glomerular injury with proteinuria occurs in Dahl salt-sensitive rats fed on a normal sodium diet (Yoneda et al. 1998). Mizuno et al. (1999) studied the ICGN mouse strain as a unique model for naturally occurring nephrotic syndrome. Focal segmental glomerulosclerosis with heavy proteinuria has been found in mice in which the Mpv17gene was inactivated (Mpv17-/-mice). Binder et al. (1999) recommended these animals as model of steroid-resistant glomerulonephritis sensitive to radical scavenger therapy.

Kimura et al. (1993) described strain specificity in the susceptibility of mice to daunomycin-induced glomerular injury. Klahr and Morrissey (1997) described the effects of ACE inhibitors and angiotensin II receptor antagonists on various parameters associated with renal interstitial fibrosis induced by unilateral ureteral obstruction in rats.

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I.E.4
Assessment of the Lower Urinary Tract

I.E.4.1
In vivo Studies

I.E.4.1.1
Micturition Studies

PURPOSE AND RATIONALE

Urinary incontinence is a major psychosocial, medical, and economic problem. The most common condition to be treated pharmacologically is incontinence due to detrusor instability. The response of the urinary bladder to filling with increasing volumes of fluid (cystometrogram) is a common procedure for evaluating bladder function in both animals and humans. The response of the vesico-urethral complex can be arbitrarily divided into the collection and expulsion phases. The nervous control of the detrusor, the internal and the external sphincter has been reviewed by Kuro (1965). A detailed description of the nervous control of the urinary bladder of the cat has been given by De Groat (1975). The pharmacology of lower urinary tract muscles and penile erectile tissues has been reviewed by Anderson (1993). Ferguson and Christopher (1996) reviewed urine bladder function and drug development. Urine storage and timely expulsion of bladder content are produced through the coordinated activation of a series of reflexes involving cholinergic, sympathetic, and, possibly, purinergic, serotonergic, and peptidergic innervation. In view of this complexity, in vivo models were developed for the quantitative analysis of the effects of drugs on the function of the vesico-urethral complex (Maggi et al. 1983, 1985, 1986, 1987a,b, 1992).

PROCEDURE

Male Wistar rats weighing 340–360 g are anesthetized. The left jugular vein is cannulated for drug injection. Body temperature is kept constant by means of a heating pad maintained at 37 °C. Through a midline incision of the abdomen, the urinary bladder is exposed and emptied of urine by application of a slight manual pressure. A 20-gauge needle is inserted through the apex of the bladder dome for 3–4 mm into its lumen. The needle is connected to a pressure transducer by means of a polyethylene tubing (1.5 mmOD and 1.0 mmID) and the whole system filled with saline. The tubing is provided with an internal coaxial polyethylene tubing (0.6 mmOD and 0.3 mmID) inserted through a side hole and sealed by a drop of epoxy resin. The second tubing serves for intravesical infusion of fluid and is connected, through a peristaltic pump, to a saline reservoir.

Intraluminal pressure signals are delivered to an amplifier and displayed on a four channel polygraph. Warm saline-soaked cotton wool swabs are laid around the exteriorized organ to maintain its temperature and to keep it moist in experiments involving the topical application of substances on the bladder dome.

After a 15-min equilibration period at zero volume, variations in intraluminal pressure are recorded in response to continuous infusion of saline at a rate of 2.8 ml/h at 37 °C for 30–40 min by means of a peristaltic pump connected to the polyethylene tubing inserted into the bladder. This infusion rate simulates the maximal hourly diuresis within the physiological range. In each preparation the infusion is continued until micturition occurs. Micturition is referred as the emission of several drops of fluid during a sustained phasic contraction of the detrusor muscle which is followed by return to zero or, in any case, to a value lower than that recorded just before micturition.

For both intravenous and topical administration, substances are dissolved in saline.

EVALUATION

In each experiment, the following parameters are evaluated:

- pressure threshold = intraluminal pressure value recorded just before micturition
- volume threshold = the volume of infused saline required to obtain micturition
- maximal amplitude of micturition contraction
- residual volume after micturition.

The effect of substances on the compliance of the bladder wall is evaluated by comparing the volume-pressure-relationship of treated animals with that of controls.

Statistical analysis of the data is performed by means of the Student's t-test for paired or unpaired data, or by means of analysis of variance followed by the Tukey test. Statistical analysis of nonparametric data is made by the chi square test.

MODIFICATIONS OF THE METHOD

Either chemical (6-hydroxydopamine, reserpine) or surgical (section of hypogastric nerves) sympathectomy produces a picture of detrusor hyperreflexia and urine dropping, mimicking cystometric finding in human disease (Maggi et al. 1987a).

Postius and Szelenyi (1983) described a model for in vivo screening of spasmolytic compounds using the rat bladder.

Dray (1985) used the spontaneous, volume-induced contractions of the urinary bladder in the anesthetized rat to assess the central activity of substances with opioid properties.

Pietra et al. (1990) studied the effects of some antidepressants on the volume-induced reflex contractions of the rat urinary bladder. The urinary bladder of anesthetized rats was filled via the recording catheter by incremental volumes of warmed saline until bladder contractions occurred as a result of central activity. Volume-induced contractions were then recorded and occurred rhythmically and reproducibly for 2–3 h. Drug activity was assessed in each animal against the background frequency of bladder contractions, for a 15-min time period following intravenous administration of different doses.

Harada et al. (1992) proposed a method for rapid evaluation of the efficacy of pharmacologic agents and their analogs in enhancing bladder capacity and reducing the voiding frequency. Conscious rats were placed in a restrainer over a urine collector. The collector was secured to an Statham UC3 strain transducer, the output of which was amplified by a Gould bridge amplifier. Data were monitored on a polygraph.

Conte et al. (1991) proposed a method for simultaneous recording of vesical and the external urethral sphincter pressure in urethane-anesthetized rats.

Angelico et al. (1992) reported in vivo effects of different antispasmodic drugs on the rat bladder contractions induced by topically applied KCl.

Oyasu et al. (1994) measured spontaneous bladder contractions caused by raising the intravesical volume in anesthetized rats.

Yaksh et al. (1986) described a chronic model for study of micturition in unanesthetized rats. A bladder catheter was implanted chronically through laparatomy and externalized percutaneously.

Horváth et al. (1994) reported an ultrasonic method to study the influence of drugs on micturition in intact rats.

Tillig and Constantinou (1996) described videomicroscopic imaging of urethral peristaltic function in anesthetized rats. Cystometrograms were performed by recording continuously the bladder pressures while detecting micturition using a sensor placed at the orifice of the urethra. Renal pelvic pressure was measured during continuous perfusion using a nephrostomy inserted through the parenchyma. A catheter was placed in the femoral vein for intravenous drug administration. The left pyelo-ureteric junction and the upper part of the ureter were visualized using a stereomicroscope equipped with a video camera and a tape recorder. One syringe pump was used for filling the bladder to perform continuous cystometrograms. Another syringe pump was used for infusion of indigo carmine to assist the visualization of the bladder pressure.

Conte et al. (1988) developed a cystometric technique for quantitative studies on physiopharmacology of micturition in conscious, freely moving rats.

Peterson et al. (1989), Noronha-Blob et al. (1991) described in vivo cystometrogram studies in urethane-anesthetized and conscious **guinea pigs**.

Moreau et al. (1983) described simultaneous cystometry and uroflowmetry for evaluation of the caudal part of the urinary tract in **dogs**.

Imagawa et al. (1989) reported an in vivo procedure for functional evaluation of sympathetically mediated responses in lower urinary tract of dogs.

Häbler et al. (1990, 1992) examined the functional properties of unmyelinated and myelinated primary afferent neurons innervating the pelvic viscera in anesthetized **cats**. The axons were isolated from the intact dorsal root and the intact or chronically de-efferented ventral root of segment S2. The responses of the neurons were studied with natural stimulation of the urinary bladder using innocuous or noxious increases of intravesical pressure.

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I.E.4.2 Studies in Isolated Organs

I.E.4.2.1 Studies on Renal Pelvis

PURPOSE AND RATIONALE

The isolated renal pelvis of the guinea pig has been used as pharmacological tool mainly by the group of Maggi (Maggi and Giuliani 1991, 1992; Maggi et al. 1992a,b,c, 1994, 1995; Giuliani and Maggi 1996; Santicioli et al. 1995, 1997; Patacchini et al. 1998; Bigoni et al. 1999).

PROCEDURE

Male albino guinea pigs weighing 250–300 g are euthanatized by exsanguination under anesthesia. The whole kidney and attached ureter are removed and placed in oxygenated Krebs solution. The renal pelvis is carefully dissected from the renal parenchyma, separated from the ureter, cut and connected to threads to record motility along the circular axis. The preparation is suspended in a 5 ml organ bath and mechanical activity recorded by means of an isotonic transducer (load 1 mN). Transmural electrical field stimulation is made by means of platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a GRASS S88 stimulator. Square wave pulses (pulse width 0.5 ms, 60 V) are delivered in trains of 10 s duration at frequencies of 5 to 10 Hz.

Experiments commence after a 60 to 90 min equilibrium period after which amplitude and fre-

quency of spontaneous activity has reached a steady state. In vitro capsaicin desensitization is made by exposure of the preparation to 10 mM for 15 min, followed by washing out and further equilibration for 30–60 min.

Concentration response curves to noradrenaline and acetylcholine are performed by non-cumulative addition to the bath at 20 min intervals. Contact time of drugs is 15 min.

EVALUATION

All values are expressed as mean ± SEM. Statistical analysis is performed by means of Student's t-test for paired or unpaired data or by analysis of variance.

MODIFICATIONS OF THE METHOD

Zhang and Lang (1994), Lang et al. (1995), Lang and Zhang (1996) Teele and Lang (1998) recommended circumferentially cut strips from the proximal renal pelvis of guinea pigs since these strips contract more frequently than strips cut from the mid region.

Kimoto and Constantinou (1990, 1991) studied contractility of smooth muscle strips from the pacemaker regions and pelviureteric junction of renal pelvis from **rabbits**.

Kondo et al. (1992) determined the effects of dobutamine and terbutaline on adenylate cyclase activity and cyclic AMP content in the renal pelvis of rabbits.

Seki and Suzuki (1990) made intracellular recordings to study the electrical properties of smooth muscle cells in the rabbit renal pelvis.

Zwergel et al. (1991) developed an intact **canine** model to measure renal pelvic pressure after complete ureteral obstruction with a balloon catheter inflated in the distal ureter.

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I.E.4.2.2 Studies on the Urinary Bladder and Internal Urethral Sphincter

PURPOSE AND RATIONALE

Several authors investigated the influence of drugs on isolated parts of the lower urinary tract. Ueda et al. (1984) studied the effects on smooth muscle of the rabbit bladder dome, trigone and proximal urethra.

PROCEDURE

Male New Zealand rabbits weighing 2–3 kg are euthantized under anesthesia and the abdomen is opened to remove the bladder and the urethra. After excess fat and connective tissue is removed, the bladder and the urethra are dissected into dome, trigone and proximal urethral preparations. All strips are cut transversely being approximately 2–6 mm unstretched.

Ligatures are placed on both ends of the strips and one end is attached to a tissue holder and the other to a strain gauge force-displacement transducer connected to a polygraph on which isometric tension changes are recorded. Each of the strips is then placed into a 20 ml tissue bath containing Krebs-Ringer solution bubbled with 95 % O_2 + 5 % CO_2 at 37 °C. Resting tension is adjusted to 1g during an equilibrium period of at least 2 h. The contractile and relaxant responses are measured as increases or decreases from the resting tension. Doses-response curves are performed in a cumulative matter. Tissues are pretreated with an antagonist 20 min before the addition of an agonist.

EVALUATION

The values are expressed or plotted as the means ± SE and pA_2 values are calculated according to Arunlakshana and Schild (1959). Data are analyzed using the t-test, analysis of variance, Dunnett's test and regression analysis.

MODIFICATIONS OF THE METHOD

Pietra et al. (1990) studied the effects of some antidepressants by the in vitro inhibition of carbachol-induced contractions of rat detrusor strip preparations. The detrusor muscle tissue (bladder dome) was cut in a semicircular direction and further dissected into strip preparations measuring approximately 2 × 20 mm.

The preparation of the isolated, innervated urinary bladder of the rat was reported by Hukovic et al. (1965). Electrical stimulation was performed by a bipolar electrode from the nerves running close to the ureter.

Mapp et al. (1990) used the isolated rat urinary bladder to study the pharmacological modulation of the contractile response to toluene diisocyanate.

Maggi et al. (1985) used isolated detrusor strips of rat bladder connected to an isometric strain gauge and stimulated by field stimulation.

Anderson (1978) recommended the **rabbit** detrusor muscle as an unique in vitro smooth muscle preparation. Rabbit detrusor muscles are thin and devoid of underlying submucosal tissue with parallel fiber orientation. The tissue exhibits autorhythmicity, characteristic of most single unit type smooth muscle preparations and can be employed in either isometric or isotonic organ bath recording systems.

Honda and Nakagawa (1986) studied the effects of the optical isomers of an alpha-1 adrenoceptor antagonist in rabbit lower urinary tract and prostate.

Khanna et al. (1977, 1981) evaluated the in vitro responses of three segments of rabbit lower urinary tract e.g., the bladder body, the bladder base and the proximal urethra.

Ferguson and Marchant (1995) studied the inhibitory actions of GABA on rabbit urinary bladder muscle strips.

Andersson et al. (1983) studied the electrically induced relaxation of the noradrenaline contracted isolated urethra from rabbit and man. In rabbits, two circular transverse sections, each 4 mm long, were taken from the middle and upper parts of the urethra. Human urethral preparations were obtained from male patients undergoing cysto-urethrectomy en bloc because of bladder cancer. Rings of tissue were taken from the membranous and supra- and infra-collicular parts of the prostatic urethra.

Andersson et al. (1992) used transversal strips from the middle and upper part of rabbit urethra to study the involvement of nitric oxide in the electrically-induced, nerve-mediated relaxation.

Weetman (1972) described the preparation of the isolated, innervated urinary bladder in **guinea pigs**. Contractions of the tissue induced by nerve stimulation could be blocked by local anesthetics and by tetrodotoxin.

Isolated innervated, rat and guinea pig hemi-urinary bladder preparations were described by Dhattiwala and Dave (1975).

Burnstock et al. (1978) used recorded isometric tension of mucosal-free strips of the detrusor of the bladder from guinea pigs in vitro after electrical field stimulation.

Von Heyden et al. (1997) tested urethral relaxation after electrostimulation in guinea pigs. Male Hartley

guinea pigs weighing 350–450 g were euthanatized and bladder, urethra and penis were dissected out. From each animal 4–6 urethral rings 1–2 mm thick were cut. The urethral rings were mounted according to their anatomical order: in channel1 the most proximal ring (near the bladder neck) and in channel 6 the most distal ring (near the penile crura). The urethral rings were stretched by two spring-wire clips (Harvard Apparatus, South Natick, MA) whose tips closed in the urethral lumen. The manner in which the urethral rings were cut and mounted ensured that only the circularly oriented, mostly striated fibers contributed to the tension measured. Detrusor muscle was cut as a horizontal ring proximal to the trigone and mounted in the same way. The clips were connected with 4-Osilk to a glass tissue support hook on one side and to an isometric force transducer (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) on the other. A double-chambered bath (Föhr Medical Instruments GmbH, D-64342 Seeheim, Germany) was used in which the working chamber was connected to a second chamber, in which the gas (95 % O_2 and 5 % CO_2) was fed. The gas flow induced fluid circulation. Forces lower than 0.1 g could be measured without bubble artifacts in the working chamber. The transducer signals were fed into a thermal array recorder (Dash 10, Astro-Med, 63110 Rodgau, Germany). For tissue stimulation, vertical, L-shaped custom-made platinum electrodes (20 mm long, 0.3 mm diameter) 10 mm apart were used with a custom-made stimulator. The tissue was mounted parallel with the electrodes. Bipolar, monophasic balance-charged rectangular pulses of 0.8-ms duration and 75 mA current were used.

Kunisawa et al. (1985) performed a pharmacological study of alpha adrenergic receptor subtypes in smooth muscle of **human** urinary bladder base and prostatic urethra.

Thornbury et al. (1992) reported on the mediation of nitric oxide of neurogenic relaxation of the urinary bladder neck muscle in **sheep**. Urinary bladders of sheep of either sex were obtained approximately 15 min after slaughter. Circularly oriented rings were cut from the region of the bladder just above the trigone. These were opened and the mucosa removed by sharp dissection to give strips with approximate dimensions of 10 × 4 × 4 mm. The strips were mounted in organ baths and perfused with Krebs solution. Tension was measured with isometric transducers after field stimulation via platinum ring electrodes.

Hills et al. (1984) used isolated strips of the bladder from female **pigs**. Bladder neck strips were cut longitudinally and horizontally from the region of the bladder just below the trigone. The preparations were stripped of mucosa and trimmed to give a muscle strip of about 2 × 15 mm.

Klarskov (1987) studied the non-cholinergic, non-adrenergic inhibitory nerve responses of bladder outlet smooth muscle from female Danish Landrace pigs in vitro. Trigone strips were taken in an oblique direction from the internal urethral orifice and medially to one of the ureteric orifices, bladder neck strips transversal from the posterior half of the borderline between bladder and urethra, and urethral strips longitudinal from the proximal posterior part.

Teramoto et al. (1997) examined the membrane potential in the proximal urethra of pigs by use of the microelectrode technique.

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I.E.4.2.3
Effects on the External Urethral Sphincter

PURPOSE AND RATIONALE

In 5 contrast to the smooth musculature of the internal urethral sphincter, the external urethral sphincter is a striate muscle and a part of the urogenital diaphragm (Kuro 1965). Judged from electromyographic studies it is generally accepted that the preponderance of continence depends on the external urethral sphincter. The external urethral sphincter shows a steady tonic discharge under resting conditions. As the bladder fills, there is initially an increase in this activity. When the rise in tension in the bladder wall leads to reflex contraction, the activity in the external urethra sphincter ceases and in remains quiescent during voiding. Parlani et al. (1992) used the external urethral sphincter of the rat as an in vitro model to evaluate the activity of drugs on the smooth and striated components of the urinary bladder outlet.

PROCEDURE

Male Wistar rats weighing 360–400 g are euthanatized under anesthesia by exsanguination. Through a midline incision of the lower abdomen, the external urethral sphincter (Watanabe and Yamamoto 1979) is isolated from the perineal muscles and surrounding connective tissue and removed in toto. The preparation is placed in oxygenated Krebs solution, and a ring is taken from its middle region. In this area the urethra is encircled by bundles of striated muscle fibers partly interlaced with urethral smooth muscle. The rings are cut to obtain strips that are suspended in a 5-ml organ bath containing Krebs solution at 37 °C. A mixture of 96 % O_2 and 4 % CO_2 is bubbled into the organ bath.

The preparations are connected by means of a silk thread to an isometric strain gauge under a constant load of 1 g. The contractile activity is recorded on a polygraph. Field stimulation is carried out by means of two platinum wire electrodes placed at the top and the bottom of the organ bath and connected to a Grass S11 stimulator. The preparations are allowed to equilibrate for at least 60 min. Square wave pulses are delivered at an intensity between 10 and 60 V, a frequency between 0.1 and 3 Hz, and a duration between 0.1 and 1 s; trains are of 5 s every 5 min. After three consecutive reproducible responses are obtained, drugs are added to the organ bath. The effect of drugs is expressed as the percent of inhibition of contractile response before exposure to drugs and is evaluated as soon as the maximum effect is reached.

EVALUATION

Means ± standard error of the mean are calculated. Statistical evaluation is performed by using Student's t-test for paired or unpaired data.

MODIFICATIONS OF THE METHOD

In some studies, denervation of the external urethral sphincter was performed (Somma et al. 1989; Parlani et al. 1992). Rats were anesthetized with 30 mg/kg penthotal i.p., then the major pelvic ganglia, known to provide both sympathetic and parasympathetic innervation to the urinary bladder and the external urethral sphincter (Hulsebosh and Goggeshall 1982; Purinton et al. 1973; Watanabe and Yamamoto 1979) are isolated and bilaterally removed through a small incision of the lower abdomen.

In the same preparations, somatic denervation of the external urethral sphincter was obtained by cutting the pudendal nerves. The paravertebral muscles were care-

fully dissected through an incision of the skin to exteriorize the sacral plexus. The pudendal nerves were isolated, and 2–3 mm of the nerve were removed. An absorbable sponge soaked with amikain solution was left in place to prevent bleeding and infection. The muscles and the skin were sutured with cat gut. The rats were allowed to recover for 10 to 15 days in individual cages with free access to water and food.

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I.E.4.2.4
Propagation of Impulses in the Guinea Pig Ureter

PURPOSE AND RATIONALE

The mammalian ureter provides one of the most clear examples of electromechanical coupling in smooth muscle. The ureter smooth muscles are electrically and mechanically quiescent but, when depolarized to threshold, they fire an action potential characterized by an unusually long-lasting potential and generate a phasic contraction (Shuba 1977; Brading et al. 1983; Meini et al. 1995). Both the action potential and the accompanying contraction critically depend upon the influx of extracellular calcium through voltage-sensitive L-type channels, which are enhanced and blocked by dihydropyridine drugs, Bay K8644 and nifedipine, respectively. The model predicts that suppression of action potentials at any site of the ureter will suppress the propagation of contraction and peristalsis. (Weiss 1992).

PROCEDURE

Male albino guinea pigs weighing 25–300 g are anesthetized and euthantized by exsanguination. The whole kidney and ureter are excised and placed in a Petri dish containing oxygenated Krebs solution for dissection. A 4–5 cm long piece of ureter is dissected from the inferior renal pole and placed in a three-compartment organ bath which enables a separate superfusion of different parts of the organs. Two Perspex partitions are used to separate the renal-, middle- and bladder-sites. They include a window covered with condom rubber: a small hole (about 300 mM) is made in the rubber to enable the passage of the ureter. Proximal to each partition, the renal and bladder ends are pinned to a Sylgard support. The distal portions of the renal and bladder ends are connected via a pulley to isotonic transducers (Basile 7006, load 2 mN) for recording of mechanical activity on a two-channel polygraph.

Each compartment is perfused by means of a peristaltic pump at a rate of 1 ml/min with oxygenated Krebs solution at 34 °C.

Electrical field stimulation is applied to either compartment by means of two wire platinum electrodes positioned in parallel with the two sides of the ureter. Square wave pulses (5–25 ms pulse width, 20 V) are automatically delivered every 100 s by means of a GRASS S88 stimulator.

Drugs are applied by superfusion at the middle site. Amplitudes of contraction are recorded.

EVALUATION

Values are calculated as mean ± SEM. Statistical analysis is performed by means of Student'st-test or by means of analysis of variance.

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Chapter I.F
Respiratory Function Assays in Safety Pharmacology

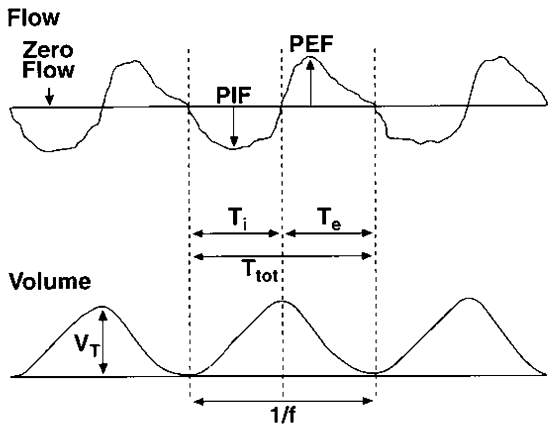
Dennis J. Murphy

I.F.1
Respiratory Function Assays – General Approach

The objectives of a safety pharmacology study of the respiratory system are to determine whether a drug has the potential to produce a change in respiratory function and to establish whether this change is a liability. Such changes can result from either the primary or secondary pharmacological properties of a drug or from organ dysfunction resulting from the toxicological properties of a drug.

The respiratory system consists of two functional units, the pumping apparatus and the gas exchange unit. As such, a complete assessment of respiratory function in safety pharmacology must include an evaluation of both of these components. The pumping apparatus includes those components of the nervous and muscular systems that are responsible for generating and regulating breathing patterns, whereas the gas exchange unit consists of the lung with its associated airways, alveoli, and interstitial area that contains blood and lymph vessels and an elastic fibrous network.

The function of the pumping apparatus is ensure the appropriate movement of gases between the environment and the central airways and is evaluated by measuring ventilatory patterns. Ventilatory parameters must include measures of respiratory rate, tidal volume and minute volume since normal ventilation requires that the pumping apparatus provide both adequate total pulmonary ventilation (minute volume) and the appropriate depth (tidal volume) and frequency (rate) of breathing. If a change in these parameters occurs, inspiratory flow (mean or peak), expiratory flow (mean or peak), fractional inspiratory time (inspiratory time/total breath time) and time between breaths (expiratory pause or apnea) should be evaluated to help define the mechanism (see Figure 1).



Functional Endpoints

V_E	-	Minute Volume (V_T x f)
f	-	Breathing Rate
V_T	-	Tidal Volume
PIF	-	Peak Inspiratory Flow
PEF	-	Peak Expiratory Flow
FIT	-	Fractional Inspiratory Time $\frac{(T_i)}{(T_{tot})}$

Fig. 1. Tracings of lung airflow and lung volume changes during spontaneous breathing in a conscious rat. Airflow was measured directly using a "head-out" plethysmograph chamber. The functional endpoints can be automatically calculated for each breath using a data acquisition and analysis software system.

The function of the gas exchange unit is to ensure that gas which enters the airways from the environment reaches the alveoli during inspiration and is removed from the alveoli during expiration. This is accomplished by maintaining patent (open) airways and elastic recoil in the parenchyma of the lung. The function of the gas exchange unit is evaluated by measuring the mechanical properties of the lung. This is most effectively accomplished in conscious animals by obtaining dynamic measurements of airway resistance or conductance (to assess airway patency) and lung compliance (to assess elastic recoil). Airway resistance (measured as total pulmonary resistance) defines the change in pleural, airway or transpulmonary pressure (ΔP) required to produce a defined change in lung airflow (ΔF) and is calculated as $\Delta P/\Delta F$, while conductance is calculated as $\Delta F/\Delta P$. To calculate dynamic resistance or conductance, the ΔP and ΔF are measured for each breath at the same lung volume during inspiration and expiration (usually between 50 and 70 % of tidal volume) (see Figure 2). By selecting isovolumetric points, the dependence of ΔP on elastic component of the lung is removed, leaving ΔP dependent only on resistance to the flow of gas in the lung and airways. Dynamic compliance is calculated by measuring the differences in airway or transpulmonary pressure (ΔP) and volume (ΔV) that occur at the beginning and end of each inspiration (i.e., at zero flow points) (see Figure 2). By selecting zero flow points, the dependence of ΔP on tissue and airflow movement is removed, leaving ΔP dependent only on the elastic component of the lung.

Supplemental studies in safety pharmacology are designed to investigate mechanisms of action or to help further characterize the liability of a drug effect. To help understand the mechanisms and liabilities associated with respiratory function changes, it is important to determine whether a drug-induced change in ventilation is acting through a mechanism involving the central or peripheral nervous system, and whether a drug-induced change in respiratory function has an effect on arterial blood gases.

Because the safety profiles defined by safety pharmacology studies can have a significant impact on the successful development of new therapeutic agents, it is important that the techniques and assays used in safety pharmacology studies minimize the occurrence of false negative and false positive results. For this reason, techniques that provide direct measures of respiratory parameters should be used. A direct measure is one that provides the endpoint of interest, in contrast to an indirect measure that provides a surrogate endpoint,

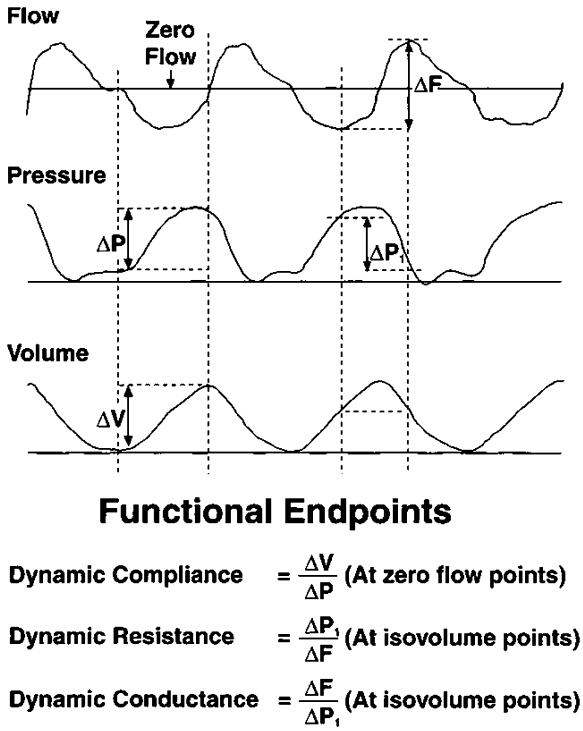


Fig. 2. Tracings of lung airflow, transpulmonary pressure, and lung volume changes during spontaneous breathing in a rat. Airflow was measured directly using a "head-out" plethysmograph chamber, while pleural pressure was measured using a pressure sensitive catheter placed into the esophagus within the thoracic cavity. The functional endpoints can be automatically calculated for each breath using a data acquisition and analysis software system.

which requires certain assumptions and/or calculations to estimate the true endpoint. Furthermore, because most drugs are intended for use in conscious patients, and most anesthetics, analgesics and sedatives can alter ventilatory reflexes, respiratory drive and airway reactivity, safety pharmacology studies evaluating the effects of drugs on respiratory function should utilize conscious animal models. Based on the above, the respiratory function assays covered in this review will focus on new technologies that provide direct measures of respiratory parameters in conscious animals and are considered to be most appropriate for use in safety pharmacology studies. Alternate techniques, which provide either indirect measures of respiratory function (e.g., barometric or whole body plethysmography) or use anesthetized models, have been described elsewhere (see references) and will not be covered in this review.

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I.F.2
Respiratory Function in Conscious Rats

PURPOSE AND RATIONALE

The rat is considered an appropriate species for general use in safety pharmacology studies of the respiratory system. The physiology of the respiratory system has been well characterized in this species and much of the information on drug-induced effects on ventilatory control and mechanisms of airway disease have been obtained in the rat. The rat is also readily available from animal vendors, is easy to handle and train, has a relatively stable breathing pattern, and has the appropriate temperament for conscious respiratory measurements. Furthermore, the techniques for measuring respiratory functions in rats are well established, and the rat is commonly used in toxicology studies. Selecting a species that is used in toxicology studies provides additional supportive information including (1) pharmacokinetic data that can be used to define the test measurement intervals, (2) acute toxicity data that can be used to select the appropriate high dose and (3) toxicology/pathology findings that can be used to help define the mechanism of the functional changes measured in safety pharmacology studies. Although this technique has been developed in the rat, it is also applicable to other small animals.

PROCEDURE

To provide a direct measure of ventilatory parameters, a head-out plethysmograph chamber is used, while measurement of pleural pressure is used to provide a direct measure of airway resistance and compliance.

Pleural Pressure Measurements

Catheter Placement

Pleural pressure is measured chronically in conscious rats by surgically implanting a fluid-filled polyurethane catheter (length = 10 cm; O.D. = 0.7 mm) attached to a pressure-sensitive radiotelemetry transmitter (Model TA11PA-C40, Data Sciences International, St. Paul, MN) beneath the serosal layer of the esophagus and within the thoracic cavity (see Figure 3).

Surgical Procedure

Surgery is initiated by anesthetizing the rat with isoflurane (2–3 %) delivered by inhalation in 100 % oxygen. The surgical area is prepared by shaving the abdomen with surgical clippers and scrubbing with a Betadine and 70 % ethanol wash. Once the area has been prepared, an abdominal incision (approximately 4 to 5 cm in length) is made along the linea alba. The lobes of the liver (within the abdominal cavity) are refracted to expose the esophagus and the lobes gently packed against the abdominal wall using moist 2 × 2 inch Versalon (or equivalent) squares. The esophagus is isolated approximately 2 cm below the Hiatus oesphagicus (junction with the diaphragm), a 22-gauge needle (1 inch in length with an approximate 90-degree bend) is inserted into the esophagus between the serosa and muscularis layers and the needle tunneled into the pleural space (see Figure 3). It is important to keep the esophagus completely straight while the needle is being inserted since the needle can re-emerge through the serosa layer and enter into the pleural space. This will generally lead to encapsulation of the catheter tip and result in a loss or dampening of the pressure signal. Care must be taken not to advance the needle too far up the esophagus (generally no more than 1.5 cm) since the needle may come into close contact with the heart, causing cardiac pressure. Once the needle is advanced to a point approximately 1 cm beyond the diaphragm junction, the needle is removed and the catheter from the telemetry transmitter unit advanced up the channel. During this step, care must

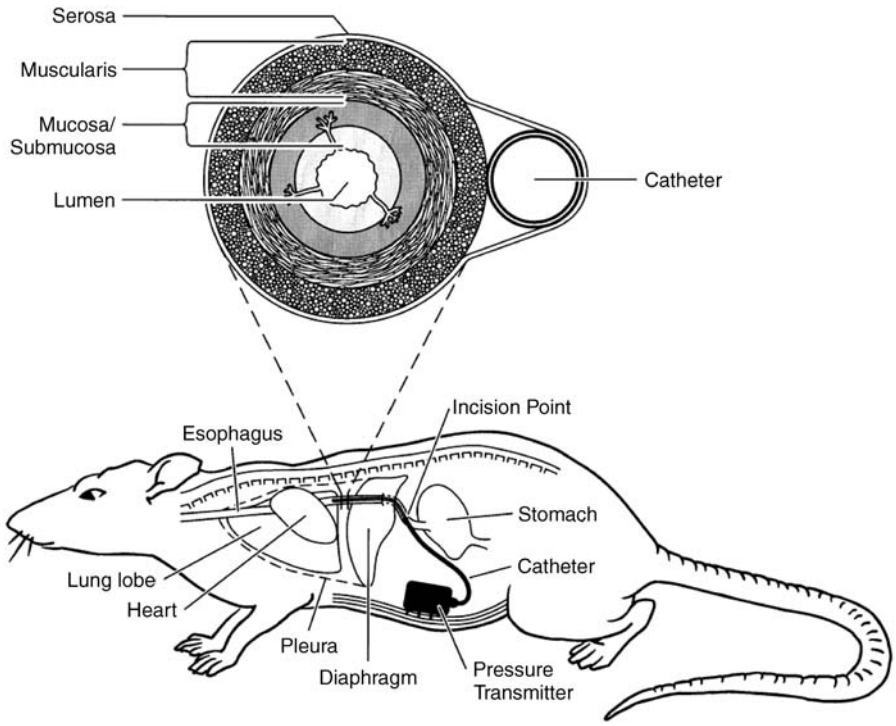

Fig. 3. Drawing of a rat showing placement of the pressure sensitive subpleural catheter and radiotelemetry transmitter for chronic measurement of pleural pressure in conscious animals. The enlargement is a cross-section through the esophagus showing the position of the catheter between the serosal and muscularis layers.

be taken not to apply pressure to the catheter for risk of damaging the fluid-filled catheter and telemetry unit. A pair of vessel cannulation forceps (5 1/8" Roboz Surgical Instruments, Rockville, MD) can be used to successfully advance the catheter without damaging the unit. Pleural pressure should be monitored during these procedures to ensure an optimal signal is obtained. Pleural pressures during isoflurane induced anesthesia should be between approximately 8 and 20 cm H_2O. The signal may be altered by slowly moving the catheter up and down the channel in the esophagus. Once a maximal and acceptable pleural pressure signal is obtained, the catheter is secured in place using medical grade tissue adhesive (Vetbond™ or equivalent) and a small cellulose patch. The body of the transmitter unit is secured to the abdominal wall with non-absorbable suture during the closure of the abdominal musculature. The skin layer is closed with absorbable suture and/or surgical wound clips.

Surgical Recovery and Training

If wound clips are used, they are removed approximately 8 to 10 days post surgery. Rats are placed in clean polycarbonate boxes with soft bedding for approximately 7 to 10 days, observed daily for any signs of distress, and weighed at least twice weekly. A rat may initially lose 10 % of its body weight after surgery, but should begin gaining weight by day 5 post surgery. Prior to the initiation of a study, all rats must be trained to the plethysmograph chamber by placement in the chamber on 3–5 occasions. The first training session should be conducted prior to surgery to eliminate any rat that does not accept the chamber. The duration of time in the chamber should be at least equal to the time selected in the study. Rats can tolerate these types of chambers for up to a maximum of approximately 6 hours.

Lung Airflow and Lung Mechanics Measurements

Changes in lung airflow are measured in conscious, restrained rats using a head-out, volume displacement plethysmograph chamber (approximately 1–3 liter capacity) (Figure 4). In this type of chamber, the head is exposed to ambient conditions, while the trunk is enclosed in the chamber. A seal is made around the neck using a neoprene collar (1/8 inch thickness). Pressure changes within the chamber are measured using a differential pressure transducer with a sensitivity of approximately +/– 2 cm H_2O (Model MP-45-14,

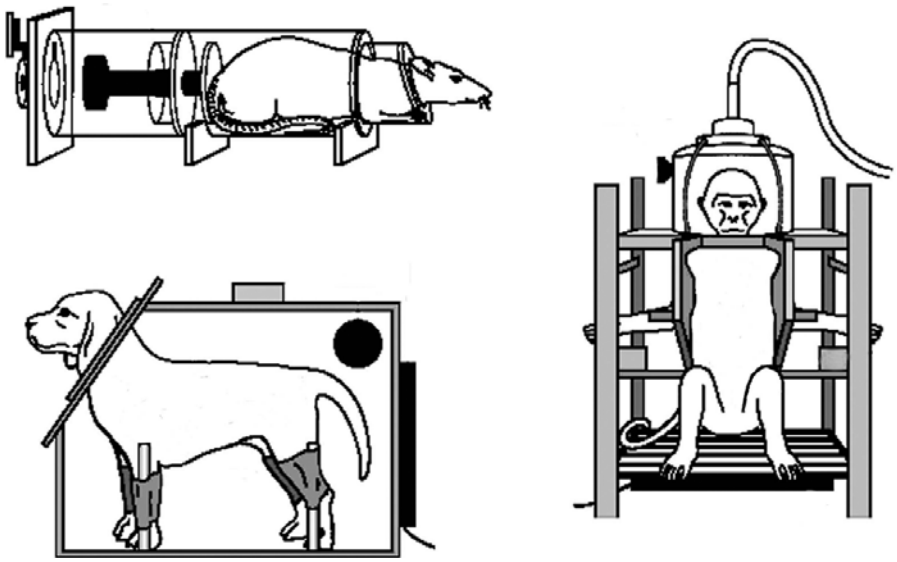

Fig. 4. Plethysmograph chambers for the direct measurement of ventilatory parameters. The rat and dog are in "head-out" chambers, while the monkey is in a "head-enclosed" chamber. For all chambers, an air tight seal is made around the neck using a 1/8 inch thick neoprene collar.

Validyne Engineering, Northbridge, CA) and the pressure changes converted to flow rates using a pneumotach port (1 inch diameter opening with six layers of 325 mesh stainless steel wire cloth). The analog flow signal is conditioned using a pre-amplifier and then converted to a telemetry (frequency) signal using a voltage analog to frequency converter (Model C12V, Data Sciences International, Inc. (DSI), St. Paul, MN). A telemetry receiver (Model RLA1020 or RPC-1, Data Sciences International, Inc., St. Paul, MN) is placed beneath the plethysmograph chamber to transmit the pleural pressure telemetry signal in parallel with the chamber flow signal to a software application (e.g., DSI Dataquest ART analog system) that converts the telemetry signals into calibrated analog voltage signals and places the signals in phase by correcting for any differences in signal transmission times. The analog signals are then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory and lung function parameters (see Figs. 1 and 2).

I.F.3
Respiratory Function in Monkeys and Dogs

PURPOSE AND RATIONALE

Species other than the rat may be required to address specific study requirements. For example, the development of humanized monoclonal antibodies or other biotechnology-derived proteins requires species that have homologous target proteins and do not produce an antigenic response to the drug. In such cases, the nonhuman primate is generally required. Since both dogs and monkeys are used in toxicology studies of new drugs, either of these species may also be required to investigate pathological changes that are suspected of having an effect on the function of the respiratory pumping apparatus or gas exchange unit. Since telemetry transmitters with sufficient power to transmit two pressure signals have been developed for larger animals, the use of monkeys and dogs also has the advantage of allowing the measurement of both respiratory and cardiovascular functions. The procedure below is described for the cynomolgus monkey (Macaca fascicularis); however, this procedure is also applicable to the dog or other large animals.

Pleural and Arterial Pressure Measurements

Catheter Placements

Pleural pressure is measured chronically in conscious monkeys by surgically implanting a fluid-filled polyurethane catheter (length = 35 cm; O.D. = 1.2 mm) attached to a pressure sensitive radiotelemetry transmitter (Model TL11M3-D70-PCP, Data Sciences International, Inc., St Paul, MN) beneath the serosal layer of the esophagus and within the thoracic cavity. Arterial pressures may also be measured by implanting a second fluid-filled polyurethane catheter (length = 35 cm; O.D. = 1.2 mm) attached to the same radiotelemetry transmitter into the abdominal aorta.

Surgical Procedure

Monkeys are premedicated with acetylcholine (0.025 mg/kg, i.m.) and flunixin meglumine (1 mg/kg, i.m.). Anesthesia is induced with ketamine (10 mg/kg, i.m.) and maintained with isoflurane (1–3 %) delivered by inhalation in 100 % oxygen. The monkey is then placed in semi-dorsal recumbency, scrubbed, and draped for aseptic surgery. A feeding tube is passed down the esophagus and into the stomach. A midline laparotomy is performed and the body of the telemetry unit is sutured to the abdominal wall. Two silastic retaining beads are placed 5 cm from the tip of one of the pressure catheters, and two retracting sutures are placed in the stomach just below the cardia. The esophagus is identified at the point where it passes through the diaphragm and an anchoring suture is pre-placed at this site. A small incision is made through the serosal layer of the esophagus and a modified groove director is inserted between the serosal and muscularis layers and advanced cranially through the diaphragm along the dorso-lateral aspect of the esophagus, using the feeding tube as a guide. The pressure catheter is advanced into the pleural cavity until the beads are located at the pre-placed suture and the groove director is withdrawn. The pleural pressure is visually verified and a negative deflection is confirmed with each respiratory effort. The catheter is either retracted or advanced until a maximal change in pressure (> 4 mmHg) was obtained. The catheter is secured using the pre-placed suture.

To monitor arterial blood pressure, a 4 cm incision is made over the right femoral region and the femoral artery is isolated. A trocar is used to pass the blood pressure catheter from the abdomen to the femoral incision. An arterotomy is made in the femoral artery and the catheter introduced 10 cm into the artery and the catheter secured using standard techniques.

Surgical Recovery and Training

After surgery, the monkeys are allowed to recover for at least three weeks prior to the start of the study. All monkeys are observed daily for signs of pain or distress and body weights are obtained at least weekly. All monkeys should be acclimated to handling, chair restraint, and the helmet used for respiratory measurements on at least 3 occasions prior to the start of each study. In addition, prior to implantation of the telemetry device, each monkey should be acclimated to the restraint chair and helmet for approximately 60 minutes on at least six occasions to ensure that the animals have the appropriate temperament.

Lung Airflow and Lung Mechanics Measurements

Changes in lung airflow are measured in conscious, restrained monkeys using a restraint chair equipped with a clear plastic helmet that seals around the neck and isolates the head from the rest of the body (see Figure 4). The helmet is adapted to serve as a volume displacement plethysmograph by attaching a pneumotachometer (Model 4500A, Hans Rudolph, Inc., Kansas City, MO). A bias flow of approximately 5 L/min of room air is used to ensure that all monkeys have an adequate air supply for breathing. The helmet is cylindrical and has a height of 13.5 cm, a diameter of 20 cm and an internal volume of approximately 4.2 liters. Room air is pulled into the helmet through the pneumotach attached to the upper side of the helmet and is exhausted through six evenly spaced openings around the base of the helmet to ensure uniform flow. A vacuum system is attached to the output line of a six port manifold at the top of the helmet using flexible plastic tubing (I.D. = 1.27 cm) with the openings at the base of the helmet connected to the manifold using flexible plastic tubing (I.D. = 0.635 cm).

Pressure changes within the helmet are measured using a differential pressure transducer with a sensitivity of approximately +/–2 cm H_2O (Model MP-45-14, Validyne Engineering, Northbridge, CA). The analog flow signal is conditioned using a pre-amplifier and then converted to a telemetry (frequency) signal using a voltage analog to frequency converter (Model C12V, Data Sciences International, Inc., St. Paul, MN). The restraint chairs are positioned in close proximity to individual telemetry receivers (Model No. RMC-1, Data Sciences International, Inc., St. Paul, MN) to ensure signal transmission. The pleural pressure signal is transmitted in parallel with the chamber flow signal to a software application (e.g., DSI Dataquest ART analog system) that converts the telemetry signals into calibrated analog voltage signals, and places the signals in phase by correcting for any differences in signal transmission times. The analog signals are then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory and lung function parameters (see Figs. 1 and 2).

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I.F.4
Distinguishing Central from Peripheral Nervous System Effects of Drugs

PURPOSE AND RATIONALE

Many drugs stimulate or depress ventilation by selective interaction with the central or peripheral nervous system. Thus, distinguishing a central from peripheral site of action is an important part of characterizing the mechanism of a drug-induced change in ventilation. Non-invasive methods for evaluating the central and peripheral effects of drugs in conscious animals have been developed and involve measuring effects of the test drug on agents known to selectively stimulate ventilation by activating either central or peripheral mechanisms. An assay developed by Murphy et al. (1995) provides a simple, non-invasive method for distinguishing the central from peripheral nervous system effects of respiratory depressant drugs in conscious rats. This technique, however, can also be applied to larger animals such as the dog or monkey. The procedure involves exposing rats for 5 minutes to an air mixture containing 8 % CO_2 (central chemoreceptor stimulant) followed by an intravenous bolus injection of 300 ug/kg sodium cyanide (peripheral chemoreceptor stimulant) and comparing the changes in minute volume and mean inspiratory flow (respiratory drive) before and after drug treatment. Morphine sulfate (3 mg/kg, intravenous), an opioid analgesic that depresses ventilation through a central mechanism, and carotid body denervation (peripheral depressant) were used to initially develop this procedure. The central respiratory depressants phenobarbital (200 mg/kg), xylazine (3 mg/kg), L-2-phenylisopropyladenosine (L-PIA) (1 mg/kg) and gamma-hydroxybutyric acid (GHBA) (300 mg/kg) were subsequently given intravenously to confirm the validity of this procedure. Using this assay, a centrally acting respiratory depressant can be identified by its inhibition of CO_2-induced stimulation of minute ventilation and enhancement of the NaCN-induced stimulation of mean inspiratory flow, whereas a peripherally acting respiratory depressant can be identified by its lack of effect on CO_2-induced stimulation of minute ventilation and its inhibition of NaCN-induced stimulation of mean inspiratory flow.

PROCEDURE

Gas and Sodium Cyanide Exposures

Rats are exposed to gas mixtures using a two chambered plethysmograph. The body of the animal is enclosed in a head-out chamber that is used to measure ventilatory parameters, while the head is enclosed in a cylindrical plastic headpiece that is attached to the front of the body chamber. A neoprene collar (1/8 inch thick) placed around the neck of the animal is used seal the plethysmograph chamber and separate the head and body chambers. Specific gas mixtures are delivered from compressed gas tanks to the head chamber at a rate of 2 liters/minute. All animals are acclimated to the chamber by placement in the chamber for at least three 15-minute periods on three separate occasions with compressed air flowing through the head chamber at a rate of 2 liters/minute. Prior to drug treatment, a catheter is inserted into a tail vein for administration of sodium cyanide. The catheter is exteriorized from the plethysmograph chamber through an opening that is made airtight by sealing with clay. Tail vein catheterization is accomplished using a 1/2 to 3/4 inch butterfly needle (23–25 gauge) attached to an extension set with syringe attachment. The gas mixtures contain either normal breathing air (21 % O_2, 79 % N_2) or elevated CO_2 (8 % CO_2, 21 % O_2, 71 % N_2). All animals are first exposed to the normal air mixture for 5–10 minutes and then to the elevated CO_2 mixture for 5 minutes. The mean value for the 5–10 minutes exposures to air and high CO_2 are calculated for minute volume and the difference used to quantify the stimulatory effect of CO_2. Following CO_2 exposure, rats are exposed to the normal air mixture until the ventilatory parameters return to normal (generally 5–10 minutes). Sodium cyanide (300 ug/kg) is then administered as a bolus injection using the tail vein catheter. The peak change in mean inspiratory flow is measured during the 1–3 minute period of ventilatory stimulation following the sodium cyanide injection.

Measurement of Ventilatory Parameters

Ventilatory parameters are measured using a head-out, volume displacement plethysmograph chamber (approximately 1–3 liter capacity). In this type of chamber, the head is exposed to ambient conditions, while the trunk is enclosed in the chamber (see Figure Figure 4). A seal is made around the neck using neoprene collar (1/8 inch thickness). Pressure changes within the chamber are measured using a differential pressure transducer with a sensitivity of approximately +/–2 cm H_2O (Model MP-45-14, Validyne Engineering, Northbridge, CA) and the pressure changes

converted to flow rates using a pneumotach port (1 inch diameter opening with six layers of 325 mesh stainless steel wire cloth). The analog flow signal is conditioned using a pre-amplifier and then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory parameters. The values for minute volume and mean inspiratory flow are determined for each breath and average values calculated for every 0.1 minute. Mean inspiratory flow (a measure of respiratory drive) is calculated by dividing tidal volume by inspiratory time and minute volume is calculated by obtaining the product of tidal volume and respiratory rate (see Figure 1).

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I.F.5 Continuous Measurement of Expired CO_2

PURPOSE AND RATIONALE

Changes in ventilatory parameters help define the mechanism and potential cause of a respiratory disorder, whereas changes in the partial pressure of arterial CO_2 ($PaCO_2$) define the physiological consequences, or liability, of a ventilatory change. A ventilatory disorder resulting in a decrease in $PaCO_2$ is defined as hyperventilation, whereas an increase in $PaCO_2$ is defined as hypoventilation. Obtaining samples of arterial blood for CO_2 analysis by needle puncture or arterial catheterization during ventilatory measurement is not considered practical since the acute insertion of the catheter or the restraint procedure needed for needle insertion and blood collection can often interfere with respiratory measurements. This is especially true for smaller animals. Furthermore, a blood sample only assesses blood gas status over the short time period that the sample is taken. Measuring changes in the peak concentration of expired CO_2 (end-tidal CO_2) during each breath has been developed as an alternative method for monitoring arterial CO_2 tension in humans and larger animals. End-tidal CO_2 measurements can be performed noninvasively and can be used for the continuous monitoring of arterial CO_2 during ventilatory measurements in conscious animals. With the use of microcapnometry, Murphy et al. (1994) developed a technique that could monitor end-tidal CO_2 in conscious rats during ventilatory measurements. The development of this technique in rats is important as the rat is a model commonly used for assessing ventilatory function in safety pharmacology. This assay was validated by showing that the changes in end-tidal CO_2 were sensitive to changes in ventilation, that a change in end-tidal CO_2 is sensitive to drug-induced respiratory stimulation or depression, and that the changes in end-tidal CO_2 are quantitatively (linearly) related to changes in arterial CO_2 and O_2 tensions and arterial blood pH.

PROCEDURE

Ventilatory Measurements

Ventilatory parameters are measured using a head-out, volume displacement plethysmograph chamber (approximately 1–3 liter capacity). In this type of chamber, the head is exposed to ambient conditions, while the trunk is enclosed in the chamber (see Figure 4). A seal is made around the neck using neoprene collar (1/8 inch thickness). Pressure changes within the chamber are measured using a differential pressure transducer with a sensitivity of approximately +/–2 cm H_2O (Model MP-45-14, Validyne Engineering, Northbridge, CA) and the pressure changes converted to flow rates using a pneumotach port (1 inch diameter opening with six layers of 325 mesh stainless steel wire cloth). The analog flow signal is conditioned using a pre-amplifier and then sent to a data acquisition and analysis software application (e.g., Life Sciences Suite Po-Ne-Mah Physiology Platform P3 with PCR application, Gould Instrument Systems, Inc., Valley View, OH) for calculation of ventilatory parameters. The values for tidal volume, respiratory rate and minute volume are determined for each breath and average values calculated for every 0.1 minute.

End-Tidal CO_2 Measurement

End-tidal CO_2 (peak expired CO_2) is measured for each breath with a microcapnometer (Microcapnometer, model 0151–003L, Columbus Instruments, Columbus, OH, USA). This microcapnometer is uniquely suited for measurement of expired CO_2 in rats and other animals with low minute volumes (200–400 mL/min) since it utilizes relatively low flow rates (5 or 20 cc/min) compared with the standard capnometers that require 150–200 cc/min. This capability is achieved by using a low pressure, high velocity principle for analyzing the sampled gas. Airflow to the microcapnometer is maintained at 20 mL/min and is collected through a Teflon catheter (I.D. = 0.76 mm). Carbon dioxide concentrations are measured spectrophotometrically

using standard infrared gas sensor technology and the values are expressed as percentage of total dry air volume. Average values for end-tidal CO_2 are calculated and expressed as numerical output every 0.1 min by the microcapnometer. The waveform and numerical data can be acquired by a computer system for storage and analysis. The microcapnometer is calibrated using an analyzed gas mixture containing 5 % CO_2 and 95 % O_2.

To convert the percentage CO_2 values measured by the microcapnometer (in dry air and at room temperature) to partial pressure values of CO_2 present in the alveoli (P_ACO_2), the following formula is used:

$$P_ACO_2(\text{mmHg}) = \%CO_2 \times [BP(\text{mmHg}) - P_AH_2O(\text{mmHg})]$$

Where BP is the barometric pressure measured at room temperature and P_AH_2O is the partial pressure of H_2O in alveolar gas at body temperature (39 °C) and saturated with water.

End-tidal CO_2 is monitored in conscious rats by using a mask that is fitted to the snout of the rat. Since rats are obligate nasal breathers, enclosure of the mouth is not necessary. The distal tip of the nasal mask has an opening (I.D. = 3.5 mm) for breathing through and an attachment site for the tube connecting to the micrcapnometer. The mask is held in place with a harness that fits over the head of the animal, and air samples are collected from the tip of the mask. To ensure a comfortable and tight seal around the snout, the inner edge of the plastic mask is coated with silicone foam (Lifecare, Lafayette, CO, USA). Dead space associated with the mask is approximately 0.06 cc. All animals should be acclimated to the plethysmograph chamber, harness and mask on at least 5 occasions prior to end-tidal CO_2 measurements.

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Chapter I.G
Metabolism Pharmacology

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I.G.1 General Considerations

Safety pharmacology is defined as those studies that investigate the potentially undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above (ICH-guideline S7A, 2001). Safety pharmacology in metabolism pharmacology is of secondary importance in comparison to the safety pharmacology of vital functions (Safety Pharmacology Core Battery: cardiovascular, respiratory and central nervous systems) and therefore listed under "Follow-up and Supplemental Safety Pharmacology Studies (ICH-guideline S7A, 2001: section 2.8.2)". In this section of the guideline, the subjects of safety pharmacology in metabolism pharmacology are listed under the topics of "gastrointestinal system" (ICH-guideline S7A, 2001: section 2.8.2.3) and "other organ systems" (ICH-guideline S7A, 2001: section 2.8.2.4).

In this book section, pharmacological methods are described for the characterization of candidate compounds on their effects on the gastrointestinal functions (gastric acid secretion, bile secretion, exocrine pancreatic secretion, gastrointestinal motility) and their gastrointestinal injury potential. In addition, there are pharmacological methods described with respect to elucidating the undesirable effect potential of compounds on intermediary carbohydrate und lipid metabolism resulting in hypo- or hyperglycaemia, hyperlipidemia, and insulin resistance, which could finally provoke life-threatening hypoglycaemia or, in the long run, diabetes, atherosclerosis and obesity.

In principle, every pharmacological assay described in detail by Vogel (2002) can be used for the safety pharmacological characterization of a candidate compound with regard to safety pharmacology in metabolism pharmacology (Vogel (2002): activity on the gastrointestinal tract, antidiabetic activity, anti-obesity activity, and anti-atherosclerotic activity). Here, in this part of the book, selections of these assays are presented which primarily meet the ICH guideline (ICH-guideline S7A 2001) and which are appropriately adapted to the characterization of candidate compounds with a different primary indication for the assessment of their pharmacological side effect potential on metabolism pharmacology.

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I.G.2 Gastrointestinal System

I.G.2.1 General Considerations

If a candidate compound with a totally different primary indication causes additionally an inhibition of gastric acid secretion, this finding may be assessed as additionally beneficial and therefore does not represent a safety concern, irrespective whether these pharmacological effects occur in the pharmacological dose range for the primary pharmacological effect or at supra-pharmacological doses as usually used for safety pharmacological studies of a candidate compound. The opposite situation, that a candidate compound for a different primary indication causes additionally a stimulatory effect on gastric acid secretion represents always a safety concern due to the risk for induction of gastrointestinal ulcers.

On the other hand an unrealized antisecretory effect on gastric acid secretion, which might occur at supra-pharmacological doses as used for toxicity studies, can become obvious during carcinogenicity studies in rats, resulting in the finding of carcinoids (ECL-cell proliferation) due to the long-lasting increase in gastric pH with subsequently elevated gastrin levels, which functionally and tropically control gastro-intestinal enterochromaffine-like cells (ECL-cells). This connection between gastric pH, gastrin level, ECL-cell proliferation, and gastric carcinoids has first been demonstrated for the proton pump inhibitor omeprazole (Arnold et al. 1986, Creutzfeldt et al. 1986, Ekman et al. 1985).

An acute stimulatory effect on gastric acid secretion as well as a direct effect on reduction of gastric mucus or bicarbonate secretion may finally result in gastric ulcers. An ulcerogenic side effect potential is always a safety issue of a candidate compound and should be carefully investigated. Since nearly 100 years it has well been known that Non-Steroidal Anti-Inflammatory Drugs (NSAID) cause gastric ulcerations, but their molecular mode of action, the inhibition of the cyclooxygenase (COX), the key enzyme in prostaglandin (PGG_2, PGH_2) synthesis, has first been proposed since the early 70 (Vane 1971). In the meanwhile different isoforms of the COX enzyme has been identified (Smith et al. 1996). The consti-

Table 1

Function	Assay/Test
Gastric secretion	Pylours-ligated rat
Gastric-intestinal injury	Gastro-intestinal ulceration in rats
Transit time	Propulsive gut motility in mice or rats (charcoal)
Gastric emptying assay	Stomach emptying in mice or rats (phenol red)

tutionally expressed isoenzyme COX-1 represents the dominant isoform in gastric mucosa. To get rid of the ulcerogenic side effect potential of NSAIDs more selective inhibitors for COX-2 have been developed (Kurumbail et al. 1996, Wolfe 1998).

In the following chapters a selection of pharmacological methods in gastroenterology is presented which primarily meet the ICH guideline (ICH-guideline S7A 2001) and exceed present practice. To date only four main tests appear to be widely used by pharmacologists to study gastrointestinal functions in safety pharmacology (Table 1, Harrison et al. 2004).

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I.G.2.2 Gastric Acid Secretion (Gastric pH Measurement)

I.G.2.2.1 Gastric Acid Secretion in Pylorus-Ligated Rats

PURPOSE AND RATIONALE

The secretory potential of a candidate compound might be much more problematic under safety aspects due to its ulcerogenic potential compared to the antisecretory potential of a candidate compound with a different primary indication.

A simple and reliable method for the measurement of gastric acid secretion and production of gastric ulcers in the rat based on ligature of the pylorus has been published by Shay et al. (1945). Gastric acid secretion can be stimulated by histamine, carbachol, or gastrin. Candidate compounds with antisecretory potential inhibit stimulated gastric acid secretion. The secretory potential of a candidate compound can be studied during basal conditions without administration of a secretogogue.

PROCEDURE

This study is performed in conscious rats with a body weight of 150–170 g. Food is withdrawn 16 hours before beginning of the study with water available ad libitum. Following pylorus-ligation, performed under ether anesthesia, the candidate compound is administered intraperitoneally (i.p.) or intraduodenally (i.d.). Gastric acid secretion is either studied under basal conditions or stimulated by subcutaneous (s.c.) injection of a secretagogue. The secretagogue is injected again 1 hour later. Three hours after the beginning of the experiment, the animals are killed, the stomach excised, and the accumulated gastric juice collected. The three different secretagogues employed are histamine (2×20 mg/kg s.c.), desglugastrin (2×400 μg/kg s.c.), or carbachol (2×40 μg/kg s.c.).

EVALUATION

The volume of the collected gastric juice is measured. Acid concentration is measured by titration against 100 mm NaOH to an endpoint of pH 7. Total acid output (mmol H^+/3 h) is calculated and percent inhibition of the treated rat group is calculated against the control group. Using various doses, dose-response curves can

be established for gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0 % corresponds to no and 100 % to maximal stimulated gastric acid output.

CRITICAL ASSESSEMENT OF THE METHOD

The pylorus-ligated rat has been proven to be a valuable method to evaluate the secretory (versus basal secretion) as well as the antisecretory potential (versus stimulation with histamine, gastrin, or carbachol) of a candidate compound with various secretory or antisecretory mechanisms of action.

For the safety pharmacological evaluation this method allows the administration of necessary high doses by i.d. or i.p. administration probably as suspension (methylcellulose) irrespective of solubility issues of the candidate compound.

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I.G.2.2.2
Gastric Acid Secretion in Anesthetized Stomach-Lumen Perfused Rats

PURPOSE AND RATIONALE

Originally Gosh and Schild (1958) introduced a method for the continuous recording of gastric acid secretion in the stomach-lumen perfused anesthetized rat. In this model, gastric acid secretion can be stimulated by histamine, carbachol or gastrin. Candidate compounds can be pharmacologically characterized for their gastric acid antisecretory potential during stimulated gastric acid secretion.

PROCEDURE

Rats with a body weight of 300–350 g are used for measuring gastric acid secretion during anesthesia. The animals are fasted for 18 hours prior to the experiment with free access to water. Anesthesia is induced by pentobarbital (priming bolus 60 mg/kg i.p. plus infusion s.c. at about 20 mg/kg/h) or i.p. injection of 5 ml/kg of 25 % urethane solution. Body temperature is artificially stabilized by means of a rectal thermometer and a heating pad. The trachea is exposed and cannulated for artificial respiration. The jugular veins are then exposed and cannulated with polyethylene tubes bevelled at the tip. The abdomen is opened through a midline incision. The oesophagus and pylorus are ligated and a double lumen perfusion cannula is inserted and fixed in the forestomach. The stomach is perfused continuously with warm (37 °C) saline at a rate of 1 ml/min. The perfusate is collected at 15 min periods and its acid concentration measured. Histamine (10 mg/kg/h), desglugastrin (100 μg/kg/h) or carbachol (30 μg/kg/h) are administered by i.v. infusion into the jugular vein after a basal period of 45 min. Ninety minutes after the onset of the secretagogue infusion, acid output has reached a stable plateau. As soon as acid secretion has reached a plateau, candidate compound or standard is injected intravenously.

EVALUATION

The perfusate is collected at 15 min periods and its acid concentration measured by titration against 100 mm NaOH to an endpoint of pH 7 and acid output (μmol H^+/15 min) is calculated. Using various doses of the candidate compound and of a standard, dose-response curves can be established and activity ratios with confidence limits can be calculated.

CRITICAL ASSESSMENT OF THE METHOD

For the specific pharmacological assessment of inhibitors of gastric acid secretion, like H_2-blockers, anticholinergics, H^+/K^+-ATPase inhibitors, this method reveals valid results with respect to the antisecretory potential of the candidate compound. Limitations of this methods with respect to safety pharmacological assessment of candidate compounds are (1) only parenteral administration of the candidate compound, preferentially i.v., is feasible and should be preferred, and (2) only antisecretory putative side effects can be investigated; this method is of limited relevance to study secretory side effect potential of candidate compounds.

For safety pharmacological assessment of a candidate compound with a totally different inherent primary indication, its antisecretory potential at supra-pharmacological doses has to be studied. Therefore, whether this method can be used for the safety evaluation depends on the solubility of

the candidate compound for i.v. administration of supra-pharmacological doses.

As different pathways of stimulation are exclusively initiated by histamine, gastrin, or carbachol, it is possible to estimate the potential interaction of the candidate compound with the secretory pathways of acid secretion (Herling and Weidmann 1996): Candidate compounds affecting

1. the H_2-receptor inhibit histamine- and gastrin-stimulated gastric acid secretion
2. the gastrin receptor inhibit only gastrin-stimulated gastric acid secretion
3. the muscarinic receptor inhibit only carbachol-stimulated gastric acid secretion
4. carboanhydrase activity inhibit gastric acid secretion, irrespective of the kind of stimulation
5. H^+/K^+-ATPase (gastric proton pump) inhibit gastric acid secretion, irrespective of the kind of stimulation.

MODIFICATIONS OF THE METHOD

Burn et al. (1952) described the evaluation of substances, which affect gastric secretion using perfusion of the stomach in anesthetized cats.

Lawrence and Smith (1974) described the measurement of gastric acid secretion in the rat by conductivity. The stomach of an anesthetized rat is continuously perfused with 2 ml/min of an isotonic (0.308 molar) glucose solution at 37 °C. The conductance of a solution depends on the total ion concentration and is therefore not specific for hydrogen ions. Since hydrogen ions have an equivalent conductance nearly 5 times greater than any other ion found in gastric juice and since they are secreted in a far greater concentration than other ions, conductivity measurements can be regarded as a relatively specific measure of hydrogen ions. Using Mullard conductivity cells (type E 791/B) and a commercially available meter (Phillips PW 9501) simultaneous measurements in 6 rats were performed.

Gallo-Torres et al. (1979) described in detail a method for the bioassay of antisecretory activity in the conscious rat with acute gastric fistula with additional collection of the biliary and pancreatic secretion by means of a catheter in the common bile duct. The gastric secretions are collected by gravity via a cannula in the most gravity dependent site of the glandular stomach.

Larsson et al. (1983) described studies in the acutely vagotomized rat. Truncal vagotomy is performed under ether anesthesia by cutting the dorsal and ventral branches of nervus vagus just below the diaphragm. The pylorus is then ligated and a polyethylene catheter (PP 200) is inserted into the duodenum, close to the pylorus. Each animal is placed in a modified Bollman cage and is allowed to recover at least 1 hour before the experiment. Gastric juice is collected by free drainage in 30 min samples.

Herling and Bickel (1986) showed that gastric acid secretion in stomach-lumen perfused rats can be stimulated in vivo on the subreceptor level by IBMX (phosphodiesterase inhibitor) and forskolin (non-receptor activation of the adenylate cyclase). H^+/K^+-ATPase inhibitors and H_2-antagonists show, according to their different modes of action, also a different inhibitory profile in this assay.

Hammer et al. (1992) used anesthetized female Sprague-Dawley rats weighing 200–320 g. After insertion of a tracheal cannula, a 3-mm silicon tubing is placed through the mouth and advanced to the stomach. The tubing is tied to the oesophagus at the neck. A 4-mm drainage tube is inserted into the stomach through a laparatomy incision and an incision in the duodenum, and ligated in place at the pylorus. Gastric perfusate (0.9 % saline at 37 °C) is collected on ice every 5 min for titration to pH 7.0.

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I.G.2.2.3
Gastric Acid Secretion in Conscious Dogs (Chronic Heidenhain-Pouch Fistula in Dogs)

PURPOSE AND RATIONALE

ICH-guideline S7A (2001: Section 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. The preparation of a chronic gastric pouch, as described by Heidenhain in 1878, is one of the classic techniques in experimental surgery. This model has much contributed to the understanding of the physiology and pathology of the stomach and to modern techniques of abdominal surgery in man. The surgical technique has been described again in detail by deVito and Harkins (1957). A preparation of chronic denervated pouches in the rat has been described by Alphin and Lin (1959). Both preparations can be used as pharmacological models for testing antisecretory drugs.

PROCEDURE

The principle technique is demonstrated in Fig. 1. Dogs weighing 15–20 kg are fasted 24 hours preoperatively. The abdominal surgery is performed during appropriate anesthesia (in former times by e.g. 30 mg/kg pentobarbital sodium; nowadays more appropriately by inhalation with halothane or isoflurane). The abdominal part is shaved with electric clippers, then with a razor. The skin is disinfected with a surface disinfectant (e.g. Zephiran®–70 % alcohol). Sterile drapes are applied to cover the whole surgical field. A midline linea alba incision from xiphoid to umbilicus provides excellent exposure and ease for closure. As the posterior sheath is divided, the large ventral fat pad present in dogs should be excised completely. A self-retaining retractor is applied and the stomach is palpated for the absence of food. Then the spleen is displaced, wrapped in warm, moist pads, and laid on the ventral wall below the incision.

The stomach is pulled into the operative field. The greater curvature is held at multiple points so that the stomach is stretched out and the line of incision for the pouch is selected. The pouch should be made from the corpus of the stomach so that true parietal cell juice can be obtained. A line projected from the incisura angularis perpendicular to the proximal lesser curvature will generally fall across the junction between corpus and antrum. Appropriate division of the gastric branches of the right gastroepiploic artery at the lower end of the proposed line of transsection clears the greater curvature for 1–2 cm. The gastroepiploic artery itself should be sectioned at this site and a long rent formed on the adjacent omentum, else the omentum vessels tend to tear during subsequent manipulations.

An index finger is then inserted through this defect dorsal to the stomach to emerge higher on the greater curvature through the gastrosplenic ligament at the upper end of the proposed line of transsection. This portion of the greater curve is cleared for 1–2 cm. Von Petz clamps with their staplings are used to control bleeding and to avoid leakage of gastric content. The stomach should be kept stretched and flattened while the clamps are applied. After division between the staples, any bleeding is controlled and the cut edges of the main stomach and pouch are then oversewn with continuous sutures of black silk. The suture should be of an inverting type. Surprisingly, leakage or excessive adhesions are not a problem when serosal apposition is neglected.

The pouch so formed is about 30 % of the corpus volume and provides adequate secretory volume for further studies (Fig. 1). A cannula, made of stainless steel, 7 cm long with a bevelled flange threaded at the other end is placed in the most ventral portion of the pouch through a small incision in the anterior wall. A single purse-string of silk holds it in place. A double sheet of omentum is then wrapped about the pouch and the cannula before being pulled through the abdominal wall, about 3 cm to the left of the midline subcostally. It is important that the cannula be held snugly by fascia, otherwise it will readily pull out of the pouch and abdominal wall. The linea alba is closed with a continuous suture of silk and the skin with subcuticular stitches of chromic catgut. On the outside of the cannula a stainless steel jacket is screwed. The cannula is always open so that secreted gastric juice does not accumulate within but is drained from the pouch.

Before recovery from anesthesia the dog receives 500 ml 5 % glucose in saline intravenously. The same volume is given for 3 days postoperatively together with oral fluid ad libitum. From the 4th day onward, normal food is given. A period of 7–10 days is required

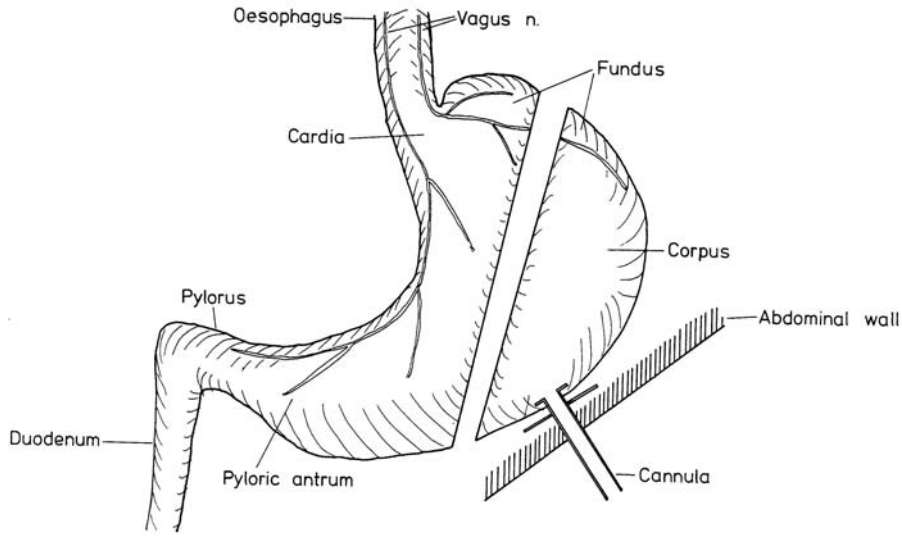

Fig. 1. Technique of Heidenhain pouch fistula in dogs

for full recovery from the operation. Special care has to be taken for each animal being kept separately in a suitable cage.

For pharmacological studies, food is withdrawn 18 hours prior to the experiment with water ad libitum. The animals are placed in Pawlow stands during the experiment of gastric secretion measurement and a tube is fitted to the cannula to collect the gastric juice from the pouch for measurement of volume and acidity by titration. To test the secretory potential of a candidate compound, which might represent a direct safety concern, the candidate compound is studied under basal (non-stimulated conditions) and administered orally or by i.v. injection or infusion and the gastric acid secretion from the pouch is measured in intervals of 15 or 30 minutes. The values are compared to the predrug secretion values and to a respective control group.

For testing the antisecretory potential gastric acid secretion is stimulated either by i.v. infusions of histamine (0.1 mg/kg/h), carbachol (10 μg/kg/h), or pentagastrin (8 μg/kg/h). When stimulated gastric acid secretion has reached a stable plateau (after 1.5 hours) the candidate compound is administered orally or by i.v. injection and secreted fluid is collected at 15 or 30 min intervals and analyzed for free HCl.

EVALUATION

The secreted volume per time interval is measured. An aliquot is used for the determination of acidity by titration against 100 mmol/l NaOH and total acid output per time interval is calculated. The effect on volume and HCl secretion at 15 or 30 min intervals after administration of the test compound is compared with the control values. Mean inhibition of stimulated gastric acid secretion can be calculated according to the formula:

$$\text{mean inhibition (\%)} = -\left(\left(\left(\frac{SAO_{postdrug}}{N_{postdrug}} \Big/ AO_{predrug}\right) \times 100\right) - 100\right)$$

$SAO_{postdrug}$ = sum of acid output per 30 min after compound administration

$N_{postdrug}$ = number of 30 min collection intervals after compound administration

$AO_{predrug}$ = acid output prior compound administration

In addition to the total acidity of the secreted juice also pepsin total activity can by determined by appropriate enzymatic methods.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described a simplified method for isolation of a portion of the stomach as compared to the original method of Heidenhain (1878).

Gastric motility can be measured by balloon manometry of the Heidenhain pouch in the conscious dog. The animals are deprived of food for 18 hours before the experiment, but water is allowed ad libitum. A latex balloon, connected via a polyethylene catheter to a pressure transducer (Statham P 23 BB), is introduced through the fistula cannula into the accessory stomach. Changes in intragastric pressure

are measured on a frequency measurement bridge and are recorded continuously. The number and height of the pressure waves are used as indices of gastric motor activity. Secretin inhibits gastric motility dose-dependently. After injection of gastrin or gastrin analogues, a dose-dependent increase of pressure is noted over a wide dose-range.

Jacobson et al. (1966, 1967) studied gastric secretion in relation to mucosal blood flow by an antipyrine clearance technique in conscious dogs with vagally denervated gastric fundic (Heidenhain) pouches. A vagally denervated fundic pouch is so constructed that the entire arterial blood supply is delivered by the splenic artery. A non-cannulating transducer (electromagnetic flowmeter) and a hydraulic occluder were implanted on the vessel.

The Heidenhain pouch preparation was used by Carter and Grossman (1978), Kauffman et al. (1980) to study the effect of luminal pH on acid secretion evoked by topical and parenteral stimulants and the effect of topical and intravenous 16,16-dimethyl prostaglandin E_2 on gastric bicarbonate secretion.

Baker (1979) and Roszkowski et al. (1986) developed a modified Heidenhain dog pouch preparation for collecting gastric juice exclusively from the pouch during experimental periods but allowed the pouch to be an integral part of the gastrointestinal tract during non-experimental periods. The pouch is prepared using conventional techniques but, instead of being fitted with a simple cannula through the abdominal wall, a three-way cannula is used which provides passage between the exterior orifice, the pouch and the main body of the stomach. By inserting an appropriate adapter, passage is available only to the pouch and not to the main stomach or vice versa.

The Heidenhain pouch technique in dogs has been used for preclinical evaluation of various drugs, such as:

- a histamine H_2 antagonist by Uchida et al. (1993)
- dual histamine H_2 and gastrin receptor antagonists by Kawanishi et al. (1997)
- a 5-HT_4 receptor antagonist by Bingham et al. (1995)
- an other 5-HT_4 receptor antagonist by Wardle et al. (1996)
- inhibition of motilin-induced phase III contractions by pentagastrin by Yamamoto et al. (1994)
- peptide YY by Zai et al. (1996)
- reversible K^+-competitive inhibitors of the gastric H^+/K^+-ATPase by Parsons et al. (1995)
- the antiulcer agent SWR-215 by Kataoka et al. (1997)
- a selective gastrin/CCK-B receptor antagonist by Yuki et al. (1997)
- Descroix-Vagne et al. (1993) used Heidenhain pouch preparations in cats and rabbits to study the effect of perfusion at pH 5.5 on acid and pepsin secretion
- For identification of the KCNQ1 protein as the K^+-channel colocalized with the H^+/K^+-ATPase at the apical membrane of the gastric parietal by studying the gastric acid inhibitory potential of the tool compound 293B (inhibitor of KCNQ1) cell by Grahammer et al. (2001).

CRITICAL ASSESSEMENT OF THE METHOD

Due to the surgical procedure the connections of the autonomic nervous system of the isolated pouch are interrupted from those of the main stomach. Therefore, basal gastric acid secretion from the pouch, which based mainly on the parasympathetic activity, is reduced.

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I.G.2.2.4
Effect of Candidate Compounds with Antisecretory Potential on Serum Gastrin Levels

PURPOSE AND RATIONALE

It is known from long-lasting and potent gastric acid inhibition caused, for instance, by the H^+/K^+-ATPase inhibitor omeprazole, that the total acid blockade initiates a gastric antral feed-back mechanism resulting in an excessive hypergastrinaemia (Arnold et al. 1986; Creutzfeldt et al. 1986; Larsson et al. 1986) which is believed to cause diffuse endocrine cell hyperplasia, characterized as carcinoids, in the gastric corpus after 2 years of treatment (carcinogenicity study) in the rat (Ekman et al. 1985).

PROCEDURE

Groups of 10–15 rats weighing 90–110 g are treated daily for 10 weeks with the candidate compound (omeprazole as standard at doses of 10 or 30 mg/kg p.o.). After treatment for 2, 4, 7, and 10 weeks, blood samples are collected under ether anesthesia by retro orbital puncture. Gastrin is determined by a commercially available radioimmunoassay kit. At the end of the study of 10 weeks, the animals are studied for their gastric acid output using the pylorus ligation (Shay technique).

EVALUATION

Serum gastrin levels are determined as pg/ml. Statistical differences ($p < 0.05$) are calculated using appropriate statistical methods.

MODIFICATIONS OF THE METHOD

Katz et al. (1987) described a five-day test to predict the long-term effects of gastric antisecretory agents on serum gastrin in rats.

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I.G.2.3 Bile Secretion

I.G.2.3.1 Bile Secretion in Mice

PURPOSE AND RATIONALE

The effect on bile secretion of a candidate compound can be studied in mice by weighing the gall bladder filled with bile. This simple method was first published by Litvinchuk (1976). With respect to the safety assessment of candidate compounds a decreased bile secretion (compound-induced cholestasis) predominantly represents a safety issue.

PROCEDURE

Groups of 10 mice weighing 15–20 g are used. Food, but not water, is withdrawn 24 hours prior to the experiment. The test compound or the control solution is administered subcutaneously or orally. After 1 hour, the animals are sacrificed and bled from the carotid artery. Laparotomy is performed, the liver exposed, and a No. 75 silk ligature is tied around the cystic duct, which is detached from the bile ducts and removed from the peritoneal cavity. If a large volume of bile has been accumulated, the full gall bladder is removed together with the bile ducts. The isolated gall bladder is weighed on a suitable balance, after which the contents are removed, the gall bladder walls are washed with distilled water, dried on filter paper, and the organ is weighed again. The difference in weight of the full and the empty gall bladder indicates the quantity of bile secreted during a measured time. The concentration of cholates, bilirubin, and cholesterol in the bile can be determined.

EVALUATION

The average of secreted bile in groups of 10 treated mice is compared with the average value of the control group using appropriate statistical methods.

CRITICAL ASSESSEMENT OF THE METHOD

The method has the clear advantage of simplicity but does not measure the true bile excretion since the outflow from the bile bladder during the test period is neglected.

MODIFICATIONS OF THE METHOD

Sterczer et al. (1996) studied the effect of cholagogues on the volume of the gallbladder in healthy dogs fasted for 24 hours by two-dimensional ultrasonography. The volume was measured immediately before the administration of each test substance and at 10-min intervals for 120 min thereafter.

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I.G.2.3.2 Bile Secretion in Anesthetized Rats

PURPOSE AND RATIONALE

In contrast to other animals, rats do not possess a bile bladder. Therefore, cannulation of the bile duct in rats can be used as a suitable model to measure choleretic (increased bile production) or cholestatic (decreased bile production) side effect potential of drug candidates. If the test compound reduces bile production, it is recommended to investigate a putative hyperlipidemic side effect potential of the drug candidate by its influence on total blood cholesterol and triglycerides in appropriate experimental methods.

In addition this method of the bile fistula rat can be used for ADME profiling of drug candidates with respect to a hepatobiliary elimination potential (high first pass effect) (Herling et al. 2002).

PROCEDURE

Bile secretion is studied in anesthetized bile fistula rats, which are anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg), tracheotomized, and one jugular vein per rat is cannulated for intravenous administration (bolus injection or infusion of the drug candidate). Anesthesia is maintained for up to 7 hours by subcutaneous infusion of pentobarbital sodium (adjusted to the aesthetic depth of the individual animal; about 24 mg/kg/h). Body temperature is monitored with a rectal probe thermometer, and temperature is maintained at 37 °C by means of a heated surgical plate.

After laparotomy, the common bile duct is cannulated in the upper half with polyethylene tubing and bile is collected every 30 minutes up to 7 hours. The drug candidate is administered at an appropriate dose by bolus injections intravenously into the jugular vein one hour after finishing surgery or by intraperitoneal administration of a 1 % carboxymethylcellulose suspension, if not adequate soluble for an intravenous

formulation. The volume of excreted bile per 30 minutes is determined gravimetrically (difference between tube weight without and with bile per collection period) with the assumption that 1 g is equivalent to 1 ml of bile. According to our experience bile flow is stable for up to 3 hours (200–300 µl/30 min) and can decline later due to the interruption of the enterohepatic circulation of bile acids, if not the secreted bile is reinjected into the ileum.

For ADME purposes the concentration of the parent test compound in the bile is measured by appropriate analytical methods and total compound excretion is calculated from the secreted volume and the measured concentration of the test compound in the bile of each sampling interval.

For determination of the side effect potential of a candidate compound on choleresis or cholestasis groups of at least 6 rats are used for control (vehicle control) and treated groups (rats receiving one dose of the test compound per group). For ADME purposes smaller groups are sufficient (n = 3–4) to determine hepatobiliary elimination of the test compound. Ideally the analytical method includes the determination of major metabolites appearing in the bile.

EVALUATION

Mean values (µl bile/30 minutes) for each group are calculated and compared to that of the control group. If bile flow is affected by the test compound detailed analysis of the bile with respect to cholesterol, bile acids and phospholipids should be performed to elucidate the underlying mechanism.

For ADME purposes the amount of hepatobiliary eliminated compound plus metabolites are calculated per collecting interval and total excreted amount over the whole experiment can be calculated and can be set into relation to the total administered dose per animal.

MODIFICATIONS OF THE METHOD

Several authors tested the choleretic activity of plant extracts and essential oils (De la Puerta et al. 1993; Peana et al. 1994; Trabace et al. 1994) and of synthetic compounds (Grella et al. 1992, Paglietti et al. 1994) in rats.

Tripodi et al. (1993) investigated the anticholelithogenic and choleretic activities of tauro-hyodeoxycholic acid by measurement of biliary flow and biliary solids content in rats.

Bouchard et al. (1993) induced cholestasis in rats by treatment with 17-α-ethinyl estradiol and studied the influence of oral treatment with ursodeoxycholic and tauroursodeoxycholic acids.

Miki et al. (1993) investigated the metabolism and the choleretic activity of homochenodeoxycholic acid in hamsters with bile fistula.

Pesson et al. (1959) recommended the guinea pig as the best choice among the common laboratory animals to study choleretic agents.

Matsumura et al. (1996) analyzed hypercholeresis in dogs with pigment gallstones after cholate infusion.

CRITICAL ASSESSEMENT OF THE METHOD

The method is simple and provides reliable results during terminal anesthetized conditions. Intraduodenal administration of the test compound, which is also reported in the literature, should be avoided due to the fact, that intestinal absorption is obviously impaired during the reduced intestinal motility during anesthesia. The interrupted enterohepatic circulation should be taken into account when extrapolating the results (choleretic and cholestatic potential) to the intact organism.

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I.G.2.3.3
Bile Secretion in Conscious Rats (Chronic Bile Fistula Rats)

PURPOSE AND RATIONALE

ICH-guideline S7A (2001: Section 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. Most of the techniques for collection of bile in rats use restrained or anesthetized animals. Such factors as well as the surgical intervention itself may profoundly influence the results. Therefore, Remie et al. (1990, 1991) developed a technique for a permanent double bile fistula in rats. The procedure is described in detail.

PROCEDURE

Preparation of cannulas

Cannulas are made of silicon rubber. The proximal bile cannula, which will be inserted into the common bile duct in the direction to the liver, is 18 cm long (Silastic tubing, Dow Corning, no. 605-135; 0.51 i.d. and 0.94 o.d.) and has one square cut and one bevelled end. Two silicon rings are wrapped around the cannula at 7 mm and 50 mm, respectively, from the bevelled end.

The distal bile cannula, which will be inserted into the common bile duct in the direction of the gut, is made of the same material, is also 18 cm long and has one square cut and one bevelled end. This cannula, however must have a smaller tip-diameter (Silastic tubing, Dow Corning, no. 605-105: 0.31 i.d. and 0.64 o.d.). To serve this purpose, the square cut end of the cannula is immersed in ether, causing the tubing to dilate. When the tubing is wide enough, a 13 mm piece of small diameter Silastic tubing is inserted. Subsequently, two silicon rings are wrapped around the cannula, one at the joint of the two tubes and the other 5 cm from the tip. The tip is then cut at a 45° angle, 7 mm from the first silicon ring.

The duodenal cannula (Silastic tubing, Dow Corning, no. 605–135) is also 18 cm long, and has one square and one bevelled end. An additional ring is placed 30 mm from the tip. Before the cannulas are fixed to the skull, they must be connected to a stainless steel needle bent in a 90° angle.

Anesthesia

The animal is anesthetized by inhalation with halothane/N_2O/O_2.

Preparation of the Crown of the Head

The head of the animal is shaved and disinfected. An incision of about 1 cm is made and the bregma exposed. Three stainless steel screws (1.0 × 4.2 mm) are mounted in the crown, two in the left and one on the right side of the bregma. The screws are tightened that approximately 2 mm is left between the skull and the head of the screws.

Double Cannulation of the Bile Duct

The abdominal wall is shaved and disinfected and the animal secured on the operation board with adhesive tape. A midline incision is made from the level of the pubic bones to the xiphoid cartilage. The abdomen is then opened by making an incision over the linea alba towards the sternum up to the distal part of the fourth sternebra, thus exposing the xiphoid cartilage.

Then, the intestines are lifted out and are laid next to the animal on moistened gauze. Using jewellers forceps, the bile duct is stripped off its surrounding tissue and ligated with a 7–0 suture. The duct is placed under tension with an artery forceps for cannulation. With the aid of a microscope, a V-shaped hole is made just cranial of the first ligature with iridectomy scissors. The sterile proximal cannula is inserted into the duct. The second ligature is tied and pulled tight ensuring that the cannula is not obstructed. The bile is now flowing into the cannula. The first ligature is released and the threads are tied behind the silicon ring. The rat is then turned and the ligature reclamped, thereby putting the distal part of the duct under tension. A third ligature is loosely introduced around the duct, distal to the first ligature. Another V-shaped aperture is made between the first and third ligature for insertion of the distal bile cannula. The third ligature is tied and pulled tight. The first ligature is released from the artery forceps and tied around the second cannula behind the silicon ring. All the loose threats are cut close to the knots. The sections of the cannulas which lie between the silicon rings are placed kink-free in the abdominal cavity. The cannulas are fixed using 7-0 silk suture to the abdominal muscle near the xiphoid cartilage.

Cannulation of the Duodenum

After location of the place where the bile duct enters the duodenum (sphincter of Oddi), a four fine-stitch purse-string suture (7–0) is made in the wall of the duodenum at the outer border at about 1 cm proximal to the sphinc-

ter. Using a 20 G needle, an incision is made inside the purse string. The cannula is inserted into the duodenum until the first, smaller silicon ring has entered the lumen, and the purse string is tightened between the first and the second ring. This cannula together with the bile cannula is placed kink-free in the abdominal cavity and anchored to the internal muscle. The abdomen is closed of resorbable sutures leaving 1 cm of the skin unclosed.

Subcutaneous Tunnelling and Anchoring of the Cannulas

From the back of the neck, a slender needle holder is pushed subcutaneously through the connective tissue in caudal direction as near as possible to the skin down to the xiphoid cartilage. The cannulas are then grasped and pulled through to emerge at the crown of the head. The abdominal wall is closed completely.

With a 5 cm piece of polyethylene tubing (0.75×1.45 mm), the two long ends of the L-shaped stainless steel adapters are connected and the short ends inserted into the respective cannulas. The cannulas together with the tubing are fixed to the skull with acrylic glue flowing under the heads of the screws.

Postoperative Care

The animals are allowed to recover in a warm and quiet place. They reach usually preoperative weight within 2–3 days, and display normal feeding and drinking behaviour. Supplementation with saline besides the normal tap water may be necessary.

Collection of Bile

The animals are housed in individual metabolic cages. For bile collection, they are attached to long swivelled PE-cannulas (0.75 × 1.45 mm). A stainless steel coil is used to protect the rats from gnawing on the tubing. For continuous collection of bile, the cannula can be connected to a fraction collector.

CRITICAL ASSESSEMENT OF THE METHOD

Among other applications, the method is suited to study the enterohepatic circulation of compounds. There should be a close health monitoring of the chronically prepared rats, and only those in a very good health conditions should be used for the study to avoid any misinterpretations of the results.

MODIFICATIONS OF THE METHOD

Castilho et al. (1990) studied the intestinal mucosal cholesterol synthesis in rats using a chronic bile duct-ureter fistula model. Male Wistar rats weighing 300–350 g were anesthetized with 50 mg/kg pentobarbital i.p. and submitted to a bile duct-right ureter fistula utilizing a PE-50 catheter after a right-kidney nephrectomy.

Cohen et al. (1992) reported a study in male black-tailed prairie dogs (Cynomys ludovicianus) weighing 1.0 ± 0.2 kg anesthetized with 20 mg/kg xylazine i.m. and 20 min later with 100 mg/kg ketamine i.m. Through an abdominal incision, the cystic duct is ligated, and gallbladder bile is aspirated. A PE-50 polyethylene cannula is inserted into the common bile duct and secured with silk sutures, thereby completely diverting bile flow for collection. The bile duct cannula is externalized, the abdominal incision closed, and the prairie dog placed in a restraining cage with access to food and water.

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I.G.2.3.4
Bile Secretion in Conscious Dogs (Chronic Bile Fistula in Dogs)

PURPOSE AND RATIONALE

ICH-guideline S7A (2001: Section 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. Herrera et al. (1968) described a special cannula, which can be used to obtain bile or pancreatic juice from a duodenal pouch after appropriate surgical procedures in conscious dogs.

PROCEDURE

The technique of chronic bile fistula in dogs is complementary to the chronic pancreatic fistula in dogs (Fig. 2) with the modification, that the duodenal pouch is formed from a duodenal segment containing the common bile duct. Male Beagle dogs weighing 15–20 kg are used. The abdominal surgery is performed during appropriate anesthesia (in former times by e.g. 30 mg/kg pentobarbital sodium; nowadays more appropriate by inhalation with halothane or isoflurane). The abdomen is opened through a midline epigastric incision. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the common bile duct is isolated. The distal stoma of the duodenum is closed and continuity restored by end-to-side duodeno-jejunostomy. The duodenal pouch is closed at both ends.

The cannula to be inserted is made of stainless steel and consists of 3 parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the pouch. When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows bile to enter the duodenum in the normal manner. For collection of bile this plug is removed and a long obturator is inserted. The latter effectively isolates the bile secretion from duodenal contents. A similar hollow obturator is reserved for use when duodenal perfusion is studied, the obturator being connected via a plastic tube to the irrigating fluid.

Through a small antimesenteric incision in the duodenal pouch, the lateral limb of the cannula is inserted; the V-shaped defect in the flange facilitates entry into the pouch. A purse string secures the cannula in position. The defunctioned loop of duodenum is then brought anterior to the pancreas, and the remaining limb of the cannula inserted through a small duodenotomy and secured by a further purse-string suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula. The cannula is left open to drain blood and secretions for 24 hours postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Daily checks of the cannula are advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 4 weeks to recover. Eighteen hours prior to the experiment food is withdrawn but water allowed ad libitum. The long hollow obturator is inserted and bile collected for 15 min periods. After 1 hour pre-test time, the test compound is given either orally or intravenously.

EVALUATION

Secretion of bile is measured at 15 min intervals and volume and bile contents are determined from 1 ml samples. The values are compared with pre-test data. The remaining bile is re-infused into the duodenum via the hollow obturator.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described several techniques for fistulae of the gall bladder and also for the fistula of the ductus choledochus in dogs.

An abdominal incision about 10 cm is made on the median line. The duodenum is pulled out and the orifice of the large (first) pancreatic duct is found. The orifice of the ductus choledochus with the orifice of the small (second) pancreatic duct is situated on the other side of the intestine some 2 or 3 cm nearer the stomach. The ductus choledochus goes straight from the gallbladder to the duodenum; further it lies parallel its end it is attached to the wall of the duodenum. The small pancreatic duct goes from the gland straight to the duodenum.

At the very beginning of the operation it is useful to cut the ligamentum that goes from the liver to the duodenum, because this facilitates orientation and operating. It is necessary to cut out a piece of the intestinal wall with the orifice of the ductus choledochus. But before this one must prepare off a little bit the intestine from the pancreas so as to be able to close conveniently and securely the hole in the intestine and divide between double ligatures the second pancreatic duct.

On the duodenum around the orifice of the ductus choledochus an incomplete oval figure is now marked with a knife, so that the duct enters this figure through the incomplete part of the oval and has its orifice in the middle of this figure. The length of the oval is about 1.5 cm and its width 1 cm. A suture is then made on the edge of this oval, which is cut out not completely but leaving a small bridge about 0.5 cm wide between the intestine and the oval; through the bridge the duct enters the oval. The mucosa of this bridge must be completely destroyed with a knife.

The oval piece of the intestine is now turned with the mucosa up and its serosa is sutured to the serosa of the intestine. The hole in the intestine is very carefully closed with two layers of sutures. Two heavy threads

are then passed underneath the intestine on either side of the place of operation; they are laid through the abdominal wall and tied after the operation is over. They serve as temporary supporting sutures. The oval piece of the intestine is now sutured with the skin of the abdominal wound and the wound is closed in the usual manner. The supporting sutures must be taken out one day or two days after the operation.

CRITICAL ASSESSEMENT OF THE METHOD

There should be a close health monitoring of the chronically prepared dogs, and only those in a very good health conditions should be used for the study to avoid any misinterpretations of the results.

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I.G.2.4 Exocrine Pancreatic Secretion

I.G.2.4.1 Exocrine Pancreatic Secretion in Anesthetized Rats

PURPOSE AND RATIONALE

The effect of a candidate compound on pancreas secretion can be measured in rats with acute pancreas fistula. For safety pharmacological assessment of candidate compounds the decrease of exocrine pancreatic secretion might be problematic due to the potential of induction of pancreatitis.

PROCEDURE

Rats weighing 150–200 g are used. Eighteen hours prior to the experiment food is withdrawn with free access to water. The appropriate size of the study groups for the control and candidate compound consists of 5–7 animals. Anesthesia is induced by pentobarbital (priming bolus 60 mg/kg i.p. plus infusion s.c. at about 20 mg/kg/h) or i.p. injection of 5 ml/kg of 25 % urethane solution. Body temperature is artificially stabilized by means of a rectal thermometer and a heating pad. The trachea is exposed and cannulated for artificial respiration. The abdomen is opened by a mid-line incision and the pylorus ligated. The proximal part of the bile duct is ligated near the hepatic porta. The bile is drained via a thin polyethylene tube into the duodenum. The distal part of the bile duct with the orifices of pancreatic ducts is cannulated with another thin polyethylene tube. The pancreatic juice is collected in Eppendorf tubes and secreted volume is measured gravimetrically or by graduated microsyringes every 15, 30 or 60 minutes. After a pre-test period of 60 min, the test compounds are applied intravenously or intraduodenally.

EVALUATION

The secretion after injection of the test compound is compared with the pre-test values. Secretin or cholecystokinin (CCK) increases pancreatic secretion volume in a dose-dependent manner and can be used as a positive standard.

MODIFICATIONS OF THE METHOD

Guan et al. (1990) inserted two separate cannulas for bile and pancreatic juice to rats under methoxyfluorane anesthesia. Both fluids were returned to the intestine. Placing the rats in modified Bollman-type restraint cages, experiments could be performed after a few days in conscious animals.

Ito et al. (1994) studied the inhibition of CCK-8-induced pancreatic amylase secretion by a cholecystokinin type-A receptor antagonist in rats.

Niederau et al. (1989) compared the effects of CCK receptor antagonists on rat pancreatic secretion in vivo. Output of amylase in pancreatico-biliary secretion was measured after various doses of caerulein. The effects of high caerulein doses were dose-dependent inhibited by CCK-antagonists.

Alvarez and Lopez (1989) studied the effect of alloxan diabetes on exocrine pancreatic secretion in the anesthetized rabbit. After a 14–15 hours fasting period, but with free access to water, rabbits weighing about 2.0 kg are anesthetized by intravenous injection of 1.0 g/kg urethane. After tracheotomy, a median laparotomy is performed, the main pancreatic duct is exposed and cannulated near its entrance to the duodenum following ligation of the pylorus and cannulation of the bile duct for deviation of bile to the exterior.

Kim et al. (1993) studied the effect of [(CH_2NH)4,5]-secretin on pancreatic exocrine secretion in guinea pigs and rats using an acute pancreatic fistula preparation.

Niederau et al. (1990), Tachibana et al. (1996) determined pancreatic exocrine secretion in mice. Because the cannulation of mouse pancreatic duct is not possible for technical reasons, the amount of amylase was determined in vivo. Five min after i.v. administration of candidate compounds, mice were sacrificed and a 5 cm-duodenal loop was removed.

The duodenal contents were washed out with 1.0 ml ice-cold saline and collected for amylase activity.

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I.G.2.4.2
Exocrine Pancreatic Secretion in Anesthetized Dogs

PURPOSE AND RATIONALE

To collect pancreatic secretion in dogs, three animal models have been developed: pancreatic fistulas, duodenal pouches (which collect the exocrine pancreatic secretion), and duodenal fistulas (through which a thin cannula is inserted into the pancreatic duct). With the exception of acute studies in anesthetized animals, pancreatic fistulas were used mainly during the first half of the 20th century and have rather historic significance. The latter two methods, duodenal pouches and duodenal fistulas, although originally developed in the 60s and 40s, respectively, are still in use today (Niebergall-Roth et al. 1997).

The duct system of the canine pancreas is different from that of the human pancreas. In dogs, the main duct is the accessory pancreatic duct (Ductus pancreaticus minor). The pancreatic duct, which joins the bile duct (Ductus pancreaticus major) and forms the major duodenal papilla in man, is small and not always present in dogs.

Here, the pancreatic fistula technique in anesthetized dogs is described. The effect of exogenous hormones, e.g. secretin or gastrin, or of vagal stimulation, on exocrine pancreatic secretion can be measured in anesthetized dogs with acute pancreatic fistulas.

PROCEDURE

Beagle dogs of either sex weighing 12–20 kg are used. The animals are fasted for a 24-hours period and then anesthetized with 25 mg/kg sodium pentobarbital i.v. After opening the abdomen along the mid-line, the pyloric sphincter is ligated and the common bile duct cannulated to prevent the entry of acid chyme and bile into the duodenum. The bile is allowed to drain. The pancreas is gently exposed and the major pancreatic duct ligated. A polyethylene tube of 2 mm diameter is inserted into the minor pancreatic duct for collection of the pancreatic juice. The left femoral vein is cannulated for continuous infusion or i.v. injection. The pancreatic juice is collected in an ice-bath in a special tapered tube with fine calibrations for measuring volumes of less than 1 ml.

At the end of each collection period, the volume is recorded, and the bicarbonate content determined titrimetrically. Furthermore, pancreatic enzymes, such as amylase, are determined in the samples. Determination of protein concentrations in the pancreatic juice can be used as end-point since the total protein concentration is proportional to the individual enzymes (Keller et al. 1958). In a pre-test period of 10 min, samples are collected every 2 min. Then, the test compound is injected intravenously and the pancreatic juice is collected every 2 min.

At the end of the animal experiment the dog is euthanized by an overdose of barbiturate.

EVALUATION

The secretion after injection of the test compound is compared with the pre-test values. Secretin increases pancreatic volume and bicarbonate secretion in a dose-dependent manner and can be ideally used as reference secretagogue.

MODIFICATIONS OF THE METHOD

Glad et al. (1996) tested the influence of gastrin-releasing peptide on acid-induced secretin release and pancreatobiliary and duodenal bicarbonate secretion in Danish country strain pigs weighing between 22 and 30 kg. The animals, starved overnight with free

access to water, were premedicated with 4 mg/kg i.m azaperone, and with 5 mg/kg i.p. metomidate. After 20 min a cannula was placed in an ear vein, and 5–10 mg/kg metomidate was given i.v., followed by intubation and artificial respiration with 50 % O_2 and 50 % N_2O. Anesthesia was maintained with an intravenous bolus infusion of 0.53 % chloralose.

Both external jugular veins were cannulated for infusion of saline or drugs. A femoral artery was cannulated for withdrawal of blood samples and recording of blood pressure. After laparotomy the cystic duct was ligated, and the common hepatic duct and the pancreatic duct were catheterized. The duodenal segment was defined as extending from the pylorus to the ligament of Treitz. A Foley catheter was passed through the pylorus into the proximal part of the duodenum and inflated. Distal to the pylorus the pancreaticoduodenal arteries, veins and nerves were dissected, and a double ligature was passed under these structures and tied around the duodenum. At the ligament of Treitz an inflated Foley catheter was placed in the distal part of the duodenum and tied with a suture around the duodenum. A catheter was placed through a splenic branch of the left gastroepiploic vein and advanced through the lienal vein to the portal vein.

The flow of pancreatic juice and bile was tested before and after the experiment by means of an intravenous bolus of 5 pmol/kg secretin. Before the experiment the duodenum was continuously perfused at a rate of 2 ml/min for 435 min with isotonic saline containing phenol red (10 mg/l) as a marker. After drug treatment (intravenous infusion of gastrin-releasing peptide or duodenal HCl perfusion) pancreatic and hepatic secretions were collected in 15-min periods and the volumes determined by weighing. Duodenal effluents were collected in 15-min periods and phenol red concentrations determined spectrophotometrically. Blood sampled were withdrawn for determination of secretin by radioimmunoassay.

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I.G.2.4.3
Exocrine Pancreatic Secretion in Conscious Dogs (Chronic Duodenal Pouches in Dogs)

PURPOSE AND RATIONALE

ICH-guideline S7 A (2001: Section 2) recommended the use of unanesthetized animals for the safety pharmacological assessment of candidate compounds. Duodenal pouches and duodenal fistulas (through which a thin cannula is inserted into the Ductus pancreaticus minor, which represents the main pancreatic duct in dogs), although originally developed in the 60 s and 40 s, respectively, are still in use today (Niebergall-Roth et al. 1997). Here, the duodenal pouch technique is described by using a Herrera-cannula. Herrera et al. (1968) described a special cannula, which can be used to obtain pancreatic juice or bile from a duodenal pouch after appropriate surgical procedures (Preshaw and Grossman 1965).

PROCEDURE

The principle technique is demonstrated in Fig. 2. Male Beagle dogs weighing 15–20 kg are used. The abdominal surgery is performed during appropriate anesthesia (in former times by e.g. 30 mg/kg pentobarbital sodium; nowadays more appropriate by inhalation with halothane or isoflurane). The abdomen is opened through a midline epigastric incision under barbiturate anesthesia. The duodenum is mobilized at the pyloric and jejunal ends, and a 5-cm duodenal segment containing the Ductus pancreaticus minor, which represents the main pancreatic duct in dogs is isolated. The proximal level of the duodenal section lies immediately distal to the opening of the common bile duct and the distal level of section lies 2.5 cm distal to the main pancreatic duct (Ductus pancreaticus minor). The duodenum integrity is restored by end-to-end duodeno-jejunostomy. The duodenal pouch is closed at the proximal end.

The cannula to be inserted is made of stainless steel and consists of 3 parts. The main casement measures 10.5 cm in length, with an external diameter of 1.0 cm. The internal end is flanged, and 1.5 cm from this point there is a short lateral limb, also 1.5 cm in length. The lateral limb is also flanged but, in addition, possesses a small V-shaped defect to facilitate insertion into the duodenal pouch.

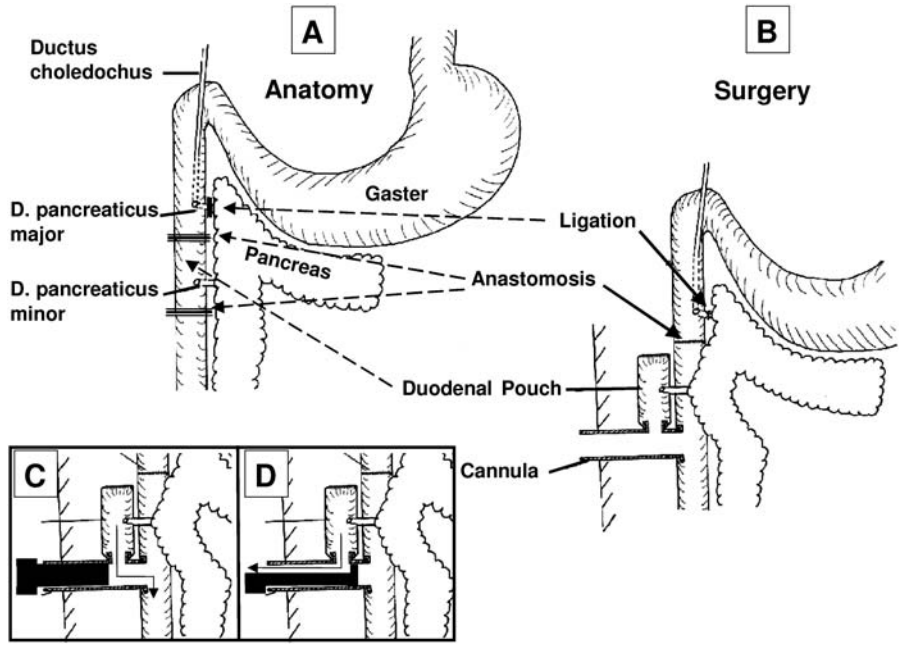

Fig. 2. Technique of chronic pancreatic fistula in dogs. Part A: shows the normal anatomic situation. Part B: diagram of the duodenal pouch preparation. Part C: demonstration of the normal flow of exocrine pancreatic juice during non-assay condition. Part D: demonstration of the flow of exocrine pancreatic juice during study condition

When not in use, the cannula is sealed from the exterior by inserting a threaded plug which allows pancreatic juice to enter the duodenum in the normal manner (Fig. 2, part C). For collection of juice this plug is removed and a long obturator is inserted (Fig. 2, part D). The latter effectively isolates the pancreatic secretion from other duodenal contents.

The lateral limb of the cannula is inserted in the distal end of the isolated duodenal segment in which the main pancreatic duct opens. The remaining limb of the cannula inserted through a small duodenotomy and secured further by a pursstring suture. The whole system is then generously wrapped in omentum and the cannula exteriorized through a stab incision toward the flank. An external collar stabilizes the cannula.

The cannula is left open to drain blood and secretions for 24 hours postoperatively, after which time the plug is inserted. Physiologic saline is administered subcutaneously for a period of 3 days, after which the animals are permitted to drink water. Checking the cannula daily is advisable to ensure that the plug remains tight. The animals receive normal kennel food and water ad libitum.

The dogs are allowed at least 2 weeks to recover. Eighteen hours prior to the experiment food is withdrawn but water allowed ad libitum. The long obturator is inserted (Fig. 2, part D) and pancreatic juice collected for 15 min periods. After 1 hour pre-test time, the candidate compound is given either orally or intravenously.

EVALUATION

Secretion of pancreas juice is measured at 15 min intervals and volume and enzyme content determined. The values are compared with pre-test data.

MODIFICATIONS OF THE METHOD

Boldyreff (1925) described details of the technique as recommended by Pavlov (1902) as well as his own modification.

Konturek et al. (1976, 1984) performed experiments with chronic gastric fistulas in cats as well as in dogs to compare the species-specific activities of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion.

Ninomiya et al. (1998) studied the effects of a cholecystokinin A receptor antagonist on pancreatic exocrine secretion stimulated by exogenously administered CCK-8 in conscious dogs with chronic pancreatic fistula.

Garvin et al. (1993) described distal pancreatectomy with autotransplantation and pancreatico-cystostomy in dogs.

Kuruda et al. (1995) developed a new technique in dogs for pancreatico-gastrointestinal anastomosis that

consists of pancreatectomy using the ultrasonic dissector and implantation of the pancreatic duct into the gastrointestinal tract without suturing the pancreatic parenchyma.

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I.G.2.5 Gastrointestinal Injury Potential

I.G.2.5.1 Gastrointestinal Injury in Rats

PURPOSE AND RATIONALE

The gastrointestinal injury potential of a candidate compound is studied in rats after oral administration. Every positive finding represents a serious safety issue for a candidate compound.

Non-Steroidal Anti-Inflammatory Agents (NASID), like indomethacin and acetyl-salicylic acid (aspirin), induce gastric lesions in man and in experimental animals by inhibition of gastric cyclooxygenase (COX) resulting in less formation of prostacyclin, the predominant prostanoid produced in the gastric mucosa.

PROCEDURE

After a 24 h starvation period groups of 8–10 rats weighing 150–200 g are used. The candidate compound is administered orally in 0.1 % Tween 80 solution. Six hours later, the rats are sacrificed in CO_2 anesthesia and their stomachs and intestines are removed. The stomach and gastrointestinal tract are removed. The mucosa is examined with a stereomicroscope and examined and assessed in relation to an ulceration index. The number of ulcers is noted and the severity recorded with the following scores:

0 = no ulcer
1 = superficial ulcers
2 = deep ulcers
3 = perforation.

E.g. Indomethacin 20 mg/kg orally or aspirin 50-100 mg/kg can be used as standard for the induction of ulcers.

EVALUATION

An ulcer index U_I is calculated:

$U_I = U_N + U_S + U_P \times 10^{-1}$

U_N = average of number of ulcers per animal
U_S = average of severity score
U_P = percentage of animals with ulcers

Ulcer index of treated animals are compared with controls. Using various doses, dose-response curves can be established for ulcer formation and gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0 % corresponds to no and 100 % to maximal stimulated gastric acid output.

Instead of an ulcer index the area of injured gastrointestinal mucosa can be measured by the following procedure. After removal of the stomach formol-saline (2 % v/v) is then injected into the totally ligated stomachs for storage overnight. The next day, the stomachs are opened along the greater curvature, then washed in warm water, and examined under a 3-fold magnifier. The lengths of the longest diameters of the lesions are measured and summated to give a total lesion score (mm) for each animal and the total injured area can be estimated, the mean count for each group being calculated.

MODIFICATION OF THE METHOD

Kitajima et al. (1993) studied the role of endothelin and platelet-activating factor in indomethacin-induced gastric mucosal injury in rats. Four hours after subcutaneous injection of 25 mg/kg indomethacin, the rats were sacrificed after ether anesthesia, and the stomach was removed. The stomach was filled with 1.5 ml of 2 % buffered formalin for 10 min and then opened along the greater curvature. The total length of the lesions was measured.

Dose- and time dependency of the ulcerogenic action of indomethacin were studied by Djahanguiri (1969).

Instead of indomethacin, gastric lesions are induced by intravenous or oral doses of aspirin which can be prevented by exogenous PGE_2 or PGI_2 (Konturek et al. 1981). Furthermore, reserpine at a dose of 8 mg/kg i.p., or cysteamine hydrochloride at a dose of 400 mg/kg s.c. induce ulcers in rats (Tarutani et al. 1985).

Wallace et al. (1989) studied the ulcerogenic activity of endothelin in indomethacin pre-treated rats using an ex vivo gastric chamber.

Scarpignato et al. (1995) evaluated NSAID-induced gastric mucosal damage by continuous measurement and recording gastric potential difference in the rat.

CRITICAL ASSESSEMENT OF THE METHOD

Absorptive and secretory changes of the gastrointestinal mucosa and microscopic damage (tight junction / brush borders) are not examined by this method.

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I.G.2.5.2
Gastric Ulcer in Pylorus Ligated Rats (SHAY Rat)

PURPOSE AND RATIONALE

A simple and reliable method for production of gastric ulceration in the rat based on ligature of the pylorus has been published by Shay et al. (1945). The ulceration is caused by accumulation of acidic gastric juice in the stomach.

PROCEDURE

Rats weighing 150–170 g are starved for 48 hours having access to drinking water ad libitum. During this time they are housed single in cages with raised bottoms of wide wire mesh in order to avoid cannibalism and coprophagy. Ten animals are used per dose and as controls. Under ether anesthesia a midline abdominal incision is made. The pylorus is ligated, care being exercised that neither damage to the blood supply nor traction on the pylorus occurs. Grasping the stomach with instruments is to be meticulously avoided, else ulceration will invariably develop at such points. The abdominal wall is closed by sutures. The test compounds are given either orally by gavage or injected subcutaneously.

The animals are placed for 19 hours in plastic cylinders with an inner diameter of 45 mm being closed on both ends by wire mesh. Afterwards, the animals are sacrificed in CO_2 anesthesia. The abdomen is opened and a ligature is placed around the oesophagus close to the diaphragm. The stomach is removed, and the contents are drained in a centrifuge tube. Along the greater curvature the stomach is opened and pinned on a cork plate. The mucosa is examined with a stereomicroscope. In the rat, the upper two fifths of the stomach form the rumen with squamous epithelium and possess little protective mechanisms against the corrosive action of gastric juice. Below a limiting ridge, in the glandular portion of the stomach, the protective mechanisms are better in the mucosa of the medium two fifths of the stomach than in the lowest part, forming the antrum. Therefore, lesions occur mainly in the rumen and in the antrum. The number of ulcers is noted and the severity recorded with the following scores:

0 = no ulcer
1 = superficial ulcers
2 = deep ulcers
3 = perforation.

The volume of the gastric content is measured. After centrifugation, acidity is determined by titration with 100 mmol/l NaOH.

EVALUATION

An ulcer index U_I is calculated:

$U_I = U_N + U_S + U_P \times 10^{-1}$

U_N = average of number of ulcers per animal

U_S = average of severity score

U_P = percentage of animals with ulcers

Ulcer index and acidity of the gastric content of treated animals are compared with controls. Using various doses, dose-response curves can be established for ulcer formation and gastric acid secretion. ID_{50} values can be calculated by probit analysis, whereby 0 % corresponds to no and 100 % to maximal stimulated gastric acid output.

CRITICAL ASSESSEMENT OF THE METHOD

The "Shay-rat" has been proven to be a valuable tool to evaluate the ulcerogenic or anti-ulcerogenic potential of a candidate compound independent of its mechanisms of action. However, due to the prolonged distress to the animals the use of this method should be ethically balanced very carefully against the expected added value of the study outcome.

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I.G.2.6 Gut Motility

I.G.2.6.1 Ileal Contraction in vitro

I.G.2.6.1.1 Isolated Ileum (MAGNUS Technique)

PURPOSE AND RATIONALE

During the profiling process of specific and safety pharmacological characterization of a candidate compound in the target-oriented research process the side effect potential is first estimated from the binding characteristics to a receptor panel in vitro. If there is a binding to a respective receptor identified in the low μmolar concentration range, which is not associated to the primary mode of action of the drug candidate, there is always the question how relevant is this finding with respect to an undesired side effect potential by receptor agonism or antagonism with respect to the receptor-mediated pharmacological effect. As a first estimate simple methods are preferred to investigate putative receptor mediated effects. A wide variety of different receptors appear in the gut their activation (using the drug candidate itself) or inhibition (using the respective receptor agonist together with the drug candidate) can be studied by isolated gut preparations.

The isolated ileum, as first described by Magnus (1904), is probably the most widely used model in experimental pharmacology. Magnus already studied simultaneously the spontaneous contractions of the longitudinal and circular musculature and the inhibiting effect of atropine. The method has been used for many purposes, such as the study on the effects of adrenaline on the lower segments causing contraction and on the segments of the upper end causing relaxation by Munro (1951) or the study on the origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips by Paton and Zar (1968) either retaining or being denervated from Auerbach's plexus. The model is used as a basic screening procedure for spasmolytic activity, whereby an anti-acetylcholine or anti-carbachol effect indicates anti-muscarinic activity and an anti-$BaCl_2$-effect indicates a musculotropic, papaverine-like effect. In addition to the isolated ileum, other parts of the gut such as the isolated duodenum and colon, have been used widely.

PROCEDURE

Guinea pigs of either sex weighing 300 to 500 g are used. They are sacrificed by stunning and exsanguination. The abdomen is opened with scissors. Just distal to the pylorus, a cord is tied around the intestine, which is then severed above the cord. The intestine is gradually removed, with the mesentery being cut away as necessary. When the colon is reached, the intestine is cut. Below the cord, the intestine is cut halfway through, so that a glass tube can be inserted. Tyrode's solution is passed through the tube and the intestine until the effluent is clear. Mesentery is cut away from the intestine that was joined to the colon. Pieces of 2–3 cm length are cut. Preferable the most distal piece is used being the most sensitive one. This piece is fixed with a tissue clamp and brought into

a 15 ml organ bath containing Tyrode's solution at 37 °C being oxygenated with 95 % O_2/5 % CO_2. The other end is fixed to an isometric force transducer (UC 2 Gould-Statham, Oxnard USA). A preload of 1 g is chosen. Responses are recorded on a polygraph. After a pre-incubation time of 30 min, the experiment is started.

The following agonists and antagonists (standards) are used (concentrations in g/ml bath fluid):

Table 2 Overview of genotoxicity assays

Agonist	Antagonist
Acetylcholine 10^{-7}g/ml	Atropine 10^{-8}–10^{-9} g/ml Scopolamine 10^{-8}–10^{-9} g/ml
Carbachol 10^{-7} g/ml	Atropine 10^{-8}–10^{-9} g/ml
Histamine 10^{-6} g/ml	Histamine antagonists, e.g. cimetidine, ranitidine, famotidine
$BaCl_2$ 10^{-4} g/ml	Papaverine 10^{-5}–10^{-6} g/ml
Serotonin 10^{-6} g/ml	Serotonin antagonists
PGE_2 2×10^{-7} g/ml	PG-antagonists

EVALUATION

Several methods for the quantitative evaluation of an antagonistic effect are available. One approach is the determination of pD_2 values according to van Rossum and van den Brink (1963). Acetylcholine or histamine is added in 1/2 $\log_{10}$ concentration increments until a maximum response is obtained. Control curves are recorded at 30 min intervals. After uniform control responses are obtained, the potential antagonist or the standard is added 5 min before the concentration-response curve is reobtained. The potency of the antagonist is obtained by calculating the pD_2 value which is defined as the negative logarithm of the molar concentration of an antagonist that causes a 50 % reduction or the maximal response obtained with an agonist.

MODIFICATIONS OF THE METHOD

Many modifications of the Magnus technique have been described in the literature, mainly with the isolated ileum (e.g., Koelle et al. 1950).

Okwuasaba and Cook (1980) dissected the myenteric plexus and longitudinal muscle free of the underlying circular muscle according to the method of Paton (1957), Paton and Zar (1968) and stimulated the preparation with trains of supramaximal rectangular pulses of 1.0 ms duration at a frequency of 0.2 Hz.

Kilbinger et al. (1995) studied the influence of 5-HT_4 receptors on [^{3}H]-acetylcholine release from guinea pig myenteric plexus.

De Graaf et al. (1983) described a fully automated system for in vitro experiments with isolated tissues. The apparatus consists of an organ bath equipped with (a) a gradient pump supplying a logarithmic concentration/time gradient of agonist; (b) pumps and valves for dispensing bath fluid, antagonist solutions, and an oxygenation gas mixture; and (c) a transducer with automatic baseline adjustment. The information coming from the preparation is fed into a mini-computer. The data of various experiments can be accumulated and Schild-plots obtained.

Furukuwa et al. (1980) studied the effects of thyrotropin-releasing hormone on the isolated small intestine and taenia coli of the guinea pig.

Paiva et al. (1988) studied the role of sodium ions in angiotensin tachyphylaxis in the guinea-pig ileum and taenia coli.

Barnette et al. (1990) used electrically stimulated strips of circular smooth muscle from the lower oesophageal sphincter of dogs to study the inhibition of neuronally induced relaxation by opioid peptides.

Griesbacher and Lembeck (1992) used the isolated guinea-pig ileum for analysis of bradykinin antagonists.

Hew et al. (1990) used field stimulated (95 % of maximum voltage, 0.1 Hz, 0.5 ms) guinea pig ileum, bathed in physiological salt solution at 37 °C in the presence of 1 mm mepyramine for determination of histamine-H_3 bioresponse. Reduction of contractile response by the test substance (>50 % relative to control 0.3 mm R-a-methylhistamine) indicates possible histamine-H_3 agonism. At a test concentration where no significant activity is seen, ability to inhibit (>50 %) R-α-methylhistamine-induced contractile reduction indicates antagonistic activity.

Feniuk et al. (1993) used the guinea-pig isolated ileum, vas deferens and right atrium to characterize somatostatin receptors. Transmural electrical stimulation was applied to guinea pig ileum (0.1 Hz, 0.1 ms continuously) and vas deferens (5 Hz, 0.5 ms for 1.5 s every 30 s) at supramaximal currents (approximately 800 mA) delivered from a Digitimer D330 multistimulator.

Radimirow et al. (1994) investigated opioid effects of short enkephalin fragments containing the Gly-Phe sequence on contractile responses of guinea pig ileum after addition of 10 nM acetylcholine or after electrical stimulation.

Coupar and Liu (1996) described a simple method for measuring the effects of drugs on intestinal longitudinal and circular muscle in rats. The preparation consists of a segment of rat ileum set up to measure the tension developed in the longitudinal

muscle and intraluminal pressure developed in the circular muscle in response to transmural electrical stimulation.

Vassilev et al. (1993) exposed Wistar rats to subtoxic doses of Co^{2+} or Ni^{2+}, receiving $Co(NO_3)_2$ or $NiSO_4$ with drinking water for 30 days, and measured the changes in the contractile responses to carbachol and in the inhibitory effects of verapamil and nitrendipine on isolated smooth muscle preparations of the ileum and the trachea.

Pencheva and Radomirov (1993), Pencheva et al. (1999) studied the effects of GABA receptor agonists on the spontaneous activity of the circular layer in the terminal ileum of cats. Segments of the terminal ileum approximately 0.5 cm long were mounted in an organ bath along the axis of the circular layer through a cotton thread with a large knot situated at the inner part of the gut wall.

Similar preparations of cat ileum were used by Kortezova et al. (1994) and Chernaeva and Mizhorkova (1995).

Vassilev and Radomirov (1992) used an isolated preparation of rat rectum. The rectal region, 1–6 cm proximal to the anal sphincter was removed and a 20 mm long segment suspended in an organ bath. The influence of prostaglandins and antagonists on spontaneous mechanical activity and electrically stimulated responses was investigated.

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I.G.2.6.2
Transit Time in vivo (Gut Motility) and Intestinal Secretion

I.G.2.6.2.1
Propulsive Gut Motility in Mice or Rats

PURPOSE AND RATIONALE

To study the side effect potential of a candidate compound on gastrointestinal motility the passage of a charcoal meal through the gastrointestinal tract in mice or rats is a simple, reliable and widely used method of safety pharmacologists.

PROCEDURE

Groups of 10 mice weighing 15 g are fed an oat diet for 3 days. Eighteen hours prior to the experiment food, but not water, is withdrawn. The animals are treated either subcutaneously 15 min or orally 60 min before administration of the charcoal meal (0.2 ml of a 4 % suspension of charcoal in 2 % carboxymethylcellulose solution). The mice are sacrificed after various time intervals, 20 min, 40 min, 60 min and 120 min. Ten animals serve as controls for each time interval. The entire intestine is immediately removed and immersed in 5 % formalin to halt peristalsis; then washed in running water. The distance the meal has travelled through the intestine as indicated by the charcoal is measured and expressed as percent of the total distance from the pylorus to the caecum.

EVALUATION

The values for the treated groups with the candidate compound for each time point are compared to those for the vehicle control group by using appropriate statistical methods. By using several doses of the candidate compound a dose-response curve can be established.

CRITICAL ASSESSEMENT OF THE METHOD

The charcoal passage test can be used for evaluation of laxative activity as well as for inhibition of intestinal motility.

MODIFICATIONS OF THE METHOD

Instead of charcoal, unsubstituted Hostapermblau (CuPcB) suspended in gummi arabicum mucilage can be used.

Carmine red (15) suspended in a 1 % tragacanth solution was used for measurement of small intestine transit in rats (Leng-Peschlow 1986).

Miller et al. (1981) measured the intestinal transit in the rat by the use of radiochromium (^{51}Cr). Female Sprague Dawley rats weighing approximately 200 g were implanted with indwelling silastic cannulas in the proximal duodenum. Following a 3 day recovery period, the animals were fasted for 18 hours and then treated with the test compounds. Thirty min later, 0.2 ml of radiochromium (0.5 mCi $Na^{51}CrO_4$) was instilled into the small intestine via the indwelling silastic cannula. Twenty-five min after chromium instillation, the animals were sacrificed. The small intestine was carefully removed and divided into 10 equal segments. The radioactivity was determined with an automatic gamma counting system. The effect of drugs could be quantified by determining the geometric center of the distribution of chromium through the small intestine.

Shook et al. (1989) used radiolabeled chromium to measure gastrointestinal transit in mice.

Megens et al. (1989) used the charcoal test to study the in vivo dissociation between the anti-propulsive and anti-diarrheal properties of opioids in rats.

Lish and Peters (1957) recommended an intestinal anti-propulsive test in intact insulin-treated rats providing certain advantages over the commonly used charcoal meal test for screening of synthetic antispasmodic and anti-propulsive agents.

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I.G.2.6.2.2
Stomach Emptying in Rats

PURPOSE AND RATIONALE

Reynell and Spray (1956) described a method for the simultaneous measurements of gastric emptying and intestinal transit of test substances in the rat using phenol red as marker. This simple method can also be ideally used for safety pharmacological assessment of candidate compounds on their side effect potential on gastrointestinal motility.

PROCEDURE

Rats weighing 200–300 g are starved for 24 hours, with free access to water before the experiment. They are treated orally or subcutaneously with the test compound 15 min prior to oral administration by gavage of 1.5 ml 0.07 % phenol red in 2 % carboxymethylcellulose solution. 15 min later the animal is sacrificed and the stomach is immediately removed. The whole stomach including the stomach content is alkalized with 1 N NaOH and homogenized. The homogenate is filtered, and after precipitation of the protein with 10 % trichloroacetic acid, centrifuged for 15 min at 3000 rpm. The concentration of phenol red in the supernatant is measured colorimetrically in a photometer at 546 nm.

EVALUATION

Percentage of stomach emptying (Se) is calculated according to the following formula:

$S_e = 100–(P_s \times P_a^{-1} \times 100)$

P_s = Concentration of phenol red in the stomach (μg/ml)

P_a = Concentration of phenol red in the initial solution after addition of equal volumes of 1 N NaOH and trichloroacetic acid (μg/ml)

MODIFICATIONS OF THE METHOD

Droppleman et al. (1980) described a simplified method for assessing drug effects on gastric emptying in rats. Three ml of a semi-solid test meal, based on methylcellulose, are given to rats fasted 24 hours prior to the experiment. At a specified time following the test meal, the rats are sacrificed, laparotomized, and the stomachs removed. The full stomachs are weighed on an analytical balance; they are opened and rinsed. Excess moisture is removed and the empty stomach weighed again. The difference is subtracted from the weight of 3 ml of the test meal, indicating the quantity emptied from the stomach during the test period. Gastric motor stimulants, e.g., metoclopramide increase, and anticholinergic compounds decrease gastric emptying.

Megens et al. (1990) used phenol red as marker to measure gastrointestinal propulsion after castor oil or paraffin oil challenge in rats.

Hedge et al. (1995) studied 5-HT_4 receptor mediated stimulation of gastric emptying in rats using a specially prepared semi-solid test meal containing charcoal.

Bonnafous et al. (1995) investigated benzodiazepine-withdrawal-induced gastric emptying disturbances in rats. Rats, weighing 200–250 g, fasted for 16 hours, received by gavage 2 ml of a test meal containing 1 μCi/ml of ^{51}Cr sodium chromate, 15 min after drug administration. 30 min later, the animals were sacrificed by cervical dislocation. The stomach, small intestine (10 segments) and the colon were excised and placed into tubes. Radioactivity was determined by placing the tubes in a gamma counter. Gastric emptying was calculated as the percentage of total counts found in the small intestine and the colon.

Varga et al. (1995) determined gastric emptying in rats 5 min after a 3 ml intragastric load of 0.9 % NaCl using phenol red as marker in order to define which bombesin receptors are involved in the delay of gastric emptying by bombesin-like peptides.

Lasheras et al. (1996) studied gastric emptying in rats. 60 min after oral administration of vehicle or test compounds, the rats received by gavage 40 steel spheroids (1 mm diameter) in 2 ml 3 % carboxymethylcellulose. 60 min later, the animals were sacrificed and the spheroids remaining inside the stomach counted.

Yegen et al. (1996) studied the inhibitory effects of gastrin releasing peptide on gastric emptying in rats using methyl cellulose and phenol red as non-absorbable marker.

Haga et al. (1994) studied gastric emptying in mice. Male mice, weighing 18–22 g, had free access to food and water before the experiment. The test compounds were administered orally in 10 ml/kg 0.5 % methylcellulose solution. The mice were deprived of food and water and sacrificed 4 hours later by cervical dislocation. The stomachs were removed and opened. The contents of the stomach were mixed with 10 % trichloroacetic acid, and centrifuged at 3000 rpm for 30 min. The weight of the sediment was taken as the food remaining in the stomach.

Ding and Hakanson (1996) examined the effect of drugs on a cholecystokinin-A receptor-mediated response by gastric emptying of a charcoal meal in mice.

Costall et al. (1987) used the to study the influence of a 5-HT_3 antagonist on gastric emptying.

Brighton et al. (1987) used scintigraphy following indium-111-labeled meals in Beagle dogs and baboons. Indium-111-labeled polystyrene beads (500 mCi per dog) were mixed into a meal consisting of 50 g of finely crushed commercial dog food and 50 ml of milk. Images of 1 min duration were taken every 5 min for a period of 1 hour using a large field of view gamma camera (ON Sigma 410).

Gullikson et al. (1991, 1993) studied gastric emptying of a solid meal in dogs.

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I.G.2.6.2.3
Enteropooling Test

PURPOSE AND RATIONALE

The enteropooling assay in rats has been developed by Robert et al. (1976) to test the diarrhoeic property of prostaglandins for prediction of this clinically relevant side effect of several synthetic prostaglandins. This method can also be used for the safety pharmacological assessment of candidate compounds on their side effect potential to induce diarrhoea.

PROCEDURE

Rats weighing 190–215 g are used. The animals are fasted overnight having free access to water. The candidate compound is administered orally, and the animals, 10–12 per group, are sacrificed one hour later. The fluid accumulation occurs in the small intestine which is cut at the pylorus and the ileocecal junction, and its contents, consisting of a thick fluid (in controls) and a very watery fluid (in prostaglandin-treated animals) are collected into a graduated test tube by milking the whole length of the small intestine with the fingers. The volume of fluid is recorded.

EVALUATION

Using various doses, dose-response curves can be established and potency ratios calculated. 16,16-dimethyl PGE_2 was found to be the most active compound and can be used as standard to estimate the diarrhoeic side effect potential of candidate compound.

CRITICAL ASSESSEMENT OF THE METHOD

Some other diarrhoeic agents, like $mgSO_4$, castor oil, bile, taurocholate and taurochenodesoxycholate cause enteropooling, whereas mineral oil and tragacanth are ineffective. The anticholinergic agent methylscopolamine partially counteracted the enteropooling. The assay, therefore, can be used to test the laxative or the antidiarrhoeal activity of drug candidates (Shook et al. 1989).

MODIFICATIONS OF THE METHOD

Beubler and Badhri (1990) used the PGE_2-induced net fluid secretion in the jejunum and colon in the rat to evaluate the antisecretory effects of antidiarrhoeal drugs. Polyethylene catheters were placed into the jejunum and colon and Tyrode solution was instilled into the loops. Net fluid transfer rates were determined gravimetrically 30 min after instillation of Tyrode solution.

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I.G.3 Carbohydrate and Lipid Metabolism

I.G.3.1 General Considerations

If a candidate compound with a totally different primary indication has additionally lipid lowering potential or causes an insulin sensitization, these findings may be assessed as additionally beneficial and therefore do not represent a safety concern, irrespective of whether these pharmacological effects occur in the pharmacological dose range for the primary pharmacological effect or at supra-pharmacological doses as usually used for safety pharmacological studies of a candidate compound. In contrast, if a candidate compound causes increases in plasma lipid parameters (free fatty acids, triglycerides, cholesterol) detailed analysis of the these side effects are highly recommended, because increased free fatty acids and triglycerides might induce insulin resistance and increased triglycerides and total cholesterol might be connected to an increased atherogenic risk.

The acute side effect potential on intermediary metabolism resulting in changes in blood glucose and lactate as well as in changes of free fatty acids can be investigated in rodents after single administration of candidate compounds, as these blood parameters physiologically vary quickly. However, changes of triglycerides and predominantly of cholesterol appear physiologically much slower. Therefore, the side effect potential of candidate compounds on these parameters are hardly to be detected in single dose studies as usually performed during initial safety pharmacological characterization. If there are additional hints, e.g. from toxicity studies, demonstrating putative side effects on triglyceride and cholesterol metabolism, additional multiple dose studies in safety pharmacology in appropriate animal models to detect an atherogenic potential are necessary to characterize the side effect potential of candidate compounds.

Due to the fact that nearly most of initial safety pharmacological studies are performed in small rodents (mainly rats and mice), one has to take carefully into consideration the differences in rat physiology compared to human physiology for characterizing the side effect potential of a candidate compound on intermediary metabolism. A normal rat is night active and takes up food predominantly during the night (8 to 10 meals per night), and to a smaller extent also during the day (3–4 meals per day). Blood glucose and liver glycogen do not vary during 24 hours (Gaertner 2001), resulting in a permanent prandial or postprandial state, but never in a postabsorptive state. In humans blood glucose and liver glycogen vary in dependence to meal intake during the day and decrease during the night to fasting values in the morning. In general, carbohydrate and fatty acid metabolism in laboratory animals are much more similar to that of humans compared to cholesterol metabolism. The functions of lipoprotein fractions (predominantly LDL and HDL) differ substantially between rodents and humans. Therefore, total cholesterol in blood is the most reliable marker in rats and mice, and the results from LDL- and HDLcholesterol in these rodents should be interpreted very carefully with respect to their role in atherosclerosis known from human pathophysiology. With respect to cholesterol metabolism the guinea pig seems to be more closely to the human cholesterol metabolism compared to mice and rats (Fernandez 2001).

Interpretation of changes in blood glucose and free fatty acids should only be performed with the additional knowledge of the corresponding serum insulin levels. This is not only important in single dose studies but also in long-term studies. Candidate compounds, which cause a slight increase in free fatty acids and triglyceride in multiple dose studies, may induce insulin resistance (normoglycaemia in the presence of hyperinsulinemia), which might provoke the progression to diabetes and obesity.

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I.G.3.2 Acute Effects on Metabolic Blood and Tissue Parameters

I.G.3.2.1 Acute Effects on Metabolic Blood and Tissue Parameters in Anesthetized Rats

PURPOSE AND RATIONALE

Anesthetized rats are used for testing the side effect potential of a candidate compound on intermediary metabolism in liver, muscle and adipose tissue with subsequent effects on metabolic blood parameters (e.g. glucose, lactate, free fatty acids, triglycerides) and insulin. The use of anesthetized rats represents more a principal assessment of the pharmacological side effect potential since the candidate compound must be administered intravenously or intraperitoneally (enteral/intestinal administration should be avoided due to the anesthesia-induced decrease in intestinal motility with subsequent impairment of enteral absorption), compared to the study in conscious rats in which the candidate compound can be studied after oral administration, which in most cases represents the clinical route of administration for small molecular drugs.

PROCEDURE

Metabolic blood parameters are assayed in anesthetized male rats using a modified method of glucose clamp studies in rodents (Terrettaz and Jeanrenaud 1983). Four to 6 rats per group (vehicle control and one dose of the candidate compound) are used. Rats are anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), tracheotomized, and one jugular vein per rat is cannulated for intravenous infusion the other vein is prepared for collection of blood samples. Anesthesia is maintained for up to 7 hours by subcutaneous infusion of pentobarbital sodium (adjusted to the anesthetic depth of the individual animal; about 24 mg/kg/h). Body temperature is monitored with a rectal probe thermometer, and temperature is maintained at 37 °C by means of a heated surgical table. Blood samples for glucose analysis (10 μl) are obtained from the tip of the tail every 15 minutes, and for lactate analysis (20 μl) every 30 minutes. The rats are allowed to stabilize their blood glucose levels after surgery for up to 2 hours. Then, the candidate compound is injected or infused intravenously or administered intraperitoneally, respectively. Blood samples (100 μl) for detection of free fatty acids, triglycerides and insulin are collected from a peripheral vein (e.g. jugular vein) immediately prior and every 1 or 2 hours after compound administration.

At the end of the experiment, the abdomen is opened, terminal blood collection is performed from the vena cava caud. or the aorta abdominalis for determination of metabolic blood parameters and insulin. A part of the liver is freeze clamped immediately as well as a part (about 1 gr) of skeletal muscle (e.g. M. gastrocnemicus). The frozen tissue is stored in liquid nitrogen for subsequent determinations of intrahepatic and intramuscular concentrations of glycogen, glucose-6-phosphate (G6P) and ATP. In liver samples hepatic triglycerides can be measured additionally. Standard enzymatic procedures were used to determine glucose, lactate, free fatty acids, triglycerides, glycogen, G6P and ATP (Bergmeyer 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA kit.

EVALUATION

The differences of the metabolic blood parameters after compound administration are calculated with respect to the pre-drug values and against a control group, which has received the vehicle only. The metabolic tissue parameters at the end of the study are assessed between control and treatment group.

Two parallel experiments in normal fed (high glucose, low free fatty acids, high triglycerides, high serum insulin) and overnight starved rats (fasting blood glucose, elevated free fatty acids, low serum insulin) as well as the complete data set of metabolic blood and tissue parameters and serum insulin levels are necessary to assess the acute putative side effect potential and its putative mechanism of the candidate compound on intermediary metabolism.

MODIFICATIONS OF THE METHOD

By using rats, which are starved longer than 16 hours before their use in the pharmacological experiment, fasting blood glucose is maintained exclusively by the process of gluconeogenesis, due to the very low glycogen content in the liver. Candidate compounds, which reduce blood glucose during this experimental setup, interfere either with the process of endogenous glucose production (hepatic and renal gluconeogenesis) either by direct interference with an enzymatic step of gluco-

neogenesis or by interference with the energy generation machinery (mitochondrial function, generation of ATP). Additional reasons of a blood glucose reduction during this experimental setup could be an insulin release from the pancreatic β-cell, or the interference with the insulin-signalling cascade in the insulin target tissues. Candidate compounds, which decrease free fatty acids, which are physiologically elevated during starvation, interfere with the process of lipolysis (antilipolytic activity) (Schoelch et al. 2004). Compounds, which increase free fatty acids (lipolytic activity), might have e.g. a stimulatory β-sympathetic potential.

It is recommended to perform a parallel experiment by using normal fed rats. Candidate compounds, which increase blood glucose, might inhibit insulin release or peripheral insulin action, or stimulate glycogenolysis. A putative lipolytic activity (increase in free fatty acids) is more pronounced during normal fed conditions, because free fatty acids are physiologically low due to elevated insulin levels. Fed rats can be also used for studying the effect of a candidate compound exclusively on the process of glycogenolysis. During this experimental setup, glycogenolysis is induced by an intravenous bolus injection of glucagon at a dose of 1 mg/rat. It can be assumed that the hyperglycaemia induced by the glucagon injection, and which lasts for about 90 to 120 minutes, is the result of the glucagon-induced breakdown of hepatic glycogen (Herling et al. 1998, 1999).

CRITICAL ASSESSEMENT OF THE METHOD

In general pharmacological studies during anesthesia should be assessed appropriately due to the possible interaction between the test compound and the used anesthetic as well as due to the reduced tone of the autonomic nervous system. Enteral administration of the candidate compound should be avoided, because enteral absorption of the test compound might be reduced due to the impaired intestinal motility during anesthesia. With respect to the effect of the aesthetic compound itself on intermediary metabolism the barbiturate pentobarbital sodium is the most inert anesthetic and does not cause alterations of metabolic blood and tissue parameters. In contrast, e.g. urethane as well as isoflurane (inhalation aesthetic) influences by itself substantially metabolic parameters over time (hours).

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I.G.3.2.2
Acute Effects on Metabolic Blood and Tissue Parameters in Conscious Rats

PURPOSE AND RATIONALE

Conscious rats are used for testing the side effect potential of a candidate compound on intermediary metabolism in liver, muscle and adipose tissue with subsequent effects on metabolic blood parameters (e.g. glucose, lactate, free fatty acids, triglycerides) and insulin after oral administration, which represents in most cases the clinical route of administration for small molecular drugs.

PROCEDURE

Rats weighing 180–240 g are kept on standard diet. Groups of 8 non-fasted animals are treated orally with various doses of the test compounds suspended in 0.4 % starch suspension. One control group receives the vehicle only. Blood is withdrawn from the tip of the tail immediately before, and 1, 2, 3, 5, and 24 hours after administration of the candidate compound. Blood glucose is determined in 10 µl blood samples collected from the tip of the tail. If pharmacokinetic data for the candidate compound are already available, when performing this test, additional blood samples (100 µl) should be taken at t_{max} by retro orbital bleeding for detection of free fatty acids, triglycerides and insulin.

At the end of the experiment, the rats are terminally anesthetized, the abdomen is opened, and terminal blood collection is performed from the vena cava caud. or the aorta abdominalis for determination of metabolic blood parameters and insulin. A part of the liver is freeze clamped immediately as well as a part (about 1 gr) of skeletal muscle (e.g. M. gastrocnemicus). The frozen tissue is stored in liquid nitrogen for subsequent determinations of intrahepatic and intramuscular concentrations of glycogen, glucose-6-phosphate (G6P) and ATP. In liver samples hepatic

triglycerides can be measured additionally. Standard enzymatic procedures were used to determine glucose, lactate, free fatty acids, triglycerides, glycogen, G6P and ATP (Bergmeyer 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA.

EVALUATION

The differences of the metabolic blood parameters after compound administration are calculated against a control group, which has received the vehicle only, for the respective time points. The metabolic tissue parameters at the end of the study are assessed between control and treatment group.

Two parallel experiments in normal fed (high glucose, low free fatty acids, high triglycerides, high serum insulin) and overnight starved rats (fasting blood glucose, elevated free fatty acids, low serum insulin) as well as the complete data set of metabolic blood and tissue parameters and serum insulin levels are necessary to assess the acute putative side effect potential and its putative mechanism of the candidate compound on intermediary metabolism.

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Geisen K (1988) Special pharmacology of the new sulfonylurea glimepiride. Arzneim Forsch/Drug Res 38:1120–1130

I.G.3.2.3
Blood Glucose Lowering Activity in Conscious Rabbits

PURPOSE AND RATIONALE

The rabbit has been used since many years for standardization of insulin. Therefore, it has been chosen as primary screening model for screening of blood glucose lowering compounds as well as for establishing time-response curves and relative activities (Bänder et al. 1969, Geisen 1988). For the safety pharmacological evaluation of candidate compounds with a different primary indication the rabbit is not the preferred animal species for first initial studies on metabolism pharmacology.

PROCEDURE

Groups of 4–5 mixed breed rabbits of either sex weighing 3.0–4.5 kg are used. For insulin evaluation, food is withheld overnight. For evaluation of sulfonylureas and other blood glucose lowering agents the animals are on a normal diet prior to the experiment. The animals are gently placed into special restraining boxes allowing free access to the rabbit's ears.

Oral blood glucose lowering substances are applied by gavage in 1 ml/kg of 0.4 % starch suspension or intravenously in solution. Several doses are given to different groups. One control group receives the vehicle only. By puncture of the ear veins, blood is withdrawn immediately before and 1, 2, 3, 4, 5, 24, 48, and 72 hours after treatment. For time-response curves values are also measured after 8, 12, 16, and 20 hours. Blood glucose is determined in 10 µl blood samples.

MODIFICATIONS OF THE METHOD

For special purposes the effect of blood glucose lowering agents is studied in glucose loaded animals. Rabbits of either sex weighing 3.0–4.5 kg are treated either once (0.5 h after test compound) or twice (0.5 and 2.5 hours after test compound) orally with 2 g glucose/kg body weight in 50 % solution.

EVALUATION

Average blood glucose values are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Mean effects at a time interval are calculated using the trapezoidal rule. The values of the experimental group are compared statistically with the t-test or the WILCOXON test for each time interval with those of the control group. Differences between several treated groups and the control group are tested using a simultaneous comparison according to Dunnett or Nemenyi/Dunnett (1966). Dose dependencies and relative activities are determined by means of linear regression analysis after Fieller (1944) and Sidak (1967). All data for statistical comparisons have to be tested for homogeneity of variances according to Levene (1960) and for normal distribution according to Shapiro and Wilk (Miller 1965). In the case of regression analyses, the lines are additionally tested for parallelism according to Tuckey (1966) and for linearity according to Scheffé (1959). The level of significance for all procedures is chosen as 5 %.

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I.G.3.2.4
Acute Effects on Metabolic Blood Parameters in Conscious Dogs

PURPOSE AND RATIONALE

Since most safety studies for safety pharmacological evaluation of candidate compounds are performed in rodents a second non-rodent species should be included to exclude any species differences in the safety pharmacological findings. Dogs are a preferred second animal species in metabolism pharmacology, because their night/day activity, their food intake behaviour and therefore their intermediary metabolism is obviously different to that of rodents and resembles more that of humans.

PROCEDURE

Male Beagle dogs weighing 15–20 kg are kept on standard diet. Food is withdrawn 18 hours prior to the administration of the candidate compound, which is given either orally, or intravenously in various doses. Control animals receive the vehicle only. Blood is collected at different time intervals up to 48 hours for determination of metabolic blood parameters (e.g. glucose, lactate, free fatty acids, triglycerides) and insulin. Standard enzymatic procedures were used to determine metabolic blood parameters (Bergmeyer 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA kit.

EVALUATION

Averages of the metabolic blood parameters (e.g. glucose, lactate) are plotted versus time for each dosage. Besides the original values, percentage data related to the value before the experiment are calculated. Similarly, plasma insulin levels are plotted versus time and compared with control values. Statistical evaluation is performed using appropriate methods.

MODIFICATIONS OF THE METHOD

Continuous blood glucose monitoring

A device for continuous blood glucose monitoring and infusion in freely mobile dogs was described by Geisen et al. (1981).

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I.G.3.3
Functional Tests

I.G.3.3.1
Oral Glucose Tolerance Test (oGTT) in Conscious Rats

Oral glucose tolerance test (oGTT) represents a simple method for estimating the effect of a candidate compound either to impair or to improve glucose tolerance. In general, impaired glucose tolerance often represents a condition of some degree of insulin resistance or impaired pancreatic insulin secretion during a glucose load. An impairment of insulin sensitivity (induction of insulin resistance) resulting in impaired glucose tolerance, which can be detected in normal animals (rarely in single dose studies, but more likely in multiple dose studies lasting for 1 or 2 weeks) represents a safety concern, since insulin resistance is associated with hyperinsulinemia and can provoke the progression to overt diabetes (type 2) and obesity. For the assessment of the glucose tolerance the additional determination of insulin is essential. As in rodents it is not possible to collect appropriate blood samples for insulin determinations at each time point of blood glucose measurements, one or two samples for corresponding insulin determinations (0.5 h or 1 h after glucose load and a the end of the study) represents a pragmatic compromise.

On the other hand normal animals are insulin sensitive and further improvement of insulin sensitivity cannot be expected. Therefore, if there are hints from

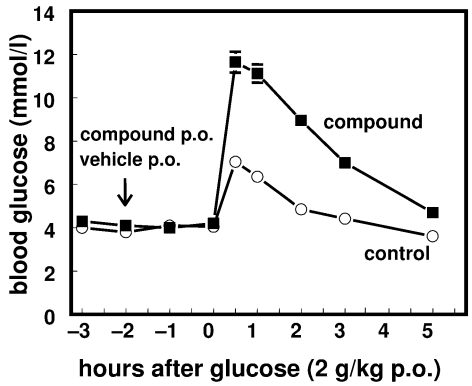

Fig. 3. Representative oral glucose tolerance test shown for a candidate compound, which impairs glucose tolerance. Candidate compound and vehicle are administered 2 hours before the oral glucose load. Values are mean +/– SEM, n = 8 rats

other studies for a candidate compound (reduction in free fatty acids, triglycerides and insulin), to have a putative insulin-sensitizing potential, which does not represent a safety concern, appropriate studies should be performed by using insulin resistant animal disease models (genetic models: e.g. Zucker Fatty (fa/fa) rat, Zucker Diabetic Fatty (ZDF, fa/fa) rat, diet-induced insulin resistant rats: e.g. high fat fed rats, high fructose-fed rats) (Vogel 2002)

PURPOSE AND RATIONALE

OGTT in conscious rats are used to assess the side effect potential of a candidate compound to impair glucose tolerance (Fig. 3).

PROCEDURE

Rats weighing 180–240 g are starved overnight. Groups of 8 animals are treated orally with various doses of the candidate compound suspended in 0.5 % HEC or Tylose. One control group receives the vehicle only. Based on the pharmacokinetic data (t_{max}), the candidate compound is administered appropriately before the glucose load (e.g. 0.5, 1 or 2 hours). Glucose is administered at a dose of 2 g/kg orally. Blood is withdrawn from the tip of the tail immediately before compound administration and immediately before glucose administration and 0.5, 1, 2, 3, 5 hours after administration of glucose. Blood glucose is determined in 10 µl blood samples collected from the tip of the tail. An additional blood sample (100 µl) should be taken at 1 h after glucose load by retro orbital bleeding for detection of insulin. Standard enzymatic procedures are used to determine blood glucose (Bergmeyer 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA.

EVALUATION

Mean values of blood glucose for each time point are calculated and time-course of control and treated groups are compared. Area under the blood glucose curve (AUC) can be calculated and compared for both groups; base-line values of blood glucose should be subtracted for the calculation of glucose AUC.

CRITICAL ASSESSEMENT OF THE METHOD

An impairment of glucose tolerance can be the result of peripheral insulin resistance, an impaired insulin secretion from the pancreatic β-cell, or both. Therefore, blood glucose levels as well as corresponding insulin levels are essential for the assessments of the glucose tolerance results. An improvement of glucose tolerance during an oGTT in normal animals can be caused by candidate compounds, which inhibit gastric emptying and thereby delay the glucose absorption from the gut.

MODIFICATION OF THE METHOD

Many authors used mice instead of rats (Gross et al. 1994, Bailey et al. 1997, Ahren et al. 2000). However, parallel insulin measurements are much more limited in mice than in rats. Also oGTT in insulin resistant animal disease models in mice (e.g. genetic: ob/ob, db/db, Ay) are described to assess peripheral insulin resistance (Xiao et al. 2001, Arakawa et al. 2001, Nagakura et al. 2003, Thorkildsen et al. 2003, Minoura et al. 2004). Other routes for glucose administration (e.g. intraperitoneal) are also reported (Xie et al. 2004).

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I.G.3.3.2
Euglycemic Hyperinsulinemic Glucose Clamp Technique in Anesthetized Rats

PURPOSE AND RATIONALE

The euglycemic glucose clamp technique represents the "gold standard" for measuring peripheral insulin sensitivity in humans and animals. This technique was first described in humans by DeFronzo et al. (1979). In this technique a variable glucose infusion is delivered to maintain euglycemia during insulin infusion. Whole-body tissue sensitivity to insulin, as determined by net glucose uptake, can be quantitated under conditions of near steady state glucose and insulin levels. Terrettaz J and Jeanrenaud B (1983) adapted this technique to anesthetized rats; Kraegen et al. (1983, 1985) developed the euglycemic glucose clamp technique for use in conscious rats

PROCEDURE

The study can be performed as described by Terrettaz J and Jeanrenaud B (1983). Briefly, overnight-fasted rats are anesthetized with pentobarbital (priming bolus 60 mg/kg i.p. plus infusion s.c. at about 20 mg/kg/h), their temperature is kept at 37.5 °C. Through left jugular and femoral vein catheters glucose and insulin are infused. Studies last 240 min. During a 120-min baseline period, blood glucose is determined every 15 min. Then, insulin is administered as a bolus (48 mU/kg/min) for 5 min, followed by a constant i.v. infusion at 4.8 mU/kg/min for further 115 min. Blood drawn from the tip of the tail is now measured for glucose every 5 min, and 15 % D-glucose infused to maintain euglycemia at about 5 mmol/l. At 120 and at 240 min, additional blood samples are drawn from the right jugular vein and placed in K-EDTA-tubes, which are immediately centrifuged at 6 °C at 5000 rpm followed by determination of plasma FFA within 60 minutes; plasma aliquots are frozen for insulin determinations.

To differentiate between peripheral (predominantly skeletal muscle) and hepatic insulin sensitivity tracer-techniques can be applied (e.g. U-^{13}C-glucose; Neumann-Haefelin et al. 2004). Additionally, the rats are given a constant infusion of U-^{13}C-glucose (1mg/kg/min) to estimate rates of glucose production and utilization (Michael et al. 2000). Every 15 min, blood samples are obtained via tail-tip bleeds for determination of glucose enrichment. Enrichments are calculated from the ratio of U-^{13}C-glucose/^{12}C-glucose during the last 30 min of the basal period and during the last 60 min of the clamp (i.e., steady state conditions). This ratio was determined by GC-MS analyses of derivatized glucose from blood samples following literature protocols (Beylot et al. 1993, Michael et al. 2000).

Blood samples were taken at 30 min for determination of baseline insulin levels and at 180, 210 and 240 min for determination of insulin levels under hyperinsulinemia. At 120 and at 240 min, additional blood samples for determination of fee fatty acids are taken. Animals were killed by pentobarbital overdose. Metabolic blood parameters are determined using standard methods (Bergmeyer 1974); insulin measurements are performed using commercially available radioimmunoassay or ELISA kits.

EVALUATION

Whole-body insulin sensitivity is calculated as the mean glucose infusion rate (GIR) during the last 60 min of the clamp study.

During steady state, the total glucose appearance in the circulation (R_a) equals the rate of disappearance of glucose (R_d), and is calculated by dividing the U-^{13}C-glucose infusion rate by the steady state value of glucose enrichment. Endogenous glucose production (EGP) is calculated as follows: EGP equals R_d minus glucose infused (GIR). For each animal, two values of EGP are obtained: one during basal conditions and one during the euglycemic-hyperinsulinemic clamp. The methods for quantification of endogenous glucose production in humans are entirely reviewed by Radziuk and Pye (2002).

MODIFICATIONS OF THE METHOD

Burnol et al. (1983) and Smith et al. (1987) used the euglycemic insulin clamp technique coupled with isotopic measurement of glucose turnover to quantify

insulin sensitivity in the anesthetized and conscious rats, respectively.

The effects of counterregulatory hormones on insulin-induced glucose utilization by individual tissues in rats, using the euglycemic hyperinsulinemic clamp technique combined with an injection of 2-[1-^{3}H]-deoxyglucose, were studied by Marfaing et al. (1991).

Lee et al. (1994) studied the metabolic effects of troglitazone on fructose-induced insulin resistance with the euglycemic hyperinsulinemic clamp technique in rats.

Hulman et al. (1993) studied insulin resistance in the conscious spontaneously hypertensive rat with the euglycemic hyperinsulinemic clamp technique.

Cheung and Bryer-Ash (1994) described a modified method for the performance of glucose insulin clamp studies in conscious rats under local anesthesia.

Xie et al. (1996) described an insulin sensitivity test using a modified euglycemic clamp in cats and rats. This test uses the amount of glucose required to be infused to maintain euglycemia over a 30-min period in rats and 60 min in cats following a bolus administration of insulin as the index of insulin sensitivity. Glucose levels are determined a short intervals and variable glucose infusion is used to hold glucose levels with-in a few percentage points of the basal pre-test glucose level. A new blood sampling technique is described that allows each insulin sensitivity test to be carried out using a total of only 0.5 ml of blood.

CRITICAL ASSESSEMENT OF THE METHOD

For the safety pharmacological assessment of candidate compounds to increase or reduce insulin resistance often long-term pre-treatment periods for 1 week or longer are necessary before an effect on insulin sensitivity can be detected. Candidate compounds causing an acute effect on lipolysis or antilipolysis of adipose tissue with subsequent changes in free fatty acids normally causes also a fast effect on peripheral insulin sensitivity, which can be measured after a relatively short (16h) pre-treatment period (Schoelch et al. 2004).

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I.G.3.4 Multiple Dose Studies

I.G.3.4.1 Effects on Metabolic Blood and Tissue Parameters in Conscious Rats (Multiple Dose Study)

PURPOSE AND RATIONALE

For studying the side effect potential of candidate compounds on blood triglycerides and cholesterol as well as on metabolic rate, insulin sensitivity and body weight development multiple dose studies for at least 1 week are necessary. For transcriptional active compounds like the PPAR agonist (peroxisome

proliferators-activated receptor) multiple dose studies lasting several weeks are necessary to detect the pharmacological activity. The fibrates (e.g. fenofibrate), known since decades as triglyceride reducing drugs, are agonist of PPARα; thiazolidinediones (or glitazones; e.g. rosiglitazone, pioglitazone) are ligands of PPARγ. These agonists exhibit their full pharmacodynamic profile after they have activated the transcription of whole gene families. Agonists of PPARα predominantly induce gene expression of enzymes involved in lipid catabolism, while agonists of PPARγ predominantly induce genes, which are involved in lipid anabolism (adipocytogenesis). PPARα agonists reduce blood lipids (triglyceride, free fatty acids) as well as tissue lipids in muscle and liver by inducing fatty acid oxidation in peroxisomes and mitochondria and thereby improving metabolic blood and tissue parameters. PPARγ agonists induce the permanent formation of new adipocytes in fat tissue and thereby maintain a permanent remodelling of the adipose tissue resulting in a redistribution of fatty acids from ectopic lipid accumulation in liver and muscle as well as reducing blood lipid parameters, however, at the expense of increased total fat mass and increased body weight. Their effects in normal rats are marginal, but impressive in animal disease models of insulin resistance, obesity and hypertriglyceridemia (e.g. Zucker Fatty (fa/fa) rats, Zucker Diabetic Fatty (ZDF-fa/fa) rats). Various animal models resembling type 2 diabetes are used for evaluation. Details of these models are described elsewhere (Vogel 2002).

At the end of such multiple dose studies the animals are killed in terminal anesthesia and maximal blood collection is possible. Therefore, not only the target metabolic parameters (e.g. glucose, lactate, free fatty acids, triglycerides, cholesterol) but also other parameters, which reflect intermediary metabolism (e.g. keton bodies, urea, uric acid) as well as safety parameters (e.g. ASAT, ALAT, AP, LDH) can be determined by clinical chemistry.

PROCEDURE

Eight rats per group (lean control, obese control, and dose groups) are treated with the candidate compound in various doses for up to 6 weeks. According to the pharmacokinetic profile ($t_{1/2}$) the candidate compound is administered once or twice a day by gavage. If the stability of the compound is sufficient the candidate compound can also be administered by food admixture. Food and water consumption as well as body weight are measured at least once a week. Blood glucose is determined in 10 μl blood samples collected from the tip of the tail and by retro orbital bleeding for detection of free fatty acids, triglycerides and insulin every 2 weeks.

At the end of the experiment, the rats are terminally anesthetized, the abdomen is opened, and terminal blood collection is performed from the vena cava caud. or the aorta abdominalis for determination of metabolic blood parameters and insulin. A part of the liver is freeze clamped immediately as well as a part (about 1gr) of skeletal muscle (e.g. M. gastrocnemicus). The frozen tissue is stored in liquid nitrogen for subsequent determinations of intrahepatic and intramuscular concentrations of glycogen, glucose-6-phosphate (G6P) and ATP. In liver samples hepatic triglycerides can be measured additionally. Standard enzymatic procedures were used to determine glucose, lactate, free fatty acids, triglycerides, glycogen, G6P and ATP (Bergmeyer, 1974); insulin is determined by using a commercially available radioimmunoassay or ELISA kits.

EVALUATION

The differences of the metabolic blood parameters of the treated groups are calculated against both control groups (lean and obese), which have received the vehicle only. The metabolic tissue parameters at the end of the study are assessed between the two control and treatment groups.

MODIFICATION OF THE METHOD

For the assessment of the side effect potential of the candidate compound on peripheral insulin sensitivity, multiple oGTTs can be performed during the treatment period or the animal study is finished by a hyperinsulinemic-euglycemic glucose clamp study.

Chang et al. (1983) studied ciglitazone in ob/ob and db/db mice, diabetic Chinese hamsters, and normal and streptozotocin-diabetic rats.

Fujita et al. (1983) investigated the effects of ciglitazone in obese-diabetic yellow KK (KK-Ay) mice and obese Zucker-fatty rats.

Diani et al. (1984) treated C5BL/6J-ob/ob and C57BL/KsJ-db/db mice for several weeks with ciglitazone and studied the morphological effects on pancreatic islets.

Fujiwara et al. (1988) performed studies in KK and ob/ob mice and Zucker fatty rats.

Moreover, Fujiwara et al. (1991) studied the effects of CS-045 on glycemic control and pancreatic islet structure at a late stage of the diabetes syndrome in C57BL/KsJ-db/db mice.

Ikeda et al. (1990), Sohda et al. (1990) used insulin resistant animals (yellow KK mice, Zucker fatty rats, and obese Beagle dogs with moderate insulin resistance).

Gill and Yen (1991) studied the effects on endogenous plasma islet amyloid polypeptide and insulin sensitivity in obese-diabetic viable yellow mice.

Hofmann et al. (1991, 1992) treated insulin resistant KKAy mice.

Stevenson et al. (1991) studied the effects of englitazone in nondiabetic rats and found no overt hypoglycaemia but an enhancement of insulin action.

Kobayashi et al. (1992) found an increase of insulin sensitivity by activating insulin receptor kinase in genetically obese Wistar fatty rats treated with various doses of pioglitazone.

Sugiyma et al. (1992) found a reduction of glucose intolerance and hypersecretion of insulin in Wistar fatty rats after treatment with pioglitazone for 10 days.

Tominaga et al. (1993) used the glucose clamp-technique in streptozotocin-induced diabetic rats.

Yoshioka et al. (1993) found antihypertensive effects in obese Zucker rats.

Lee et al. (1994) studied the metabolic effects on fructose-induced insulin resistance in rats.

Apweiler et al. (1995) administered BM 13.09143 to lean and obese Zucker rats and performed hyperinsulinemic-euglycemic clamp studies in these animals.

Fujiwara et al. (1995) found a suppression of hepatic gluconeogenesis in long-term troglitazone treated diabetic KK and C57BL/ksJ-db/db mice.

Lee and Olefsky (1995) studied the effects of troglitazone in normal rats with the euglycemic glucose clamp technique.

CRITICAL ASSESSEMENT OF THE METHOD

The results of metabolic tissue parameters in liver, and muscle must be interpreted carefully, when a hyperinsulinemic-euglycemic glucose clamp study is performed at the end of the treatment period. Under clamp conditions these tissue parameters are mainly influenced by the hyperinsulinemic condition during the clamp study than by the compound's effect itself.

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I.G.3.4.2 Cholesterol-Diet Induced Atherosclerosis in Rabbits and Other Species

The additional anti-atherosclerotic potential of a candidate compound with a different primary indication does not represent a safety concern. In contrast, the atherogenic potential of a candidate compound, identified during safety pharmacological evaluation, represents a serious safety issue. Development of atherosclerosis needs time and therefore multiple dose studies are necessary to detect a putative anti-atherosclerotic or atherogenic side effect potential of a candidate compound.

Experimental atherosclerosis was first successfully induced in rabbits by Saltykow (1908) and Ignatowski (1909). During the following years, various scientists found that dietary cholesterol was the responsible stimulus for development of atherosclerosis. Other species are also susceptible to diet-induced atherosclerosis (Reviews by Kritchevsky 1964; Hadjiinky et al. 1991). A unifying hypothesis of the pathogenesis of atherosclerosis has been proposed by Schwartz et al. (1991).

PURPOSE AND RATIONALE

Rabbits are known to be susceptible to hypercholesterolemia and arteriosclerosis after excessive cholesterol feeding (supplemented with 0.3–2 % cholesterol in the diet). Therefore, this approach has been chosen by many authors to study the effect of potential anti-atherosclerotic drugs. For studying the atherogenic potential of a candidate compound a low cholesterol concentration in the diet (0.1–0.3 %) is recommended (pro-atherogenic).

PROCEDURE

Several modifications of the protocol have been described. Usually, male rabbits from an inbred strain, e.g., white New Zealand, at an age of 8–10 weeks are used. Body weight variation should be as low as possible. At the beginning of the experiment, blood is withdrawn from the marginal ear vein for determination of total cholesterol, total triglycerides, and blood glucose. Groups of 10 animals are used for treatment with drugs or as controls. The rabbits are switched from commercial food to a diet supplemented with 0.3–2 % cholesterol and kept on this regimen for a period of 10–12 weeks. One group is kept on normal diet. During and at the end of the experiment blood is taken for analysis. Usually, cholesterol and triglyceride levels increase several-fold over the original values.

The animals are sacrificed and the thoracic aorta is removed, cleaned of surrounding tissues, and longitudinally cut and opened for fixation with formaldehyde. The tissue is stained with oil red. The percentage of the intimal surface covered by the oil red positive lesions is calculated with a computerized planimeter. In animals fed a normal diet, the aorta does not show any staining, whereas in cholesterol-fed rabbits the aorta shows severe atherogenic lesions.

EVALUATION

The areas of the aortic lesions are compared between control and treatment groups. Appropriate statistical evaluation is performed and percent inhibition (anti-atherogenic effect) or percent increase of areas with lesions (atherogenic effect) can be calculated.

MODIFICATIONS OF THE METHOD

Shore and Shore (1976) studied two different strains of rabbits (New Zealand White and Dutch Belt) as models of hyperlipoproteinemia and atherosclerosis.

Studies of Kritchevsky et al. (1989) on experimental atherosclerosis in rabbits fed cholesterol-free diets revealed a greater influence of animal protein and of partially hydrogenated soybean oil on development of atherosclerosis than plant protein and unsaturated soybean oil.

Cockerels (Tennent et al. 1960) and turkeys (Simpson and Harms 1969) are very susceptible to cholesterol feeding and develop marked hypercholesterolemia in rather short periods. Atherosclerosis could also be induced in cockerels by high doses of oestrogen without atherogenic diet (Caldwell and Suy-dam 1959).

Spontaneous arteriosclerosis in pigeons has been described by Clarkson and Lofland (1961).

The Japanese sea quail (Coturnix coturnix japonica) is highly susceptible to the rapid development of severe experimental atherosclerosis (Day et al. 1975, 1977, 1979, 1990; Chapman et al. 1976).

Out of 13 strains of mice, Roberts and Thompson (1976) selected the C57BR/cdJ and the CBA/J strain und used these strains and their hybrids as models for atherosclerosis research.

Paigen et al. (1987) described quantitative assessment of atherosclerotic lesions in mice. After 14 weeks on an atherogenic diet C57BL/6J female mice had aortic lesions at each of the coronary arteries, at the junction of the aorta to the heart and in scattered areas of the aortic surface. The lesions increased after

9 months of atherogenic diet. Methods of evaluating the number and size of lesions were compared including sizing with a microscope eyepiece grid and computer-assisted planimetry.

Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice were described by Paigen et al. (1990).

Yamaguchi et al. (1993) found that addition of 10 % linoleic acid to a high-cholesterol diet enhanced cholesterol deposition in the aorta of male ICR strain mice.

In rats hypercholesterolemia can be induced by daily administration by gavage of 1 ml/100 g body weight of a cocktail containing in 1 l peanut oil: 100 g cholesterol, 30 g propylthiouracil, and 100 g cholic acid over a period of 7 days. The test compounds are administered simultaneously with the cocktail (Fillios et al. 1956; Lustalot et al. 1961).

Inoue et al. (1990) induced experimental atherosclerosis in the rat carotid artery by balloon de-endothelialization and atherogenic diet. A balloon catheter was introduced into the rat's carotid arteries from the iliac arteries and the endothelium was denuded.

The hamster is susceptible to atherosclerosis. Nistor et al. (1987) fed male hamsters a hyperlipidemic diet consisting of standard chow supplemented with 3 % cholesterol and 15 % commercial butter for 12 months. Serum total cholesterol doubled after 3 weeks and attained a 17-fold value after 10 months. Up to 6 months, smooth muscle cells in the intima and media of the aorta as well as endothelial cells began to load with lipids. After 10 months the affected zones looked like human atherosclerotic plaque with huge cholesterol crystal deposits, calcium deposits and necrosis.

Especially the hybrid hamster strain Bio^{TM} F_1B (Bio Breeders Fitchburg, MA, USA) is more susceptible to dietary induced atherosclerosis than other strains (Kowala et al. 1991). Early atherosclerotic lesions can be induced within a 3-months-feeding of a cholesterol/butter-enriched diet. In these animals simvastin dose-dependently inhibited the development of hyperlipidemia and the plaque formation by cholesterol synthesis inhibition The histopathological examination of the aortas showed that the cholesterol/butter fed F_1B hamster developed atherosclerotic lesions and functional changes in the aorta which are closely related to man (Schäfer et al. 1999).

Soret et al. (1976) studied the diet-induced hypercholesterolemia in the diabetic and non-diabetic Chinese hamster.

Beitz and Mest (1991) used cholesterol-fed guinea pigs to study the antihyperlipemic effects of a potentially anti-atherosclerotic drug.

Malinow et al. (1976) recommended the cynomolgus monkey as a model for therapeutic intervention on established coronary atherosclerosis.

This species was used by Hollander et al. (1978) to study the development atherosclerosis after a cholesterol and fat enriched diet.

Beere et al. (1992) described experimental atherosclerosis at the carotid bifurcation of the cynomolgus monkey by a cholesterol-enriched diet.

Eggen et al. (1991) studied the progression and the regression of diet-induced atherosclerotic lesions in aorta and coronary arteries on rhesus monkeys.

Howard (1976) recommended the baboon as model in atherosclerosis research because of the similarity of cholesterol metabolism and composition of the lipoproteins to man.

Kushwaha et al. (1991) determined the effect of oestrogen and progesterone on plasma cholesterol concentrations and on arterial lesions in ovarectomized and hysterectomized baboons fed a high-cholesterol/high-saturated-fat diet.

Blaton and Peeters (1976) reported studies on the chimpanzee, the baboon and the rhesus macacus as models for atherosclerosis.

Ming-Peng et al. (1990) studied high density lipoproteins and prevention of experimental atherosclerosis in tree shrews (Tupaia belangera yunalis). In contrast to rabbits, no increased lipid deposition in aortic intima after cholesterol feeding was found in tree shrews.

CRITICAL ASSESSEMENT OF THE METHOD

Diet-induced hypercholesterolemia is useful only for detection of agents interfering with the adsorption, degradation and excretion of cholesterol. Agents interfering with cholesterol biosynthesis are less probable to be detected.

The use of normal adult marmosets, a species with a lipoprotein profile similar to that of man, may be an alternative (Crook et al. 1990, Baxter et al. 1992).

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I.G.3.5 Acute Effect on Food Consumption

The additional anorectic effect of a candidate compound with a different primary indication does not represent a safety concern. In contrast, an orexigenic potential of a candidate compound, identified during safety pharmacological evaluation, represents a serious safety issue. Compound-mediated increased food consumption (orexigenic effect) might lead to obesity, insulin resistance and to overt type 2 diabetes. Food and milk consumption can be measured in acute experiments in mice and rats as well as in multiple dose studies over weeks in parallel to the measurement of body weight development and changes in body composition.

I.G.3.5.1 Acute Effect on Milk Consumption in Mice

PURPOSE AND RATIONALE

Milk intake is measured in acute experiments in normal or obese mice after an overnight fast. Milk intake serves as a surrogate for food consumption.

PROCEDURE

According to Bickel et al. (2004) male, fasted (24 hours) mice, which are placed individually in cages having free access to sugared milk (1 ml = 3.27 kJ) in a graduated cylinder, are used to monitor the amount of milk consumed by the animals. Milk is offered at time 0 min. Cumulative milk consumption is measured at 2, 4 and 6 hours after drug or vehicle administration. The experiments are performed during the light phase. During the experimental session, the animals have no access to solid feed.

EVALUATION

Average milk consumption is recorded per control and treatment groups and can be expressed as means per time point or cumulative over the whole study period of 6 hours.

MODIFICATIONS OF THE METHOD

Yamada et al. (1993) studied the interaction of the leptin-induced suppression of milk consumption with the serotonergic pathway.

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I.G.3.5.2 Acute Effect on Food Consumption in Rats

PURPOSE AND RATIONALE

Food intake is measured in acute experiments in normal or obese rats. Additionally, in semichronic experiments body weight gain is recorded.

PROCEDURE

Female Zucker (fa/fa) rats weighing 250–350 g are maintained under standard conditions (temperature, light-dark cycle, ground rodent pellet chow, tap water). Measurements of food intake begin on day –2. The food is offered in special dishes to reduce spillage. Food intake and body weight are measured daily between 8:00 and 9:00 A.M. At this time any spilled food from the collecting paper under the cage is gathered, air-dried if necessary, and weighed. Individual food intakes in grams are recorded. The tests compounds are either administered with the food or injected intraperitoneally. Groups of 8–10 animals are used for control or treatment with various doses of the test compound or the standard. Mazindol, 3 mg/kg i.p., or 10 mg/kg p.o. can serve as standard. Treatment is continued for 7 days.

EVALUATION

Average food intake and body weight is recorded for each day. Average values of candidate compounds are compared statistically for each day with the control group. Results after oral administration have to be confirmed by parenteral route in order to exclude errors due to palatability.

MODIFICATIONS OF THE METHOD

Bickel at al. (2004) reported the use of an online-feeding monitoring system. Animals were placed individually in cages (Macrolon-cage (Type 4), size: 44×26×15 cm) equipped with a device for continuous monitoring of feed consumption. This device is a container, filled with feed and hanging on an electronic balance. The balance transmits the weight changes of the feed container continuously to a central unit; data were stored and processed at the end of the experiment. This system can measure feed consumption up to 3 weeks from > 100 feeding sensors simultaneously. The feeding monitoring system and data processing hard- and software (Release V3.07–03/2001) were obtained from TSE, Technical & Scientific Equipment GmbH, Bad Homburg, Germany. Software options: The data are processed from a matrix. The matrix delivers cumulatively or sequentially feed consumption in grams (g) for given time intervals for each individual animal. Cumulative and sequential feed consumption is normalized to g feed/time/100 g body weight. A program for microstructural analysis calculates the parameters for feed consumption over a given time (24 hours). Parameters delivered are: number of meals (Nm/time), intermeal breaks (IMB), (min), average meal size (g) and average meal duration (min). A meal was defined to be 0.5 g and the IMB 15 min. Details of this method are described by Bickel et al. (2000).

Hull and Maher, Maher and Hull (1990) used male Sprague-Dawley rats placed on a mush diet composed of equal parts of ground rodent chow and of 4 % nutrient agar solution. The agar-based chow allows a more accurate measurement of food intake and has been shown to be sufficient for maintaining normal growth in rats. The rats were made hyperphagic by food deprivation for 4 hours at the beginning of the dark cycle.

Mennini et al. (1991) and Anelli et al. (1992) studied the anorectic activity of various compounds in different species, such as mice, rats, and guinea pigs.

Bowden et al. (1988) used metabolism cages equipped with automated feeding monitors. Food was provided as 45 mg pellets which were singly delivered to a feeding trough. A photodetector sensed the removal of the pellet, and the number of pellets delivered over a specified time interval was recorded.

Samanin et al. (1979) described anorexia in rats induced by the central serotonin agonist m-chlorophenylpiperazine.

Blavet et al. (1982) studied food intake in fasted rats after treatment with several typical anorectic agents.

Dourish et al. (1985) investigated the effects of the serotonin agonist 8-OH-DPAT on food intake in non-deprived male rats. This effect was prevented by p-chlorophenylalanine (Dourish et al. 1986).

The anxiolytics gespirone, buspirone and ipsapirone increased free feeding in rats and did not inhibit feeding induced by 8-OH-DPAT (Gilbert and Dourish 1987).

Jackson et al. (1997) investigated the mechanisms underlying the hypophagic effects of the 5-HT and noradrenaline reuptake inhibitor, sibutramine, in the rat.

Simansky and Vaidya (1990) tested the anorectic action of a serotonin uptake inhibitor by measuring the volume of milk consumed by food-deprived rats.

Stevens and Edwards (1996) induced anorexia by subcutaneous injection of 5 mg/kg 5-hydroxytryptamine in Wistar rats habituated to a restricted feeding regime and tested the effects of a 5-HT_3 antagonist.

Rouru et al. (1992) investigated in genetically obese male Zucker rats the effect of subchronic metformin treatment on food intake, weight gain and plasma insulin and corticosterone levels and somatostatin concentrations in the pancreas.

Cooper et al. (1990a, b) used non-deprived rats to study anorectic effects in a test of palatable food consumption and in nocturnal free-feeding.

Cooper et al. (1990c) tested not only food consumption but also the frequency of feeding bouts and duration of individual feeding episodes.

Eberle-Wang and Simansky (1992) studied the influence on the anorectic action of CCK and serotonin by measuring the uptake of sweetened mash mixture in rats.

Voigt et al. (1995) studied the involvement of the 5-HT_{1A} receptor in CCK induced satiety by recording food intake during a 2 hours test meal in food deprived and in freely feeding rats.

Influence on postprandial satiety in rats was tested by Rosofsky and Geary (1989). Rats were given pelleted chow and water ad libitum. Near the middle of the bright phase of the light-dark cycle, pellets were removed, and the animals treated and condensed milk presented 30 min later. Milk consumption was measured at 4-min intervals for 40 min.

Rats show a dramatic and reliable reduction of food intake if they are pre-fed a low-protein basal diet and then offered a diet that is imbalanced in any of the essential amino acids (Leung and Rogers 1969). This anorectic response has been used by Hammer et al. (1990) to test serotonin3 receptor antagonists.

Thurlby and Samanin (1981) studied the effect of anorectic drugs on food-rewarded runway behaviour.

Ferrari et al. (1992) studied the effects on anorexia induced by ACTH and immobilization in rats in an X-maze with alternate open and covered arms, each baited with laboratory chow.

Cooper et al. (1993) studied dopamine D-1 receptor antagonists in rats with chronic gastric fistula, which were trained to sham-feed a 10 % sucrose solution in a 60 min test.

In wild rodents, hoarding of food covers the long-term alimentary need. In the laboratory, hoarding behaviour does not occur in ad libitum fed rats. On the contrary, rats whose energy balance is threatened by previous food restriction hoard as soon as experimental conditions allow to do so. When such a rat gets free access to a food stock (placed outside its usual territory), it carries food into its shelter and accumulates an amount proportionate to its body weight. Fantino et al. (1980, 1986, 1988), Nishida et al. (1990) used the reduction of the amount of food hoarded during a period of 3 hours as parameter for anorectic activity of drugs.

Caccia et al. (1993) studied the anorectic effect of D-fenfluramine in the marmoset (Callithrix jacchus).

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Chapter I.H
Peripheral Nervous System

H. Gerhard Vogel

I.H.1
Tolerance of Local Anesthetics

I.H.1.1
General Considerations

One generally has to distinguish between surface anesthesia, infiltration anesthesia and conduction anesthesia (Fromherz 1922; Schaumann 1938; Camougis & Takman 1971). Special local tolerance tests have been developed for each of these applications including peridural and intrathecal injections.

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I.H.1.2
Irritancy after Surface Anesthesia

PURPOSE AND RATIONALE

Surface anesthesia is used to anesthetize the cornea and conjunctiva of the eye and the mucous membranes in the mouth. The classical pharmacological test is the blockade of the rabbit corneal reflex as described by Régnier (1923) that has become a standard test method for evaluating local anesthetics (Fußgänger and Schaumann 1931; Ther 1953a; Quevauviller 1971; Muschaweck et al. 1986). These pharmacological methods are only partially suitable to determine the irritancy potential of local anesthetic on mucus membranes. Luduena et al. (1960) compared the mucus membrane irritancy of mepivacaine and lidocaine by the eye irritation method according to Hoppe (1950) and Draize et al. (1944).

For further description of the method see chapters I.N (Ocular Toxicity Tests) and I.P (Safety Assays in Skin Pharmacology).

PROCEDURE

For the rabbit eye irritation tests, the rabbits are placed in ventilated boxes with only the head free. Solutions of local anesthetics in 0.9 % saline are prepared and a volume of 0.2 ml is instilled into the conjunctival sac of one eye, leaving the other as control. The lower lid is retracted gently and held away from the cornea fro 1 min, thus insuring exposure to the solution.

EVALUATION

The medicated eye is examined in comparison with the control eye at 1, 2, 4, and 8 h after medication and scored according to the method of Draize et al. (1944).

Threshold irritant concentrations are calculated. The threshold irritant concentration is taken as that

concentration, expressed in percent, which produces no more than a mild irritation.

According to Draize et al. (1944), injuries to the cornea, conjunctival and palpebral mucosae and the iris are scored separately. The severity of ocular lesions has been graded in a scale of weighted scores.

Cornea

A. Opacity – Degree of density (area, which is most dense is taken for reading),

- scattered or diffuse area, details of iris clearly visible 1
- Easily discernible translucent areas, details of iris slightly obscured 2
- Opalescent areas, no details of iris visible, size of pupil barely discernible 3
- Opaque, iris invisible 4

B. Area of cornea involved

- One quarter (or less) but not zero 1
- Greater than one quarter, but less than one-half 2
- Greater than one half, less than three quarters 3
- Greater than three quarters, up to the whole area 4

Score equals A × B × 5
Total maximum = 80

Iris

Values

- Folds above normal, congestion, swelling, circumcorneal injection (any one or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive) 1
- No reaction to light, hemorrhage, gross destruction (any one or all of these) 2

Score equals A × 5
Total possible maximum = 10

Conjunctivae

A. **Redness** (refers to palpebral conjunctivae only)

- Vessels definitively injected above normal 1
- More diffuse, deeper crimson red, individual vessels not easily discernible 2
- Diffuse beefy red 3

B. **Chemosis**

- Any swelling above normal (includes nictitating membrane) 1
- Obvious swelling with partial eversion of the lids 2
- Swelling with lids about half closed 3
- Swelling with lids about half closed to completely closed 4

C. **Discharge**

- Any amount different from normal (dos not include small amount observed in inner canthus of normal animals) 1
- Discharge with moistening of the lids and hairs just adjacent to the lids) 2
- Discharge with moistening of the lids and considerable area around the eye 3

Score (A + B + C) × 2
Total maximum = 20

The **maximum total score** is the sum of all scores obtained from the cornea, iris and conjunctivae.

CRITICAL ASSESSMENT OF THE METHOD

The Draize ocular irritation test is the accepted standard as part of evaluating chemical substances for eye safety. Justified by an exigency for public protection, the Draize test became a governmentally endorsed method to evaluate the safety of materials meant for the use in or around eyes. Because of discomfort to test animals that may be associated with a positive response, continued use of the Draize test has been criticized and alternative methods proposed (Durham et al. 1992; Prinsen and Koeter 1993; York and Steiling 1998; Curren and Harbell 1998; Kulkarni et al. 2001; Wilhelmus 2001; Abraham et al. 2003; Perrot et al. 2003). With the development of alternative methods to replace the Draize test, the data generated in the Draize test are still being used as a "gold standard" against which the performance of alternative procedures is measured.

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I.H.1.3 Irritancy after Intradermal Injection

PURPOSE AND RATIONALE

Intradermal tolerance of local anesthetics can be used as a screening selection criterion for finding an optimal local anesthetic. Luduena & Hoppe (1952), Luduena et al. (1960) determined intradermal irritancy by the trypan blue method according to Hoppe et al. (1950).

PROCEDURE

Albino rabbits of either sex weighing 2 to 3 kg are fastened securely in a supine position and the hair carefully clipped from the abdominal area with a fine-bladed clipper. The bare abdominal surface is marked off into 6 to 8 areas of approximately 20 cm^2 each by a soft wax pencil. In preparing solutions for injections, the highest concentration was prepared first and subsequent dilutions are made therefrom in ratios of 1/2, 1/4, 1/8, 1/16, etc., in order to find the submaximal irritation range. A volume of 0.3 ml of each dilution is injected intracutaneously into randomly designed areas of the abdominal skin of the rabbit. A dose of 1.0 ml/kg of a 1 % solution of trypan blue in normal saline is injected 10 to 20 min after the last intracutaneous injection of test solutions. The sites of injection are examined at one-half, one and three hours after injection of trypan blue.

EVALUATION

The intensity of staining by trypan blue at the site of injection is scored as follows:

• No color	0
• Faint but discernible color	2
• Distinct blue color throughout	4
• Deep blue color throughout	8
• Ischemic central area surrounded by deep blue halo	16

The scores are rated as follows:

• Average score	Rating
0	none
1–3	mild
4–7	moderate
8 or greater	marked

The threshold concentration is taken as that concentration expressed in %, which produces no more than a mild irritation.

MODIFICATION OF THE METHOD

Henn & Brattsand (1966), using the trypan-blue test, reported that intradermal irritancy of mepivacaine in rabbits was less than with tetracaine, whether the solution contained adrenaline or not.

Using the trypan-blue test, Luduena et al. (1972) found that racemic, (+)- and (–)-mepivacaine had the same intradermal irritancy.

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I.H.1.4 Irritancy after Subcutaneous Injection

PURPOSE AND RATIONALE

The pharmacological effect of local anesthetics after subcutaneous injection is determined with the method of Bülbring and Wajda (1945) in guinea pigs and modifications thereof (Ther 1953b).

Irritancy of local anesthetics after subcutaneous injections can be determined by subcutaneous injection into the ears of rabbits (Ulfendahl 1957). This method can be used not only as a screening selection criterion for finding an optimal local anesthetic but also as test method for evaluation of production batches (Hergott 1965).

PROCEDURE

Rabbits of either sex weighing 2.5 to 3.5 kg are used. A volume of 0.1 ml of the test solution is injected in the outer part of the rabbit's ear avoiding hitting any blood vessels. The same volume of saline is injected into the contralateral ear. A pale discoloration of the skin appears immediately, which disappears within 1 h in the control.

EVALUATION

The injection site is inspected after 2 and 24 hours. Reactions are scored as absent, slight, moderate or marked (necrosis).

MODIFICATION OF THE METHOD

The method has been used for evaluation of several local anesthetics (Ther 1953a; Muschaweck and Rippel 1986).

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I.H.1.5 Irritancy after Intramuscular Injection

PURPOSE AND RATIONALE

Local anesthetics can cause irritation and necrosis in muscular tissue. Most studies are performed in rabbits (Luduena et al. 1960; Baeder et al. 1974).

PROCEDURE

Groups of three rabbits of either sex weighing 2 to 3.5 kg are injected intramuscularly in the glutaeal muscle with one ml solution of the local anesthetic with and without vasoconstrictor in various concentrations. Each muscle injection site is used only once. The animals are sacrificed at 1-, 2-, or 7-days intervals. The injection sites are examined macroscopically, fixed in Zenker-formalin and embedded in paraffin. Histologic preparations are made in the usual way for microscopic examination.

EVALUATION

The slides are checked for inflammatory signs, such as edema, leukocytic infiltration or foci of coagulative necrosis.

MODIFICATION OF THE METHOD

Several studies were performed on myotoxicity of local anesthetics.

Basson (1978), Basson & Carlson (1980) described myotoxicity after single and repeated injections of mepivacaine in the **rat**. Young rats received single or repeated injections of 2 % mepivacaine into the tibialis anterior or extensor digitorum longus muscles. Repeated injections consisted of 6 injections of the anesthetic (100 µl per injection into the tibialis anterior) on different schedules, at intervals of 2 1/2 hours, 24 hours, or 4 days. The muscles were examined histologically for evidence of myotoxicity at 0 to 7 and 20 days after the last injection. Single injections showed that mepivacaine is a myotoxic

drug, producing a lesion, which ultimately results in a degeneration and subsequent regeneration of large amounts of muscle. A similar picture was seen with repeated injections except that greater tissue destruction was noted. Long-term studies following a single injection of mepivacaine showed restoration of the original muscle structure whereas after repeated injections some muscles showed persisting foci of interstitial connective tissue. The damage produced by 2 % mepivacaine is restored to a large extent by the regeneration of new muscle fibers.

Benoit & Belt (1972) described effects of local anesthetic agents on skeletal muscle and the correlation between local anesthetic-induced myotoxicity and disturbances of intracellular calcium distribution. The gracilis anticus and posticus muscles of albino rats were exposed to various local anesthetic agents (lidocaine, procaine, bupivacaine, cocaine, mepivacaine or prilocaine). Clinically used concentrations of most agents appeared to be specifically destructive to skeletal muscle. Muscle regeneration from single myoblasts was found to be rapid and complete by 2 weeks. One experiment (experiment 3) was designed to assess only extent of damage but not sequence of changes. Muscles were removed 1 day after a single injection of local anesthetic. No muscle damage was observed with 0.5 % lidocaine, 0.5 % mepivacaine or 2 % procaine. After 2 % prilocaine and 2 % mepivacaine, the muscle damage was more extensive.

In another study (Benoit et al. 1980) the local anesthetic mepivacaine and various other drugs known to perturb sarcoplasmic calcium metabolism and/or sarcolemmal sodium conduction were injected into the gastrocnemius or subcutaneously over the gracilis muscle of rats. After 48 h, all of the agents which are capable of increasing the intracellular concentration of free calcium (mepivacaine HCl, quinidine gluconate, A23187, caffeine, and 2,4-dinitrophenol) produced extensive and qualitatively similar myonecrosis. To assess the influence of a calcium antagonist on local anesthetic-induced myonecrosis, verapamil HCl was administered s.c., both alone and in combination with mepivacaine. Although verapamil itself resulted in some minor surface injury, it almost completely blocked the damage produced by mepivacaine. It was concluded that the myotoxicity of local anesthetics is related to a disturbance of intracellular calcium homeostasis.

Carabot et al. (1988) tried to explain the myotoxicity by effects of mepivacaine on the microcirculation of the skeletal muscle. Mepivacaine was inoculated directly into the anterior tibialis muscle of rats. Capillaries were examined through an electron microscope and capillary density in the damaged area was calculated by the alkaline phosphatase technique. Degenerative changes were observed in the capillaries one hour post-inoculation. These changes were more significant at 5 days, and were no longer visible after 20 days, when the regeneration of the muscle fibers was almost complete. Capillary density was reduced when ultrastructural changes were most intense.

Since local anesthetics are widely used in ophthalmic surgery, several studies on extraocular muscle regeneration after local anesthetic-induced lesions were performed by Carlson & Rainin (1985), Okland et al. (1989) in rats and by Carlson et al. (1992) in primates. In the first study, rats were given an injection of 50 µl of a local anesthetic (mepivacaine, lidocaine or bupivacaine) into the retrobulbar space of the left eye and injection of the same volume of sterile saline into the retrobulbar space of the right eye. The point of needle entry was posterior to the eyeball. Rats were sacrificed at 3, 7, and 30 days after treatment. The eyes and orbital content were removed and fixed in Bouin's fluid for paraffin sectioning. Sections were stained with hematoxylin-eosin. All three anesthetics produced massive degeneration of the extraocular muscles. Muscle degeneration is followed by regeneration on the damaged muscle fibres. In addition to muscular damage, severe damage was also seen in Harderian glands. In an other study, adult rats were given single retrobulbar injections of 50 µl of 2 % mepivacaine and the lateral rectus muscles were examined ultrastructurally from 15 min to 30 days post-injection. The lateral rectus muscle was massively damaged by exposure to the anesthetic, with membrane lesions seen as early as 15 min after the injection. Intracellular damage was followed by the phagocytic removal of the remnants of the damaged muscle fibers. The activation of satellite cells to myoblasts began during the phase of phagocytosis, and between 3 and 4 days after injection multinucleated myotubes actively forming sarcomeres appeared. The myotoxic effect of retrobulbarly applied local anesthetics in rats seemed to be much greater than they are in primates.

In a study in **Rhesus monkeys**, retrobulbar administration of local anesthetics resulted in a low incidence of muscle fiber lesions in the extraocular muscles closest to the site of injection. Most lesions resulted in the degeneration and regeneration of muscle fibers on the surface of the muscles, but occasionally a massive internal lesion was seen.

Hagiwara & Ozawa (1985) investigated toxicity of local anesthetics on **chick myogenic cells** (mononu-

cleated myoblasts and multinucleated myotubes) in culture. Following treatment with the drugs, myogenic cells showed some morphological changes and finally detached from the culture dishes. The toxic effect was estimated by the amount of cells detached and by the DNA and creatine kinase activity of the cells remaining on the dishes. Dibucaine was more toxic than bupivacaine, mepivacaine, tetracaine and procaine.

Carlson et al. (1990) studied local anesthetic-induced skeletal muscle fiber degeneration and regeneration in the **monkey** by light microscopy, Komorowski et al. (1990) by electron microscopy. Intramuscular injections of 0.75 % bupivacaine, 2 % mepivacaine, or 2 % lidocaine + epinephrine were given into the abductor pollicis muscles of Rhesus monkeys. The muscles were examined from 2 to 28 days. Severe muscle damage, consisting of breakdown of sarcolemma and myofibrils, was seen as early as 2 h. Phagocyte mediated fragmentation of the degenerating muscle fibers was at its peak during the third and fourth days. Myoblasts were abundant during the fourth day. Early myotubes appeared on the fifth and sixth days, and they matured during the second week. Satellite cells appeared alongside mature myotubes.

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I.H.1.6 Irritancy after Intraneural and Perineural Injection

PURPOSE AND RATIONALE

Nerve injuries are well recognized complications of regional anesthesia (Selander 1993; Borgeat & Ekatodramis 2001). Kalichman et al. (1986, 1988, 1998) studied neurotoxicity of local anesthetics in rat sciatic nerve.

PROCEDURE

Female Sprague Dawley rats (250–400 g) were anesthetized by intraperitoneal injection of a mixture of sodium pentobarbital 50 mg/ml, diazepam 5 mg/ml and 0.9 % saline in volume proportions of 1:1:2. Both sciatic nerves were exposed by lateral incision of the thigh and reflexion of the fascia and underlying muscle. Commercial preparations of low and high potency ester and amide local anesthetics (among them 2 % mepivacaine) were administered using a 30-gauge needle adjacent to the nerve, but external to the epineurium. The wounds were closed and the rats allowed to recover. After 48 hours, nerves were excised after anesthesia and prepared for light and electron microscopy. They were fixed by immersion in 2.5 % glutaraldehyde in 0.1 M phosphate buffer at a pH of 7.4. After glutaraldehyde fixation for 24 h, nerves were rinsed in buffer and postfixed for 3 h in 1 % osmium tetroxide. The tissue was subsequently dehydrated in serial alcohol solutions and later in propylene oxide prior to infiltration with araldite. Blocks were sectioned for light microscopy and stained with either paraphenylenediamine or methylene blue azure II. Electron microscopy was performed on selected bocks after preparation of ultra thin sections stained with uranyl acetate and bismuth subnitrate. Sections were examined in Siemens 1001 ultramicroscope operation at 80 kV.

EVALUATION

Concentration dependence of nerve injury was tested using a subjective scoring system of 0,1, or 2. Max-

imum severity was assigned a score of 2, a score of 0 indicated no injury, and moderate or equivocal injury was indicated by a score of 1. The 3 measures of interest were edema ("structureless space"), nerve fiber injury (degeneration and demyelinization) and lipid droplets (osmophilic inclusions in epineurial, perineurial and endoneurial cells).

MODIFICATION OF THE METHOD

Henn & Brattsand (1966) tested tissue irritancy after intraneural and perineural injection in rabbits. The test solution – 0.25 ml intraneural and 1.0 ml perineural, respectively – were injected into/around the exposed N. ischiadicus of adult mixed bred rabbits anesthetized with thiopental sodium. Four and eight days after the operation, tissue specimens were excised. The nerve tissues were treated by two different methods – according to the Marchi technique and according to the hematoxylin-eosin staining procedure after fixation in Bouin's solution. At all injection sites, some connective-tissue proliferation was found, and after intraneural injection some loosening-up of neurofibrils was observed. These reactions were also seen among the controls with NaCl and were therefore due to the trauma caused by the operation and injection. In no case were there pathological findings in the axons of the nerves. On the other hand, at high concentrations some moderate degenerative effects upon the myelin sheaths were observed. At concentrations of 0.5 %–3 % mepivacaine used clinically, no such signs were found.

Knox et al. (1961) studied nervous tissue toxicity in anesthetized rabbits. Using a sterile technique, the right and left sciatic nerves were exposed. In one series of 15 animals 0.25 ml of lidocaine and mepivacaine in concentrations of 0.5, 1.0, 1,5 and 2 % were injected directly into the right and left sciatic nerves. In a second series, 3 animals received 0.25 ml mepivacaine 4 % directly into both nerves. Controls received 0.25 ml normal saline. In a fourth series of 5 animals, 1.0 ml of test drugs was deposited around the nerve utilizing the same concentrations. All animals except the controls had a satisfactory sciatic nerve block as evidenced by hind limb paresis on recovery from the ether anesthesia. The rabbits were sacrificed on the second, fourth or eighth day following injections, and the sciatic nerves were excised and prepared for histologic examination utilizing hematoxylin and eosin, Luxol fast blue and Masson trichrome staining techniques. In none of the microscopic sections of sciatic nerves which were exposed to the 0.5 through the 2.0 % concentrations of drugs was there evidence of nerve damage (myelin and/or axon degeneration). No demonstrable difference was noted between nerves blocked with lidocaine and those blocked with mepivacaine. The usual reaction seen in these sections was a connective tissue proliferation which was believed to be caused by the trauma of surgery or the injection, and which did not differ markedly from control. Nerve degeneration was seen in the right sciatic nerves of 2 of the 3 rabbits that received mepivacaine 4 % in both sciatic nerves. This reaction was noted in the rabbits sacrificed on the fourth and eighth days.

Gentili et al. (1980) performed a light and electron microscopic, fluorescent microscopic, and horseradish peroxidase study in rats on nerve injection injury with local anesthetic agents: the sciatic nerve of pentobarbital-anesthetized adult Wistar rats was exposed in the upper thigh with the aid of an operating microscope; care was taken to avoid damage to the vascular supply. Using a 30-gauge needle, the nerve was injected at a standard location 1 cm distal to its exit from under the piriform muscle. The agents were injected either directly into the nerve fascicle or applied to the surrounding epineural tissues (extrafascicular). The nerve was examined at 1 to 2 hours and 9 to12 days after injection. Extrafascicular injections did not result in any significant nerve injury or disturbance of the blood-nerve barrier. After intrafascicular injection, the degree of injury varied significantly depending on the specific agent injected. Minimal damage was seen after the injection of 1 % mepivacaine and 0.5 % bupivacaine. The most severe injury with widespread axonal and myelin degeneration was seen after the injection of 2 % procaine and 1 % tetracaine.

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I.H.1.7 Irritancy after Epidural Anesthesia

PURPOSE AND RATIONALE

Activity and tolerability of new local anesthetics after intrathecal injection have to be studied in various animal species in order to predict both parameters for spinal (subarachnoid) anesthesia in patients. Myers & Sommer (1993) published a survey on methodology for spinal neurotoxicity studies. Studies in rats were reported by Blomberg & Rickstein (1988), in guinea pigs by Siems & Soehring (1952), Akerman et al. (1988), rabbits by Hughes et al. (1993), Chernyakova et al. (1994), dogs by Defalque & Stoelting (1966), Feldman & Covino (1988), Raner et al. (1994), Kamibayachi et al. (1995), cats by Ide et al. (2001), laboratory pigs by Richer et al. (1998), sheep by Lebeaux (1975), Feldman et al. (1997). Kief & Bähr (1970) described epidural tolerance of local anesthetics in dogs.

PROCEDURE

Epidural tolerance of artecaine with and without addition of Suprarenin was studied in Beagle dogs weighing 9 to 12 kg. For pre-operative sedation, the dogs received 0.03 ml/kg Combelen (= Propionylpromazine) intravenously. The fur of the lumbosacral area was shaved and the skin disinfected. A single dose of 5 ml of a 2 % articaine solution was administered epidurally under sterile conditions. All dogs showed the typical symptoms of spinal anesthesia, which subsided after a few hours. The dogs were sacrificed after 1 or 3 days. The portion of the vertebral column with the site of injection in the middle was removed and placed in 8 % buffered formalin. When semifixed, the vertebral arches were opened and the spinal cord as well as the roots of the spinal nerves with the adipose tissue of the epidural space were removed. After embedding in gelatin and Paraplast, the serial sections from the area of injection were stained with fast red 7B, hematoxylin-eosin and myelin sheath staining according to Olivecrona was performed. Furthermore, the PAS and iron reaction was performed in one section.

EVALUATION

The presence or absence of nerve damage and of inflammation was noted.

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I.H.1.8 Irritancy after Intrathecal (Spinal) Injection

PURPOSE AND RATIONALE

Transient neurological symptoms have been observed in patients after spinal anesthesia (Hampl et al. 1995). Activity and tolerability of new local anesthetics after intrathecal injection were studied in various animal species in order to predict both parameters for spinal (subarachnoid) anesthesia in patients.

Myers & Sommer (1993) published a survey on methodology for spinal neurotoxicity studies. Studies in **rats** were performed by Yaksh & Rudy (1976), Hylden & Wilcox (1980), Bahar et al. (1984a), Akerman (1985), Ossipov et al. (1988), Cole et al. (1990), Wang et al. (1991), Dirksen et al. (1992), Mestre et al. (1994), Omote et al. (1995), Chanimov et al. (1997), Grouls et al. (1997), De la Calle & Palino (2002), in **mice** by Hylden & Wilcox (1980), Akerman (1985); Akerman et al. (1988a,b), Langerman et al. (1994), in **rabbits** by Bieter et al. (1936a,b), Luduena et al. (1960), Langerman et al. (1991), in **dogs** by Wagner et al. (1940), Feldman & Covino (1981), Kozody et al. (1985), Dohi et al. (1987), in **marmosets** by Bahar et al. (1984b), in **rhesus monkeys** by Denson et al. (1981), in **sheep** by Lebeaux (1975), Kyles et al. (1992).

For detailed description of the pharmacological effects see the respective Chapter in "Drug Discovery and Evaluation – Pharmacological Assays".

Kirihara et al. (2003) compared neurotoxicity of intrathecal and epidural lidocaine in rats.

PROCEDURE

Kirihara et al. (2003) compared neurotoxicity of intrathecal and epidural lidocaine in rats**.** Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and 1.5 % halothane. A catheter of stretched polyethylene tubing PE-10 was introduced into the subarachnoid or epidural space using an aseptic technique. Catheters were passed through the L4-L5 intervertebral space and advanced 1.3 cm in the caudal direction. Rats were allowed 4 days to rest for recovery from the operation.

To measure the response to noxious heat stimulus, a tail flick test was performed. A 100-W projector lamp was focused on the distal segment of the tail approximately 5 cm from the tip. The time at which rats withdrew the tail was defined as the tail flick latency. A cut-off time of 10 sec was used to avoid damage of the tail.

To measure the response of legs to noxious mechanical stimulus, a paw pressure test was applied to the dorsal surface of both hind paws using a device capable of progressively increasing the pressure at a rate of 15 g/s. The pressure at which the rat withdrew the paw from the device wad defined as the paw pressure threshold, and the mean of both paws was used for analysis. A cut-off pressure of 400 g was used to prevent damage to the paws.

Motor function in the lower limbs was assessed by grading: 0 = none, 1 = partially blocked, and 2 = completely blocked.

Various concentrations of local anesthetic or saline are injected intrathecally in a volume of 20 μl or epidurally in a volume of 100 μl followed by 10 μl saline to flush the catheter. Tail flick test, paw pressure test and motor function were performed 10, 20, 30, 60, 120, 180, and 240 min after injection and continued daily for 4 days.

After the last experiments, the rats are euthanized by injection of an overdose of pentobarbital and then perfused intracardially with a phosphate-buffered 2.0 % paraformadehyde – 2.5 % glutaraldehyde fixative. Methyl green solution was injected to confirm the location of the catheter after the perfusion. The spinal cord and nerve roots were dissected out and immersed in the same fixative for 4 h. Two specimens (10 mm rostral and caudal to the conus medullaris from each rat were postfixed with cacodylate-buffered 1 % osmium tetroxide dehydrated in a series of graded alcohol solutions, and embedded in epoxy resin. From the embedded tissue, 1-μm transverse sections were obtained and stained with toluidine blue dyes. Sections obtained from 10 mm rostral to the conus (caudal spinal cord) were used for qualitative evaluation. Quantitative analysis of nerve injury was performed using the sections obtained form 10 mm caudal to the conus. Each fascicle present in the cross section was assigned to an injury score 0 to 3. The injury score for each cross section was then calculated as the average score of all fascicles present in the cross section.

EVALUATION

Data are presented as mean ± SEM. Tail flick latencies and paw pressure thresholds were converted to the percentage of maximal possible effect. The area under the time-effect curve was calculated by accumulating the effect measured at discrete time intervals using the trapezoidal integration method. The results were analyzed by ANOVA with repeated measures followed by Scheffe and Dunnett tests. The injury score for each technique and each solution was compared using two-way ANOVA followed by the Scheffe test. The frequency (i.e., the number of rats with lesions) in each group was analyzed by chi-square test.

MODIFICATION OF THE METHOD

Wakamatsu et al. (1999), Ohtake et al. (2000), Oka et al. (2001) studied the effects of intrathecally administered local anesthetics on glutamate release and neuronal injury in rabbits. New Zealand white **rabbits** were anesthetized with isofluorane. With the rabbits in prone position, midline skin and subcutaneous fascia were incised between the third lumbar and the first

sacral spinous process after infiltration with 0.25 % bupivacaine. Muscles were dissected; the third to seventh processes, ligamentum flavum and epidural fat were sequentially removed; and the underlying dura was exposed. Using an operating microscope, a small slit was made in the dura and arachnoid membrane at the L3-4 interlaminar space. A loop-type dialysis probe was then implanted. A PE-10 catheter for the administration of saline or the local anesthetic to be tested was implanted intrathecally through the slit made at the L6-7 interlaminar space so that the tip of the catheter was located at the level of the cauda equine. The implanted dialysis probe was perfused with artificial cerebrospinal fluid bubbled with 95 % oxygen and 5 % CO_2 at pH 7.2. Samples were collected before and after administration of test substance and analyzed for glutamate.

After collecting the last sample (90 min after intrathecal administration of test substance) the catheters were removed, and all incisions were sutured. Isoflurane was discontinued, and the lungs were ventilated with 100 % oxygen. Extubation of the trachea was performed when adequate spontaneous ventilation occurred. The animals were allowed to recover with infusion of Ringer's solution and antibiotic treatment.

The animals were neurologically assessed daily until 1 wk after test drug administration by an observer unaware of the treatment group. Sensory function was evaluated by seeking an aversive response to pinprick stimulation with a 23-gauge needle, progressing from sacral to thoracic dermatomes. The score of the sensory function was assessed by a three-point grading scale. The hind-limb motor function was assessed by a five-point grading scale.

After completion of the neurologic function scoring at 1 wk, the animals were re-anesthetized, and transcardiac perfusion and fixation were performed. The spinal cord was removed and refrigerated in phosphate-buffered formalin 10 % for 48 h. After dehydration in graded concentrations of ethanol and butanol, the spinal cord was embedded in paraffin. The coronal sections of the spinal cord at L3, L4, and L5 levels were cut at a thickness of 8 μm and stained with hematoxylin and eosin. The degree of the spinal cord damage was assessed for the vacuolation of the dorsal funiculus with a four point grading scale and the chromatolytic changes of the motor neuron. The neurons with chromatolytic appearance were identified by round-shaped cytoplasm with loss of Nissl substance from the central part of the cell and eccentric nuclei. The motor neurons with chromatolytic appearance were counted in two sections for each animal and averaged.

Parametric data were presented as mean ± SD. To determine differences in glutamate concentrations, a repeated-measures analysis of variance was performed. The cutaneous sensation, hind-limb motor function, and morphological changes of the spinal cord were analyzed with a non-parametric method (Kruskal-Wallis test) followed by the Mann-Whitney U-test.

Muschaweck et al. (1971) performed comparative intrathecal tolerance studies in dogs. **Beagle dogs** weighing 8 to 12 kg were anesthetized with 30 mg/kg sodium pentobarbital intravenously. The animals were intubated and submitted to artificial respiration. The fur on the neck was shaved and the skin disinfected. All further procedures were carried out under sterile conditions. The spinal canal was punctured through the foramen magnum at the atlanto-occipital joint. Successful entry of the spinal cervical canal was checked by withdrawal of cerebrospinal fluid. Five ml of cerebrospinal fluid was withdrawn and used as solvent for the tested local anesthetics. The same volume was injected intrathecally either as solution of local anesthetics in concentrations used in therapy or as control (saline solution). Artificial respiration was continued until spontaneous breathing resumed. Motor performance was checked during 24 hours. Two days later, the animals were sacrificed under anesthesia. Dissection included the cerebellum, medulla oblongata, sections from the cervical, thoracic and lumbar cord, carefully observing that always the same segments were taken including the injection site. Segments were semi-fixed in 8 % buffered formaldehyde for 2 days. When semi-fixed, the vertebral arches were opened, the spinal cord as well as the roots of the spinal nerves removed and completely fixed. After embedding in gelatin and Paraplast, a fat red 7B and myelin sheath staining according to Olivecrona were performed on serial sections from the site of injection as well as from cervical, thoracic and lumbar marrow. Hematoxylin-eosin staining as well as PAS and iron reaction was performed in one section. The presence or absence of nerve damage and of inflammation was noted.

Yaksh et al. (1995) studied the safety of chronically administered neostigmine methylsulfate in rats and **dogs**. Adult beagle dogs weighing 13–17 kg were adapted for 5 days to experimental protocols and placement of a nylon vest. For placement of the spinal catheter, the dogs were sedated (atropine 0.04 mg/kg and xylazine-Rompun 1–2 mg/kg i.m.) given an i.m. injection of penicillin G and procaine, and brought an anesthetic depth by mask administration of halothane

(3–5 %) and then the trachea was intubated. The dog was maintained under spontaneous ventilation with 1–2 % halothane and 50 % N_2/50 %O_2. Surgical areas on the back of the neck and head were shaved and prepared with alcohol and a povidone iodine scrub, and the dog was placed in a stereotaxic head holder. After draping and using sterile technique, the cisterna magna was exposed, and a small incision (10–2 mm) was made. The intrathecal catheter (polyethylene rubbing PE-50 stretched by 30 %, making the nominal diameter 0.6 mm) was inserted and passed caudally at a distance of 40 cm, to a level corresponding approximately to the L3-L4 segment. Presence of the catheter in the intrathecal space was confirmed by free withdrawal of cerebrospinal fluid. A small stainless steel screw was placed in the skull and the catheter fixed to the screw. The catheter was tunneled subcutaneously and caudally to exit on the upper left back at the level of the scapula. The incision was closed by sutures, the halothane turned off and the animal allowed to recover. An analgesic was administered for postoperative pain medication. At this time, the catheter was connected to the infusion pump placed into a vest side pocket, and an infusion of sterile saline (2 ml/day) was started.

For a 28-day infusion study, dogs were randomly assigned to receive saline or neostigmine (4 mg/4 ml). After 28 days of infusion, the dogs were sacrificed. After induction of a deep anesthesia, the animal was manually ventilated to maintain adequate oxygenation. A percutaneous puncture of the cisterna magna was performed and cerebrospinal fluid withdrawn for analysis. The chest was opened and a large-bore cannula placed in the aortic arch through which was perfused saline followed be 10 % formalin. After fixation, the dura was exposed by an extensive laminectomy of the spinal canal and the lower brainstem, being careful to leave the catheter and the dura undisturbed. Dye was injected through the catheter to determine its integrity, visualize the position of the intrathecal catheter, and determine the spread of dye around the catheter, the spinal cord was removed in four blocks (cervical, thoracic, caudal, and rostral from the catheter tip), taking care to keep the dura intact, and placed in formalin. After fixation, tissue blocks were embedded in paraffin and then decalcified overnight, embedded in paraffin, sectioned at a thickness of 6–7 μm and stained with hematoxylin and eosin. Particular attention was given to the presence or absence of fibrosis and other reactions around the catheter, dural thickening or other reaction; inflammation in the epidural space, leptomeninges/subarachnoid space, or spinal cord parenchyma, microglial nodules, demyelinization, or gliosis. The degree of chronic and/or acute inflammation was graded as normal, mild, moderate or severe.

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I.H.1.9 Studies on Porphyrogenicity

PURPOSE AND RATIONALE

Acute hereditary porphyrias are disorders of heme synthesis in which overproduction of heme precursors is often accompanied by severe clinical manifestations. Most of the time these diseases remain clinically latent and only occasionally result in acute abdominal and neuropsychiatric symptoms. Occurrence of the symptoms often follows exposure to drugs, such as barbiturates, sulfonamides, estrogens and some local anesthetics (Blanloeil et al. 1989).

De Verneuil et al. (1983), Deybach et al. (1987), Blanloeil et al. (1989) studied according to the method of Anderson (1978) the propensity of various drugs to induce hepatic porphyria. A situation analogous to the latent stage of human hepatic porphyria could be produced in chick embryos by injecting an inhibitor of ferrochelatase (the last enzyme in the heme biosynthesis pathway). The simultaneous injection of a porphyrinogenenic drug (e.g. barbiturate) results in a strong increase of the porphyrin levels in the chick embryo. This method is used for drug screening: drugs that are found porphyrinogenic in this test were considered to be potentially dangerous to patients with hepatic porphyria.

PROCEDURE

Fertilized 16-day-old chick embryos are kept in an incubator at 37 °C with 70 % humidity. Drugs are dissolved in a small volume (0.1–0.3 ml) of 0.15 M NaCl. According to Anderson (1978) a small dose of DDC (= 1,4-dihydro-3,5-dicarbethoxycollidine) is added that leads to the formation of N-methylprotoporphyrin, which is the inhibitor of ferrochelatase, the last enzyme in heme biosynthesis. Sterile injections are made when the eggs are 18 days old. After 24 h, the embryos are killed by decapitation, the livers removed, separated from the gall bladder and rinsed with saline prior to homogenization with 3 vol of 0.25 M sucrose/0.02 M Tris buffer, pH 7.4. At least six embryo livers are pooled for each determination.

For determination of porphyrins, the livers are extracted from the whole homogenate with 1 N perchloric acid:methanol (1:1 v/v). The type and the concentrations of porphyrins are studied with a spectrofluorimeter (Grandchamp et al. 1980). The type of accumulated porphyrins is confirmed by high-pressure liquid gas chromatography (de Verneuil et al. 1978).

For enzyme assays the homogenates are centrifuged at 800 g for 15 min and the supernatant is sonicated

3 times for 15 sec. Activity of δ-aminolevulinate synthase (ALA-S) is measured following the radiochemical method of Strand et al. (1972) with [^{14}C]succinic acid and a succinyl-CoA-generating system. Enzyme activity is expressed as pmoles δ-aminolevulinic acid per 30 min per mg of protein. Ferrochelatase activity is measured using a modified radiochemical method (Bonkowsky et al. 1975; Deybach et al. 1981).

To measure cytochrome P-450, 10 ml of the 800 g supernatant are centrifuged at 12 000 g for 15 min; 5 ml of the supernatant are then centrifuged at 105 000 g for 30 min, after washing twice, microsomes (pellet) are resuspended in phosphate buffer and the protein concentration adjusted to 2 mg/ml. The level of cytochrome P-450 is measured in the microsomal fraction using the method of Omura & Sato (1964).

EVALUATION

The tests allow judgement of the propensity of local anesthetics to induce symptoms of hepatic porphyria. The local anesthetics lidocaine, bupivacaine, etidocaine, mepivacaine, prilocaine and pyrocaine belong to this group, but procaine, butacaine, oxybuprocaine, proxymethacaine and tetracaine had no (or very slight) porphyrinogenic effect.

MODIFICATION OF THE METHOD

Schütz and Fuchs (1985a,b) studied the porphyrinogenic potential of the local anesthetic articaine (= Ultracain). Rats and dogs were given 25–30 intravenous and intramuscular injections of ultracaine without a vasoconstrictor in sublethal or maximum tolerated doses. No signs of any hepatotoxic effect were observed. Rabbits found to have porphyrinuria following administration of allyl-isopropyl-acetyl-carbamide (= Apronal) were injected the local anesthetic intramuscularly on five successive days in a dosage (50 mg/kg) which caused cramps without resulting in urinary porphyrin secretion or hepatic damage.

Bayar & Sümer (1995) investigated the effect of some local anesthetics on methemoglobin levels and erythrocyte enzymes.

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I.H.2 Tolerance of Neuromuscular Blocking Agents

I.H.2.1 General Considerations

Neuromuscular transmission is mediated by nicotinic acetylcholine receptors, for which various subtypes are described (Sargent 1993; McGehee and Role 1995; Karlin and Akabas 1995; Alexander et al. 2001).

Neuromuscular blocking agents are distinguished by whether or not they cause depolarization of the motor end plate. They are classified either as competitive (stabilizing) agents, of which d-tubocurarine is the classical example, or as depolarizing, desensitizing agents such as succinylcholine.

For safety evaluation, the absence of cardiovascular side effects is important (Vizi et al. 2003).

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I.H.2.2 Evaluation of Autonomic Margins of Safety

PURPOSE AND RATIONALE

Savarese (1979) determined not only the potencies of metocurine and d-tubocurarine but also the autonomic margins of safety in anesthetized cats.

PROCEDURE

Adult cats of either sex are anesthetized with α-chloralose, 80 mg/kg, and pentobarbital 7 mg/kg, given intraperitoneally. Cannulas are placed in the left femoral vein and artery for drug injection and recording blood pressure and heart rate. The lungs are mechanically ventilated through a tracheostomy and a small animal ventilator set to deliver 15 ml/kg tidal volume and 20 breaths/min.

The right vagus nerve and the right sympathetic trunk are exposed and divided in the neck. The distal ends are placed on the same shielded platinum wire electrode to permit preganglionic stimulation of both nerve trunks. The left sympathetic trunk is also dissected along its postganglionic portion at the base of the skull, and cut distal to the superior cervical ganglion to permit postganglionic stimulation through another electrode. Trains of square wave pulses (20 Hz for 10 sec) are delivered at supramaximal voltage every 4 min simultaneously to all three autonomic nerve trunks. The resulting bradycardia and hypotension (the vagal response) are measured. Contractions of both nictitating membranes, one (the right) elicited pregangionically and the other (the left) elicited postganglionically, are recorded. The maximal vagal response (i.e., cardiac arrest for 10 sec) is achieved by stimulation of the right vagus nerve or of both vagus nerves.

Twitches of the right tibialis anterior muscle are elicited at 0.15 Hz via the peroneal branch of the right sciatic nerve, to which square-wave shocks of 0.2 msec are applied at supramaximal voltage. Twitch recording is done via a transducer. All nerves and tendons are kept moist in small pools of mineral oil or in cotton pledges soaked in mineral oil. Tibialis anterior and esophageal muscle temperatures are monitored and kept between 35 °C and 38 °C by heat lamps.

Simultaneous recordings of heart rate, arterial pressure, pre- and postganglionic elicited contractions of the nictitating membrane, and twitches of the tibialis anterior muscle are made on a polygraph. Cumulative dose – response curves for inhibition of neuromuscular, vagal (parasympathetic) and sympathetic functions are determined simultaneously for each animal. The mechanism of vagal inhibition is localized at parasympathetic ganglia or cardiac muscarinic receptors by determining whether the bradycardic response to methacholine (20 μg/kg) is blocked as well as the neurally-elicited bradycardia.

A single-bolus dose of the neuromuscular relaxants producing the delayed depressor response plus tachycardia (Paton 1957) is determined in each animal. This response being pathognomonic for histamine release is defined as sudden hypotension to less than 80 % of the control arterial pressure within 2 min of relaxant injection and with tachycardia to more than 25 % above the baseline value.

Test drugs are given intravenously.

EVALUATION

Data analysis is done by the method of Litchfield and Wilcoxon. Mean dose – response curves are plotted on log-probit paper. Best fit to straight lines on these scales is determined by computerized regression. The cumulative ED_{50} values for vagal and sympathetic inhibition and the cumulative ED_{95} values for neuromuscular blockade are determined from the lines and 95 % confidence limits are calculated. Differences in potency are considered significant when $P < 0.05$.

The occurrence of histamine release is also treated as an all-or-none response to permit log-probit plotting. The delayed depressor response plus tachycardia is judged to have or have not occurred after each single bolus injection of the drugs. The percentage of animals responding at each dose level is then determined and the data handled by the Litchfield-Wilcoxon method.

The autonomic margins of safety of the test drugs are calculated as the ratios of cumulative doses producing 50 % block (ED_{50}) of vagal (parasympathetic) and sympathetic transmission and the ED_{50} for histamine release, each divided by the ED_{50} of neuromuscular blockade.

MODIFICATION OF THE METHOD

Clutton et al. (1992) studied the autonomic and cardiovascular effects of neuromuscular blockade antagonism in the **dog**. Neuromuscular blockade was antagonized with various anticholinesterase-antimuscarinic drug combinations including atropine, neostigmine and glycopyrrolate.

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Chapter I.I
Safety of Intravenous and Inhalation Anesthetics

H. Gerhard Vogel

I.I.1 Determination of Safety of Intravenous Anesthetics

I.I.1.1 General Considerations

PURPOSE AND RATIONALE

The first agents used as intravenous anesthetics were **barbiturates**. Barbiturates with a duration of action appropriate to the requirements of surgery became available with the introduction of hexobarbital and thiopental (Volwiler and Tabern 1930; Miller et al. 1936). The studies with barbiturates were extended (Butler and Bush 1942; Christensen and Lee 1973). Intravenous anesthetics from other chemical groups were developed, such as **acetamido-eugenol** (Estil, Domenjoz 1959), steroid derivatives (Presuren=**hydroxydione sodium**, Laubach et al. 1955, **alfaxolone**, CT1341, Child et al. 1971), **propanidid** (Epontol, Goldenthal 1971), **ketamine** (CI-581, Chen et al. 1966; Reich and Silvay 1989), **etomidate** (Janssen et al. 1975), **propofol** (ICI 35868, Glenn 1980), **midazolam** (Pieri 1983; Reilly and Nimmo 1987).

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I.I.1.2 Tests for Safety of Intravenous Anesthetics

PURPOSE AND RATIONALE

Besides determination of the ratio between anesthetic and lethal dose, intravenous anesthetics have to be tested for their influence on the cardiovascular and

pulmonary system. Borkowski et al. (1990) described a method to compare intravenous anesthetics in rabbits.

PROCEDURE

Adult New Zealand White rabbits with a mean weight of 4.5 kg are used. To provide access for direct blood pressure measurement and arterial blood samples, an 18-gauge catheter is implanted into the left carotid artery under halothane anesthesia. Following a minimum 24-h recovery period, the rabbit is placed in a sling and a pneumograph is fitted around the rabbit's caudal thorax at the level of 10th to 12th ribs to monitor respiratory rate and pattern. From the arterial catheter blood is withdrawn for blood gas analysis. Then the catheter is connected to a blood-pressure transducer. A 10-min acclimatization period is allowed before control measurements are recorded. Each rabbit serves as its own control in that cardiopulmonary parameters and responses to noxious stimuli are determined before anesthesia is induced. The right marginal ear vein is catheterized with a 22-gauge catheter, which is secured with adhesive tape, flushed with physiologic sterile saline and used for the administration of the anesthetic agents.

One third of the dose of the anesthetic to be tested is injected manually over a 1-min period. When the rabbit is relaxed it is removed from the sling and is placed in left lateral recumbence on a heating blanket. The degree of muscle tension and reaction to noxious stimuli are determined while the rabbit is in the sling and at 15 min intervals following anesthesia. The assessments performed include those of jaw tone, leg muscle tone, palpebral reflex, corneal reflex, ear pinch reflex and pedal withdrawal reflex. Jaw tone is evaluated subjectively by pulling the lower jaw open by an index finger. Leg muscle tone is evaluated by flexion and extension of the right rear leg according to subjective scores. The corneal reflex is tested by placing a moistened cotton swab on the cornea. The palpebral reflex is tested by touching the medial canthus with a dry cotton swab. Assessment of the ear pinch reflex is performed by applying a compression force with an alligator clip. The pedal withdrawal reflex is determined by applying the same clip on the right rear fifth digit at the distal phalanx.

Cardiopulmonary parameters and rectal body temperature are determined while the rabbit is in the sling and also at 15 min intervals following induction of anesthesia with the rabbit in lateral recumbency. Heart rate, mean arterial blood pressure, respiratory rate and respiratory pattern are calculated from tracings from the physiological recorder. Arterial blood pH, partial pressure of oxygen (PaO_2), and partial pressure of carbon dioxide ($PaCO_2$) are determined from arterial blood samples.

EVALUATION

The heart rate, mean arterial blood pressure, respiratory rate, pH, PaO_2 and $PaCO_2$ are analyzed using a two-factor analysis on repeated measures. The control values are treated as covariate to allow standardization of the inherent variation between rabbits. The single *t*-test for paired differences is used to compare control values to data obtained during the later testing intervals. The standard error of the mean (SEM) is calculated for each variable at each time interval. Data for muscle tone and responses to noxious stimuli are calculated as frequency percentages. The Fisher's exact test is used to compare between treatments. For all of the statistical analyses, a *p*-value of less than 0.05 is considered significant.

MODIFICATIONS OF THE METHOD

Details of anesthesia in the rabbit were also described by Murdock (1969).

Peeters et al. (1988) performed a comparative study of four methods for general anesthesia in rabbits.

Glenn (1980) examined the anesthetic activity of propofol (ICI 35868) in mice, rats, rabbits, cats, pigs and monkeys, including cardiovascular and respiratory parameters and EEG studies.

Korkmaz & Wahlström (1997) developed the electroencephalographic (EEG) threshold test in rats to determine the central nervous sensitivity to several depressant drugs, mainly intravenous anesthetic drugs. The test drugs were administered by continuous intravenous infusion until a defined EEG criterion indicating deep anesthesia was reached. The criterion was a burst suppression which lasted 1 s or more, the 'silent second' (SS). The dose of the drug needed to induce the SS, the threshold dose, was the dependent variable. In the intact animal, it is influenced by the potency of the drug and the dose administration rate of the infusion. With the method it is possible under in vivo conditions, to monitor continuously the electrical changes in the CNS.

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I.I.2 Determination of Safety of Inhalation Anesthetics

I.I.2.1 General Considerations

PURPOSE AND RATIONALE

The efficacy and safety of new inhalation anesthetics has to be evaluated in pharmacological experiments. Robbins (1946) defined the anesthetic AD_{50} as the concentration of anesthetic at which 50 % of mice failed to right themselves for 15 s when placed in a rotating bottle with a known concentration of anesthetic. The concentration of the anesthetic that caused apnea in 50 % of the mice in 10 min was defined as the LD_{50} and the ratio LD_{50}/AD_{50} as and an index of safety.

Wolfson et al. (1972) recommended brain anesthetic concentration for construction of anesthetic indices.

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I.I.2.2 Safety Margin of Inhalation Anesthetics

PURPOSE AND RATIONALE

To assess the safety margin of an inhalation anesthetic, not only should the ED_{50} values but also the maximally effective dose and the dose with a minimal danger of fatal outcome be determined. In particular, **cardiovascular parameters** are observed (Kissin et al. 1983).

PROCEDURE

Male Sprague Dawley rats weighing 300–350 g are placed into a clear chamber with the tail protruding from a special opening. An anesthetic-oxygen non-humidified mixture is directed into the chamber at a rate of 4 l/min. The inhalation anesthetics, e.g., halothane or isoflurane, are vaporized in Draeger vaporizers and the level in the chamber is monitored with a gas analyzer which is calibrated with a mass spectrometer. Rectal temperature is monitored and maintained at 37 °C with a heating pad. Each rat is exposed to only one pre-determined concentration of anesthetic for 30 min, at which time the presence or absence of the end point of anesthesia is determined. For the lethal end point, rats are tracheotomized and ventilated at 60 strokes/min through an endotracheal catheter. Tidal volume is adjusted to maintain Pa_{CO2} at 40 ±5 mm Hg.

Endpoints of anesthesia used:

1. Loss of righting reflex. The test is regarded as positive if the animal fails to right itself with all four feet on the floor within 15 s after being placed in a side position.
2. Prevention of purposeful movements response to a noxious stimulus. The animals are stimulated for 60 s by placement of a 1-kg weight on the middle of the tail. Only the purposeful movement of the head or legs is considered to be a response.
3. Prevention of the heart rate increase to a noxious stimulus (ECG signals). An increase in heart rate of greater than 1 % is regarded as a positive response.
4. The end point for the lethal effect is 7 mm Hg in the femoral artery with artificial respiration.

With each of the anesthetics, four series of experiments are performed: to determine the righting reflex, purposeful movement response, heart rate response, and lethal effect. The concentrations of the test compounds and the standard are spaced equally between the above-mentioned doses.

After determination of the heart rate effect and the lethal effect, the rats are sacrificed for determinations of brain tissue concentrations. The whole brain is removed and tissue anesthetic concentration is determined by gas chromatography.

EVALUATION

For calculation of the dose-effect curves, the probit method of statistical analysis is used.

For the assessment of anesthetic safety, not only the therapeutic ratio (LD_{50}/ED_{50}) but also the standard safety margin

$$SSM = (LD_5 - ED_{95})/ED_{95} \times 100$$

is used. This represents the percentage by which the ED_{95} has to be increased before LD_5 is reached.

CRITICAL ASSESSMENT OF THE METHOD

The standard safety margin has definitive advantages over therapeutic ratio. In contrast to the LD_{50}/ED_{50} index, the standard safety margin is influenced not only by the distance between central points of the anesthetic

and lethal dose-effect curves, but also by the slope of these curves.

MODIFICATIONS OF THE METHOD

A similar concept, based on response to tail clamping, respiratory arrest and cardiovascular failure in the **rat**, was published as an anesthetic index by Wolfson et al. (1973).

Another attempt to determine anesthetic requirements in rats was published by White et al. (1974).

Kissin et al. (1984) studied the morphine-halothane interaction in rats.

Fukuda et al. (1996) investigated the effects of sevoflurane and isoflurane on bupivacaine-induced arrhythmias and seizures in rats.

Kanaya et al. (1998) Compared myocardial depression by sevoflurane, isofluorane, or halothane in **cultured neonatal rat ventricular myocytes**. Changes in beating rate and amplitude during exposure to the anesthetics were measured.

Chaves et al. (2003) used non-invasive electrocardiography in **mice** to study the effects of intravenous and inhalation anesthetics and of age.

Krantz et al. (1941, 1953) described an anesthetic index between surgical anesthesia (cornea and wink reflexes abolished) and respiratory failure in **dogs**.

Van Poznak and Artusio (1960a,b) determined the anesthetic properties of fluorinated compounds in dogs using a face mask for the induction of anesthesia and a cuffed endotracheal tube later on. ECG (lead II) and EEG were monitored.

Steffey and Howland (1978) determined the potency of enflurane in dogs in comparison with halothane and isoflurane.

Johnson et al. (1998) compared isoflurane with sevoflurane for anesthesia induction and recovery in adult dogs.

Salmempera et al. (1992) studied in dogs the potency of remifentanil, a short acting opioid analgesic, which is used as anesthetic adjunct by variable-rate infusion. Enfluorane minimal alveolar concentration was measured by the tail-clamp method in dogs before and after sequential infusion of various doses of remifentanil. The plasma concentration causing a 50 % reduction of enfluorane minimal alveolar concentration was determined.

Kataoka et al. (1994) studied the negative inotropic effects of sevoflurane, isoflurane, enflurane and halothane in canine blood-perfused papillary muscles.

Hirano et al. (1995) compared the coronary hemodynamics during isoflurane and sevoflurane anesthesia in dogs.

Mutoh et al. (1997) compared the cardiopulmonary effects of sevoflurane with those of halothane, enflurane, and isoflurane, in dogs.

Hashimoto et al. (1994) examined the effects of sevoflurane and halothane on the effective refractory period and ventricular activation in a canine myocardial infarction model.

The effects of desflurane, sevoflurane and halothane on postinfarction spontaneous dysrhythmias in dogs were examined by Novalija et al. (1998).

Cardiopulmonary effects in **cats** were studied for desflurane by McMurphy and Hodgson (1996), for sevoflurane by Hisaka et al. (1997).

Saeki et al. (1996) determined the effects of sevoflurane, enflurane, and isoflurane on baroreceptor-sympathetic reflex in **rabbits**.

Hanagata et al. (1995) found that isoflurane and sevoflurane produce a dose-dependent reduction in the shivering threshold in rabbits.

Antognini & Eisele (1993) determined anesthetic potency and cardiopulmonary effects of enfluorane, halothane, and isofluorane in **goats**.

The effects of multiple administrations of sevoflurane to **cynomolgus monkeys** were evaluated by Soma et al. (1995).

The effect of inhalation anesthetics on the **respiratory system** was investigated in several studies:

Mazzeo et al. (1996) compared the relaxing effects of desflurane and halothane at various *MAC*s on isolated proximal and distal airways of dogs precontracted with acetylcholine.

Hashimoto et al. (1996) compared the bronchodilating effect of sevoflurane, enflurane and halothane in dogs using a superfine fiberoptic bronchoscope. The dogs were anesthetized with pentobarbital, paralyzed with pancuronium, and the lungs were mechanically ventilated. The endotracheal tube had an additional lumen to insert the superfine fiberoptic bronchoscope (outer diameter 2.2 mm) which was located between a second and third bronchial bifurcation to monitor continuously the bronchial cross-sectional area of third or forth generation bronchi. Bronchoconstriction was produced by histamine injection and infusion. The bronchial cross-sectional area was printed out by a video-printer at the end of expiration and was calculated on a computer using an image program after various *MAC*s of the different inhalation anesthetics.

Mitsuhata et al. (1994) induced systemic anaphylaxis in dogs sensitized to *Ascaris suum* by intravenous injection of the antigen and measured pulmonary resistance and dynamic pulmonary compliance.

Sevoflurane was as effective as isoflurane in attenuating bronchoconstriction associated with anaphylaxis in dogs.

Cervin and Lindberg (1998) examined the short-term effects of halothane, isoflurane and desflurane on mucociliary activity in the rabbit maxillary sinus in vivo.

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I.I.2.3 Determination of Minimal Alveolar Anesthetic Concentration (*MAC*)

PURPOSE AND RATIONALE

The term "minimum alveolar anesthetic concentration" (*MAC*) was coined by Merkel and Eger (1963) as an index to compare various anesthetic agents.

The use of *MAC* which represents the partial anesthetic pressure in the brain has gained wide acceptance (Eger et al. 1965; Quasha et al. 1980).

For **man**, Saidman and Eger (1964) defined *MAC* as the point at which 50 % of the patients moved in response to a surgical incision.

A method for determining minimum alveolar concentration of anesthetic in the **rat** was published

by Waizer et al. (1973). Kashimoto et al. (1997) determined the minimum alveolar concentration of sevoflurane in rats. Eger et al. (1999) studied minimum alveolar anesthetic concentration of fluorinated alkanols in rats and discussed the relevance to theories of narcosis. Eger et al. (2003) studied additive minimum alveolar concentration effects of halothane and isofluorane in rats.

Issues in the design and interpretation of minimum alveolar anesthetic concentration studies were discussed by Sonner (2002).

PROCEDURE

Minimum alveolar anesthetic concentrations are determined in Sprague Dawley rats weighing 300–450 g. Each rat is placed in an individual gas tight plastic cylinder closed at both ends by rubber stoppers. The stoppers are pierced with holes for various purposes. A rectal temperature probe (temperature maintained between 36 °C and 38.5 °C) and the rat's tail are drawn separately through holes in the rubber stopper closing the distal end of the cylinder. Delivered gases at an average inflow rate of 1 L/min to each rat enter through ports at the head (proximal) end of the cylinder and exit at the tail (distal end), a flow to minimize rebreathing (inspired $CO_2 < 10$ mm Hg). Exiting gases are scavenged.

The anesthetics are introduced from conventional vaporizers. For the determination of *MAC*, an initial concentration is used that permits movement of the rats in response to noxious stimulation. A tail clamp is applied for 1 minor until the animal moves, and the anesthetic partial pressure is measured by gas chromatography. If the animal moves, the partial pressure is increased by 0.2 % or 0.3 % atmospheres. After equilibration for 30 min, the tail clamp is applied again and the anesthetic partial pressure measured by gas chromatography. This procedure is repeated until the partial pressures bracketing movement-nonmovement are determined for each rat.

EVALUATION

MAC is defined as the average of the partial pressures that just prevented movement in response to clamping of the tail. Differences between anesthetics are accepted at $P < 0.05$.

MODIFICATIONS OF THE METHOD

Fang et al. (1997) found that maturation decreases ethanol minimum alveolar anesthetic concentration more than desflurane *MAC* in rats.

Gong et al. (1998) assessed the effect of rat strain on susceptibility to anesthesia and convulsions produced by inhaled compounds in five different rat strains. Strain minimally influenced anesthetic and convulsant requirements of inhaled compounds in **rats**.

Doquier et al. (2003) studied the minimum alveolar anesthetic concentration of volatile anesthetics in rats as tools to assess antinociception in animals.

Davis et al. (1975) Determination of the minimal alveolar concentration of halothane in the white New Zealand **rabbit** was published.

Determination of an anesthetic index (Apnea/*MAC*) in experiments in dogs has been proposed by Regan and Eger (1967).

Murphy and Hug (1982), Hall et al. (1987) used the reduction of enflurane *MAC* values in dogs as parameter for the anesthetic potency of fentanyl or sufentanyl, respectively.

Seifen et al. (1987) used *MAC* values for comparison of cardiac effects of enflurane, isoflurane, and halothane in the dog **heart-lung preparation**.

Ide et al. (1998) used airway occlusion in **cats** as a noxious respiratory stimulus that induces a visceral sensation of choking for determination of minimum alveolar anesthetic concentrations during halothane, isoflurane, and sevoflurane anesthesia. These values were compared with *MAC* values for somatic noxious stimuli such as toe pinch or tetanic stimulus. The authors recommended this method as a new concept for *MAC* determination.

Eger et al. (1988) determined minimum alveolar concentration of fluorinated anesthetics in **pigs**.

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Chapter I.J
Side Effects of Central Analgesic Drugs

Charles P. France

I.J.1 General Considerations

Morphine, as the prototype of a central analgesic drug, has characteristic adverse effects that are relevant to the treatment of patients. Important among these effects are the following:

- Respiratory depression,
- Decrease of body temperature,
- Tolerance,
- Physical dependence,
- Abuse liability.

Since the ratio between therapeutic effect and adverse effects varies among opioids, great effort has been made to synthesize compounds with a better ratio of antinociceptive activity versus adverse side effects. This effort to discover better, safer compounds necessitated the development of laboratory methods that are sensitive to and, therefore, could be used to quantify the adverse effects of opioids. Moreover, the discovery of several distinct types of receptors that can interact with opioids or with endogenous peptides (e.g. μ, κ, and δ receptor) allows a more selective classification of agonists and antagonists.

Contributed by Charles P. France, Ph.D., on the basis of Chapter H.1.3: Side effects of central analgesic drugs in the book "Drug Discovery and Evaluation – Pharmacological Assays", second Edition, Springer Verlag 2002.

I.J.2 Test for Respiratory Depression

PURPOSE AND RATIONALE

Respiratory depression is one of the most prominent adverse effects of μ opioid agonists (e.g., morphine). The frequency of breathing and the inspiratory volume can be affected differently by drugs and have to be measured.

PROCEDURE

Male or female rabbits with a body weight between 2.5 and 3 kg are placed in restraining cages. A mask is placed over the nose of the animals which is connected with a valve opening on exhaling and closing on inhaling. The frequency of breathing is recorded from the opening and closing of the valve. The inspiratory volume is measured with a gasometer. Intravenous injection of morphine in doses between 1 and 10 mg/kg results in a dose-dependent decrease in respiratory frequency and respiratory volume. The doses are increased logarithmically after the effect of the preceding dose has subsided.

EVALUATION

Three animals are used for the test compound and the standard. Dose-response curves of the effect on respiratory frequency and volume are compared. While μ opioid agonists decrease respiratory function, κ opioid agonist either increase or have no effect on respiratory function. The magnitude of respiratory depression produced by μ opioid agonists is related to their efficacy at opioid receptors with low efficacy agonists such as nalbuphine having much less effect on respiration as compared to morphine.

MODIFICATIONS OF THE METHOD

Nelson and Elliott (1967) compared the effects of morphine, morphinone and thebaine on respiration and

oxygen consumption in rats and Murphy et al. (1995) provided a method for distinguishing central and peripheral mechanisms of respiratory depression in rats.

Ling et al. (1983, 1985) measured blood gas values (pO_2, pCO_2, pH) in unrestrained rats via an arterial cannula. pO_2 and pH decreased whereas pCO_2 increased after morphine.

Studies in rhesus monkeys have compared the respiratory depressant effects of opioids to their effects on other behavior (Howell et al. 1988; Butelman et al. 1993; Gerak et al. 1994).

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I.J.3 Decrease of Body Temperature

Opioids can have profound effects on body temperature; however, depending on the particular conditions under which the compound is evaluated either increases or decreases in body temperature can be observed. Route of administration, dose, age of the subject, as well as ambient temperature contribute to the type of response opioids exert on body temperature. Thus, in rats a single i.p. injection of morphine can produce hypo- or hyperthermia; restraint exaggerates the former response and attenuates the latter. Since this adverse side effect of opioid drugs is shared by several other compounds with no central antinociceptive activity, the test can not be regarded as specific.

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I.J.4 Methods for the Study of Tolerance

PURPOSE AND RATIONALE

Repeated treatment with opioid agonists can decrease sensitivity to drug effects, thereby limiting the effectiveness of analgesics and requiring an increase in dose for recovery of antinociceptive effects. The radiant heat or the hot plate method for testing antinociceptive activity in mice is adapted to measure drug induced changes in the sensitivity to a noxious stimulus.

PROCEDURE

Male mice (10–12 per condition) with an initial weight of 18–20 g are used. They are placed in restraining cages. A noxious stimulus is produced by an intense light beam directed to the proximal part of the tail. The subject can respond to this stimulus by flicking its tail. The reaction time, the interval between stimulus onset and response, is automatically measured. A maximum time of exposure to the stimulus (e.g. 12 s cut off time) prevents tissue damage. Prior to drug administration, two control measures of reaction time are obtained for each animal. After administration of the drug the test is repeated 15, 30, and 60 min after subcutaneous injection or 30, 60 and 120 min after oral administration. In this way, time of peak activity can be determined. Mice showing a reaction time of the average control value plus 2 times the standard deviation in the control experiment are regarded as positive. Complete dose-response curves are determined and ED_{50} values are calculated. Subsequently, the animals are treated for 5 days once every day with a dose which is 4 times

higher than the ED_{50} in the first experiment. On the following day, dose-response curves are determined using at least 3 doses. The ED_{50} is calculated again.

EVALUATION

Reduced effectiveness of a fixed dose and/or the need for larger doses to obtain a constant response indicates the development of tolerance. ED_{50} values obtained before and after repeated daily treatment are compared to assess the magnitude of tolerance.

CRITICAL ASSESSMENT OF THE METHOD

Tolerance is observed not only with opioid agonists but also with many other drugs including barbiturates, benzodiazepines and ethanol. The measurement of antinociception after single and repeated administration, therefore, has to be regarded as a primary test. Moreover, a decrease in the potency of a drug after daily drug treatment, while providing evidence for tolerance, does not give insight to the mechanism by which tolerance has developed (i.e., pharmacodynamic or pharmacokinetic). Demonstration that the antinociceptive effects of a new drug do not decrease after repeated daily treatment with high doses indicates that it is not necessary to escalate dose in order to maintain effectiveness and represents the first step for establishing the absence of tolerance liability.

MODIFICATIONS OF THE METHOD

Other authors (e.g. Glassman 1971) injected the dose which induced a full antinociceptive effect in mice twice daily for a period of 21 days and evaluated the stepwise decay of effectiveness. After 21 days, the effect of 10 mg/kg morphine or 30 mg/kg meperidine i.p. decreased to approximately 50 % of the value of the first day.

Langerman et al. (1995) evaluated the acute tolerance to continuous morphine infusion up to 8 h in the rat with various doses using the hot plate and the tail flick assay. Tolerance was observed with the hot plate assay but not with the tail flick assay suggesting tolerance development at a supraspinal site.

Smith et al. (2003) used twice daily injections of morphine and implantation of morphine-containing pellets to study mechanisms of opioid tolerance in mice.

Riba et al. (2002) showed that the role of δ opioid receptors in modifying the antinociceptive effects of μ opioid agonists changes during morphine tolerance.

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I.J.5 Tests for Physical Dependence

PURPOSE AND RATIONALE

Withdrawal phenomena, either after abrupt cessation of chronic treatment or after administration of a pharmacologic antagonist (e.g. naltrexone), can be observed in a variety of non-human species. Importantly, the withdrawal that emerges in non-humans topographically resembles important features of withdrawal in humans. On this basis, tests for drug dependence and withdrawal have been developed for monkeys (Seevers, 1963a; Seevers and Deneau 1963b; Aceto, 1990; Woods et al. 1993), dogs (Martin et al. 1974, 1976), rats (Buckett 1964; Cowan et al. 1988), and for mice (Way et al. 1969; Von Voigtlander and Lewis 1983). Two general approaches are used to evaluate physical dependence potential: primary physical dependence and single-dose substitution. In the former, the test substance is administered repeatedly over days and the assessment of dependence (i.e., withdrawal) occurs either after discontinuation of drug treatment or by administration of a pharmacologic antagonist. Precipitated withdrawal studies are warranted only when the mechanism or site of action of the test substance is known and when an appropriate pharmacologic antagonist is available. In a single-dose substitution study, a reference substance (e.g. morphine) is administered repeatedly over days; after discontinuation of treatment with the reference substance, and when reliable withdrawal signs have emerged, the test substance is assessed for its ability to attenuate withdrawal signs. In this type of study, the test substance can be administered just once to assess its acute withdrawal-reversing effects, or can

be administered repeated over days (i.e., replace the reference substance) and subsequently discontinued followed by assessment of withdrawal signs.

A well-established in vitro procedure has also been used to test for opioid dependence (i.e., antagonist-precipitated withdrawal) in opioid-treated guinea pig ileum (Villarreal et al. 1977; Rodríguez at al. 1978; Collier et al. 1979; Cruz et al. 1991).

Opioid Withdrawal Responses in the Guinea Pig Ileum Made Dependent in vitro

A 40-cm long segment of the small intestine of male guinea pigs weighing 600–900 g is removed and placed in a low-magnesium Krebs solution. The terminal section of the guinea pig ileum is used after discarding the portion of 10 cm closest to the ileo-caecal junction. The ileum is cut in eight 3-cm long segments. The intestinal content is gently removed with the aid of an glass rod. To produce opioid dependence, segments are incubated in 500 ml Erlenmeyer flasks containing 480 nM morphine in 250 ml Krebs solution saturated with a 95 % O_2/5 % CO_2 gas mixture at a temperature ranging between 4° and 6 °C for 1 to 48 h. One h before completion of the incubation time, the segments are removed, placed in glass chambers with 50 ml Krebs solution bubbled with 95 % O_2/5 % CO_2 gas mixture at 36 °C, mounted on a vertical nickrome electrode with one edge fixed to the chamber plug and the opposite fixed to an isometrical force transducer (Grass FT 03) connected to a polygraph for recording the contractile activity of the longitudinal muscle. The ilea are set up with an initial tension of 1 g and left for a period of 30 min for stabilization. Thereafter, all segments are electrically stimulated with supramaximal rectangular pulses (10–40 V) of 0.5-ms duration at a frequency of 0.1 H.

Five min before naloxone administration, the electrical stimulation is suspended. The response to naloxone is recorded by administration of up to 100 nM. The response to the antagonist is recorded for 20 min and thereafter the electrical stimulation is reinitiated and maintained for 10 min.

Thirty-five min after naloxone administration, various doses of nicotine are administered to provide a positive control. For comparisons, a concentration-response curve for nicotine (1, 31., 5.6, 10, 31, and 56 μM) is obtained in untreated ilea. Moreover, the concentration-response curve for nicotine is obtained in ilea that are: 1) exposed to 10 nM naloxone for 20 min; 2) exposed to 480 nM of morphine for 1 h; or 3) pretreated for 10 min with 3 or 10 nM of naloxone and exposed to 480 nM of morphine for 1 h. The response to nicotine is attenuated after pretreatment with morphine and this attenuation is dose-dependently antagonized by naloxone.

There is a correlation between the response to supramaximal electrical stimulation and the withdrawal response (contraction) precipitated with 100 nM naloxone as well as a correlation between withdrawal and nicotine response after long-term exposure (12–48 h) with 480 nM morphine.

Test for Physical Dependence in Rats

Male albino rats receive either morphine or saline i.p. twice daily. The starting dose of morphine is 20 mg/kg followed by 40 mg/kg increments daily until by day 11 the level is 420 mg/kg. Maintenance at 400 mg/kg is continued through day 20. The test compound is similarly administered to groups of 10 rats each. The daily increments have to be adjusted to a maximum level that is not lethal for the duration of the experiment.

Primary Physical Dependence capacity is measured on days 11 and 17 when all animals receive an injection of 10 mg/kg of naltrexone or naloxone i.p. in the morning. Signs of withdrawal are recorded during a 30 to 60 min period. Rats are scored for the presence or absence of withdrawal signs (e.g. diarrhea, wet-dog-type shaking) using standardized scoring.

A Single-Dose Substitution study substitutes either a single dose or multiple doses (from day 20 through day 23) of the test compound in morphine-dependent rats; scoring for suppression of withdrawal occurs on days 20–23 and after discontinuation of the test substance.

Test for Physical Dependence in Monkeys

Groups of 3–4 rhesus monkeys (3–6 kg body weight) receive morphine 4 times daily (s.c. or i.m.) beginning with a dose of 1.0 mg/kg. Progressively the unit dose is increased to a final dose of 3.2 mg/kg/6 h. The test substance is similarly administered to groups of 3–4 monkeys. For the test compound, the daily increments in drug administration are adjusted to a maximally-tolerated (non-toxic) dose and frequency of injection. Both groups of monkeys are then maintained at their appropriate dose levels for a minimum of 112 days. On days 35, 60, and 91, 1 mg/kg of naltrexone or naloxone is administered (s.c. or i.m.) in the morning. On days 50 and 112 all doses are omitted for 24 h. Signs of withdrawal are recorded during a 30 to 60 min period using standardized scoring (e.g. Katz 1986; Brandt and France 1998).

CRITICAL ASSESSMENT OF THE METHOD

The emergence of withdrawal signs after discontinuation of drug treatment is dependent of the duration of action of the treatment compound. Thus, after discontinuation of morphine treatment withdrawal reliably emerges within 24 h. For drugs with an unusually long duration of action (e.g. buprenorphine), observations for withdrawal signs need to occur over longer periods of time (e.g. several days). Opioid antagonists will precipitate withdrawal in animals treated with opioid agonists. If a test substance has actions at non-opioid receptors, a negative result with naltrexone or naloxone in a precipitated withdrawal study will not provide useful information regarding dependence potential. Thus, both precipitated withdrawal and abstinence-induced withdrawal need to be studied for test compounds.

Rhesus monkeys have been used extensively for assessing physical dependence potential of opioid agonists. An excellent correlation between humans and rhesus monkeys has been shown regarding the physical dependence liability of opioids, although there are some compounds for which the relative potency between humans and monkeys is not what is predicted from other data. Non-human species can also be used to assess physical dependence potential of other classes of drugs, including sedative/hypnotics. Physical dependence potential alone can not be assumed to predict abuse liability because some drugs that are not abused (e.g. κ opioids) can produce marked physical dependence (Gmerek et al. 1987).

MODIFICATIONS OF THE METHOD

Mouse jumping as a simple screening method to estimate the physical dependence capacity of opioid agonists has been recommended by Saelens et al. (1971). Mice receive seven i.p. injections over 2 days. The test compound is given at doses increasing in multiples of two until a maximally tolerated dose is reached. Two hours after the last injection the animals receive an i.p. injection of 100 mg/kg naloxone and are placed individually into glass cylinders. The number of jumps is recorded during 10 min.

Kest et al. (2002) compared naloxone-precipitated withdrawal jumping in several strains of mice after acute or multiple injections of morphine or after chronic infusion of morphine with osmotic minipump.

Yoshimura et al. (1993) studied the physical dependence on morphine induced in dogs via the use of mini-osmotic pumps. Naloxone-precipitated withdrawal signs were recorded such as hyperactivity, biting, digging, tremors, nausea, hyperthermia, and increased wakefulness, and by EEG activation in the amygdala and hippocampus, followed by a dissociation of the EEG in the cortex (fast wave) from that in the limbic (slow wave) system, increased heart rate, and raised blood pressure. Withdrawal signs were more severe in animals with mini-osmotic pumps than in those receiving the same dose by syringe injections.

Pierce and Raper (1995) studied the effects of laboratory handling procedures on naloxone-precipitated withdrawal behavior in morphine-dependent rats and Gellert and Holtzman (1978) used access to drug in drinking solutions to study morphine dependence and withdrawal in rats.

Pierce et al. (1996) used slow release emulsion formulations of methadone to induce dependence in rats. Withdrawal was induced following i.p. challenge with either naloxone or saline, and dependence was assessed in terms of the presence or absence of characteristic withdrawal signs.

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I.J.6 Tests for Abuse Liability

I.J.6.1 General Considerations

Drug abuse often occurs in the absence of physical dependence. Various terms have been used to describe abuse-related phenomena that are not specifically linked to physical dependence (Deneau 1964a), e.g. psychological dependence, and laboratory procedures have been developed that are predictive of abuse-related effects in humans. For example, based on the early observations of Olds (Olds et al. 1956; Olds 1979) on intracranial self-stimulation, procedures have been developed for studying drug-induced changes in brain-stimulation reward (Kornetsky and Bain, 1990). Moreover, self-administration procedures are used widely to study the reinforcing effects of a variety of drugs (Deneau et al. 1969; Hoffmeister 1979; Littmann et al. 1979; Woolverton and Schuster 1983; Bozarth 1987; Meisch and Carroll 1987; Weeks and Collins 1987; Yokel 1987; Woolverton and Nader 1990) and more recently conditioned place preference has been used in studies on abuse liability. Furthermore, drug discrimination procedures can be used to complement other assays of abuse liability; discrimination procedures have the advantage of having a high degree of pharmacologic selectivity (Holtzman 1983; Brady et al. 1987; Colpaert 1987; Overton 1987; Hoffmeister 1988; Holtzman 1990). A general discussion of abuse liability assessment, as it relates opioid analgesics as well as non-analgesic drugs, appears elsewhere in this volume (Porsolt et al.).

I.J.6.2 Drug Discrimination Studies

PURPOSE AND RATIONALE

Many laboratories have used simple two-choice discrimination methods to investigate the mechanism or site of action of new compounds by examining those compounds in animals trained to discriminate a reference substance and known drug of abuse (Shannon and Holtzman 1976; Holtzman 1983; Brady et al. 1987; Shannon and Holtzman 1986; Colpaert 1987; Overton 1987; Hoffmeister 1988; Carboni et al. 1989; Holtzman 1990).

PROCEDURE

Rats are trained to press one of two choice levers to avoid or to escape electric foot shock which is delivered intermittently beginning 5 s after the start

of the trial. The occurrence of a trial is signaled by the illumination of a light in the operant chamber. A third (observing) lever is mounted in the wall of the chamber opposite the two choice levers and must be pressed before the choice response is made. This contingency prevents the rat from persevering on a single response lever; thus, the choice response in each trial is relatively independent of the consequences of choice responses in the preceding trials of the session. The rats are tested in 20-trial sessions. Animals are trained to discriminate a prototype of the drug of interest. Morphine and fentanyl have served well as training drugs for exploring the discriminative effects of classical μ opioid drugs. Training often occurs more rapidly when the dose of the training drug is the largest dose that does not disrupt behavior. For discrimination training, the animal is placed in the operant chamber and trained to perform the required response, initially under a schedule of continuous reinforcement where a single response on either lever postpones or terminates shock. As performance increases, the response requirement is increased progressively across days (e.g. to a maximum of 10 [fixed ratio 10]) and discrimination training commences whereby responding on one lever postpones or terminates shock. In two-choice procedures, the left and the right choice lever are designated for drug and vehicle, respectively, for half of the animals in a group; the lever designation is reversed for the other half of the animals. Acquisition of the discrimination is a function of the drug, training dose, and the number of training sessions. Training continues until the subject reaches predetermined performance criteria, which typically could be as follows: at least 80 % of the total session responses on the injection (drug or vehicle) appropriate lever and less than one fixed-ratio value (e.g. 10) of responses on the injection-inappropriate lever prior to delivery of the first reinforcer (e.g. shock postponement or termination) for 6 consecutive training sessions. A morphine discrimination can be established in rats, according to these criteria, in 6–12 weeks. Once stable discrimination performance has been achieved, tests of generalization to novel drugs can be interposed among the training sessions. During test sessions the reinforcer is available after completion of the response requirement on either lever. Complete dose-response curves for the training drug (e.g. morphine) and the test drug are obtained. In cases where the test drug does not produce responding on the training-drug appropriate lever, the test drug should be evaluated up to doses that that decrease rates of lever pressing or until other behavioral effects are observed, in order to insure that the compound is evaluated up to behaviorally-active doses.

EVALUATION

Results of the stimulus-generalization test usually are evaluated with the quantitative or graded method, whereby the amount of responding on the training-drug associated lever is expressed as a percentage of the total number of responses during a test (i.e., responding on the drug-appropriate lever plus responding on the vehicle-appropriate lever). This percentage is than compared with the percentage of drug-appropriate responses normally engendered by the training dose of the training drug (reference standard). The discriminative stimulus effects of the test drug substitute for those of the training drug if the maximal percentages of drug-appropriate responding are not significantly different from each other. When stimulus control of behavior transfers from one drug to another, it can be inferred that the test drug produced discriminative effects that are similar to those of the training drug. An advantage of this procedure is that it is pharmacologically very selective and that the discriminative stimulus effects of drugs are related to and predictive of subjective effects in humans.

CRITICAL ASSESSMENT OF THE METHOD

Drug discrimination procedures display a high degree of pharmacologic selectivity. While test drugs that resemble the training drug result in dose-dependent drug-appropriate lever selection, drugs that are pharmacologically dissimilar to the training drug typically cause responding on the choice lever that is appropriate for the drug vehicle, up to behaviorally-active doses. The pharmacologic selectivity of these procedures permits differentiation not only among compounds acting on different receptors or neurochemical systems (e.g. dopamine receptors versus opioid receptors) but also among compounds acting on different subtypes of receptors within the same receptor class (e.g. μ versus κ opioid receptors). Because of the importance of pharmacokinetic factors to the overall abuse liability of drugs, and because drug discrimination procedures are relatively insensitive to pharmacokinetic factors, as compared to self administration procedures, positive results from a drug discrimination study are not in themselves sufficient to predict abuse liability. Along with other measures of drug action, results of drug discrimination studies are used to predict the likelihood of new compounds having abuse liability. Typically self administration data are used along with

drug discrimination data, since these two assays are sensitive to different, though related aspects of drug activity.

MODIFICATIONS OF THE METHOD

Drug discrimination studies are performed in a variety of species including squirrel monkeys, rhesus monkeys, pigeons, gerbils and mice (Hein et al. 1981; Herling and Woods 1981; Bertalmio et al. 1982, 1987; Dykstra et al. 1987, 1988; France and Woods, 1993; France et al. 1994, 1995; Jarbe and Swedberg 1998; Shelton et al. 2004; Stolerman et al. 2004). Operant responding can be maintained with different reinforcers, including food, liquids, and aversive stimuli (e.g. electric shock). The pharmacologic selectivity of drug discrimination procedures is particularly evident with opioid agonists that bind selectively to different receptor subtypes. For example, monkeys trained to discriminate injections of the μ agonists codeine, etorphine, or alfentanil generalize to other μ agonists and not to non-opioid drugs, to opioid antagonists, or to opioid agonists that produce their behavioral effects through other (non μ) opioid receptors. Conversely, monkeys trained to discriminate a κ agonist such as ethylketocyclazocine or U-50,488 generalize to κ opioid agonists and not to μ opioid agonists, opioid antagonists, or non-opioid drugs (Woods et al. 1993; France et al. 1994).

Meert et al. (1989) used drug discrimination studies to characterize risperidone as an antagonist of LSD.

Meert and Janssen (1989), Meert et al. (1990) showed differences between ritanserin and chlordiazepoxide in drug discrimination procedures.

Δ^9-Tetrahydrocannabinol discrimination in rats has been proposed as model for cannabis intoxication in humans (Balster and Prescott 1990).

An attempt was made to measure opiate abstinence responses in the guinea pig ileum made dependent in vitro (Cruz et al. 1991).

The drug discrimination method has also been applied to study anxiolytic drugs using pentylenetetrazol at subconvulsive doses (Sherman and Lal 1979, 1980; Sherman et al. 1979; Lal and Sherman 1980).

The conditioned taste aversion procedure has been described as a more rapid alternative to two-lever operant procedures in drug discrimination research (Garcia et al. 1955; van Heest et al. 1992).

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I.J.6.3 Conditioned Place Preference Paradigm

PURPOSE AND RATIONALE

The conditioned place preference paradigm has been used widely to examine behavioral actions that are thought to be related to positive reinforcing effects as measured by other procedures, such as self administration (van der Kooy 1987; Hoffman 1989; Tzschenke 1998; Self and Stein 1992). Particular environmental stimuli are paired with the presence or absence of a presumed reinforcer (e.g. drug or food) and later, in the absence of that reinforcer, animals are tested for their preference for either environment.

PROCEDURE

To induce place preference with food, a food-restricted animal is exposed to an experimental chamber that consists of two compartments (which differ in floor texture and wall color) and that are separated by a removable barrier. In some iterations of this procedure the two compartments are joined by a small tunnel or a third (neutral) compartment. On alternate days the animal is confined to one or the other compartment, with food available in only one of the compartments. Thus, food is selectively paired with one of the distinctive environments. After several (e.g. 4 in each compartment for a total of 8) conditioning sessions, the animal is placed in the same chamber without the barrier in place (for procedures that use a third [neutral] compartment the animal is placed in that compartment and otherwise in the middle of the chamber). In the absence of the reinforcer (e.g. food), animals demonstrate a relative increase in the amount of time spent in the environment that was paired with food as compared to the compartment that was not paired with food. Place conditioning with drugs is conceptually similar and involves the differential pairing of drug effect with one compartment and the absence of drug effect (vehicle) with the other. Drugs can be administered by various different routes (Amalric et al. 1987; Bals-Kubik et al. 1998, 1990; Iwamoto 1988; Shippenberg and Herz 1987) and usually animals are placed in the chamber immediately after drug administration for a 40-minute conditioning session.

Male Sprague-Dawley rats weighing 250–300 g are used for these studies. When drugs are to be administered intracerebroventricularly, rats are anesthetized with 60 mg/kg i.p. sodium hexobarbital and 23 gauge guide cannulae aimed at the lateral ventricle (AP = –0.9 mm, L = +1.5 mm, DV = 3.5 mm) (Paxinos and Watson 1982) are stereotaxically implanted; conditioning commences one week later.

The apparatus consists of 30 × 60 × 30 cm plexigas boxes with a clear Plexiglas front. For conditioning sessions, each box is divided into two equal-sized compartments by means of a sliding wall. One compartment is white with a textured floor, the other black with a smooth floor. For testing, the central wall is raised 12 cm above the floor to allow passage from one compartment to the other.

Conditioning sessions are conducted once a day for 8 days and consist of administering drug or its vehicle on alternate days. The rats are immediately confined to one compartment of the box following drug injection and to the other compartment following vehicle injection. All conditioning sessions last 40 min. Test sessions are carried out one day after the last training session and in the absence of drug. The rats are placed in a neutral position (either in the center or in the neutral compartment) of the test box and allowed free access to both sides of the box for 15 min. A video-camera with integrated stop-watch is used for data recording. Alternatively, photocells mounted along the sides of each compartment can be used to electronically monitor the location of the subject in the apparatus. The time spent in each compartment is assessed by visual analysis of the recorded videotape or by data collected through photocell beam breaks.

For intracerebroventricular injections, a 30 gauge injection needle is attached to a microsyringe via polyethylene tubing. The drug solutions are administered over a 60 s period and the injection needles are left in place for and additional 30 s to ensure complete delivery of the solution. For antagonism tests, groups of rats receive an intracerebroventricularly injection of the antagonist (naltrexone or naloxone) or vehicle 10 min before the microinjection of the conditioning drug. At the end of the experiments, the rats are anesthetized and sacrificed by decapitation. The brains are removed and sectioned in a cryostat to verify the location of the cannulae. Alternatively, antagonists can be administered systemically.

EVALUATION

Conditioning scores represent the time spent in the drug-paired place minus the time spent in the vehicle-paired place, and are expressed as means ± SE. In cases where animals show a bias towards one compartment prior to conditioning, drug conditioning can be established with the non-preferred compartment, thereby increasing the confidence that preference for that compartment is specifically related to drug administration. Dose-response curves are analyzed with a one-way ANOVA. The Wilcoxon test, in which the time spent in the drug associated place is compared to that in the vehicle-paired place, is used to determine whether individual doses produce significant conditioning. A one-way ANOVA followed by the Student Newman-Keul's test is used to determine the statistical significance of effects of the antagonist pretreatment.

Unlike drug discrimination, conditioned place preference is not pharmacologically selective insofar as drugs from many different classes (e.g. opioids, ethanol, stimulants) generate positive results. Generally there is a strong positive correlation between drugs that can be used to establish conditioned place preference and those that are positive reinforcers by other measures (e.g. i.v. self administration); however, one of the most effective reinforcers in self administration studies, that is also widely abused by humans, does not unanimously generate strong conditioned place preference in non-humans – cocaine. Thus, results from conditioned place preference studies should be used in concert with results from other measures of reinforcing effects (e.g. self administration). For the purpose of opioids, in general μ agonists are effective for establishing place preference whereas κ agonists are not. In fact, μ antagonists or κ agonists can generate place aversion (e.g. Sante et al. 2000).

MODIFICATIONS OF THE METHOD

In order to distinguish place preference and place aversion, place conditioning behavior can be expressed by a difference in preference pre and post conditioning, where post and pre values are the difference in time spent in the preferred and the non-preferred sides in the post-conditioning and pre-conditioning tests, respectively. Positive values indicate preference and negative values aversion (Kitaichi et al. 1996). For non-biased procedures, where animals do not show an inherent preference for either compartment, results are presented simply as a difference score (i.e., time spent in the drug-paired compartment minus time spent in the vehicle-paired compartment).

In addition to place preference, others (Mucha and Herz 1985; Broadbent et al. 2002) used taste preference conditioning.

Cunningham (Bormann and Cunningham 1998; Gabriel et al. 2004) used the same chamber for training and testing with the exception that floor texture varied according to treatment condition. Thus, drug and vehicle were paired with different floor textures and during test sessions the time spent on each section of a floor comprising the two different textures (half of the floor with each) was used as an index of preference or aversion. This procedure has the advantage that the size of the test chamber is not different from the size of the training chamber.

Perks and Clifton (1996) used sucrose solution to generate a place preference which was subsequently devalued using a LiCl taste aversion procedure.

Brockwell et al. (1996) described a computerized system for the simultaneous monitoring of place conditioning and locomotor activity in rats consisting of 4 independent conditioning boxes, each equipped with 6 pairs of photosensors connected to an Experiment Controller, an electronic board containing a microprocessor and a programmable timer for storing both instructions and data.

Steinpreis et al. (1996) investigated place preference in Sprague Dawley rats treated with graded i.p. doses of methadone. Place preference for methadone peaked at 4 mg/kg and aversion was produced at 10 mg/kg.

Using the conditioned place preference paradigm, Mamoon et al. (1995) assessed the rewarding properties of butorphanol in comparison to morphine after unilateral microinjections into the ventral tegmental area of male Lewis rats.

Gaiardi et al. (1997) assessed rewarding and aversive effects of buprenorphine by place preference and taste aversion conditioning. After subcutaneous doses of 0.025, 0.050 and 0.100 mg/kg, buprenorphine caused a significant increase in the amount of time spent on the drug-paired compartment, but no significant decrease of saccharin consumption. Rewarding and aversive effects did not occur within a similar dose range.

Contarino et al. (1997) found no tolerance to the rewarding effects of morphine.

Tsuji et al. (1996) studied the effect of microinjections of GABA agonists and antagonists into the ventral tegmental area of Sprague Dawley rats on morphine-induced place preference.

Sufka (1994) recommended the conditioned place preference paradigm as a novel approach for assessing effects of opioids in chronic pain induced in rats by

unilateral injections of Freund's adjuvant into the hind paw.

Conditioned place avoidance was found after naloxone which was attenuated by clonidine (Kosten 1994).

In addition to morphine and other μ opioid agonists, other drugs with known or putative abuse liability were tested in the place conditioning paradigm, e.g. cocaine (Lepore et al. 1995; Suzuki and Misawa 1995; Calcagnetti et al. 1996; Martin-Iverson and Reimer 1996; Martin-Iverson et al. 1997), caffeine (Brockwell et al. 1991; Brockwell and Beninger 1996), cannabinoids (Lepore et al. 1995; Sañudo-Peña et al. 1997), LSD (Parker 1996), methamphetamine (Suzuki and Misawa 1995), amphetamine (Hoffman and Donovan 1995; Turenne et al. 1996), methylphenidate (Gatley et al. 1996), fenfluramine (Davies and Parker 1993), 7-OH-DPAT (Khroyan et al. 1995; Chaperon and Thiébot 1996), gamma-hydroxybutyric acid (Martellotta et al. 1997), propofol (Pain et al. 1997), and NMDA receptor antagonists (Steinpreis et al. 1995; Papp et al. 1996).

Furthermore, 5-HT_3 receptor antagonists (Acquas et al. 1990), 5-HT_3 receptor agonists (Higgins et al. 1993), dopamine release inhibitors (Schechter and Meehan 1994), D1 receptor antagonists (Acquas and Di Chiara 1994), D3 receptor preferring agonists (Khoyan et al. 1997), antiemetic agents (Frisch et al. 1995) were studied in the place-conditioning paradigm.

Suzuki et al. (1991, 1993) and del Pozo et al. (1996) studied opioid-induced place preference in and Bechtholt et al. (2004) studied the effects of handling on conditioned place aversion and conditioned place preference by ethanol in mice.

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Chapter I.K
Safety Pharmacology of Antiinflammatory Drugs

H. Gerhard Vogel

I.K.1 General Considerations

Gastrointestinal side effects are among the most frequent of the untoward or adverse reactions associated with orally ingested anti-inflammatory or anti-arthritic agents. Nonsteroidal anti-inflammatory agents, like indomethacin and acetyl-salicylic acid, induce gastric lesions in man and in experimental animals by inhibition of gastric cyclo-oxygenase resulting in less formation of prostacyclin, the predominant prostanoid produced in the gastric mucosa.

The risk of gastrointestinal ulceration, bleeding and even perforation with non-steroidal anti-inflammatory drug therapy is well known (Hawkey 1994; Lanas et al. 2003). The mechanisms by which these drugs cause gastro-intestinal irritation are complex (Rainsford 1989). Deleterious effects may result from local actions, which cause injuries to the submucosal capillaries with subsequent necrosis and bleeding, or from inhibition of the formation of protective prostaglandins.

Unfortunately, methods in safety pharmacology and safety toxicology were not able to predict the risk of serious cardiovascular events (myocardial infarction and stroke), which became apparent after treatment of patients for more than 18 months with a selective COX-2 antagonist (VIGOR study group, Bombardier et al. 2000, FDA Drug Information September 30, 2004). The mechanism of action of these effects remains to be clarified, since other long-term studies did not show an increased cardiovascular risk (Silverstein et al. 2000, Weir et al. 2003, Clark et al. 2004, Spektor & Fuster 2005, Mitchell & Warner 2006).

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I.K.2 Ulcerogenic Effect in Rats

PURPOSE AND RATIONALE

Gastric irritation properties of orally administered compounds are evaluated in fasted rats. Following treatment the animals are sacrificed after predetermined time in-

tervals. The stomachs are removed and inspected for irritation and ulcers.

PROCEDURE

Groups of 10 male Wistar rats with a weight between 150 and 175 g are used. They are starved 48 h (water ad libitum) prior to drug administration. The test drugs are administered orally in 10 ml/kg as aqueous solution or suspension. Doses are chosen which are highly active in the anti-inflammatory tests in rats. The animals are sacrificed 3, 5 or 7 h post drug. Control animals are sacrificed after 7 h. Stomachs are removed and placed on saline-soaked filter paper until inspection. A longitudinal incision along the greater curvature is made with fine scissors. The stomach is inverted over the index finger and the presence or absence of gastric irritation is determined. The presence of a single or of multiple lesions (erosion, ulcer or perforation) is considered to be positive. The number of ulcers and the occurrence of hyperemia is noted.

EVALUATION

The number of animals with one or more lesions of the stomach is calculated as a percentage of the animals of the test group. Running various doses, an ED_{50} value can be calculated. Standard compounds which produce gastric lesions are: Acetylsalicylic acid (10–100 mg/kg); naproxene (5–50 mg/kg); indomethacin (1–10 mg/kg).

CRITICAL ASSESSMENT OF THE METHOD

A good correlation between gastro-intestinal side effects in man and the ulcerogenic effects in rats has been found. The anti-inflammatory compounds of the pyrazolone type, like phenylbutazone, are almost devoid of these effects, whereas non-steroidal anti-inflammatory compounds of the classical phenyl acetyl type show ulcerogenic properties, which parallel their therapeutic effect.

MODIFICATION OF THE METHOD

Gastro-intestinal irritation afflicts not only the stomach but also the small intestine. These lesions are easier to detect after repeated doses than after a single administration. Therefore, the animals are treated once a day orally for 4 days. During this period, they have free access to food and water. On day 5 (24 h after the last dose being given), the animals are sacrificed and the stomachs and small intestines removed. A longitudinal incision along the lesser curvature is made. The stomach and the small intestine are rinsed in running water and the presence or absence of ulcers is determined.

A computer-planimetric analysis of the areas of lesions has been published by Szabo et al. (1985). The stomachs are formalin fixed *in situ* by oral dosing upon death and after opening are pinned on to a small cork base with mucosa upwards. The cork base and stomach are placed in a Petri dish under a stereomicroscope and illuminated from above with a cold-light source on flexible fiberoptic leads. An extension tube from the microscope projects the image from this onto an adjacent planimeter (Microplan II), which is essentially a digitized tablet with built-in microprocessor enabling data to be calculated for subsequent printout digitally. The output indicates area, number, length, or width of the lesions as totals.

In addition to evaluation of gastric lesions, Goburdhun et al. (1978) measured the decrease of intestinal tensile strength, which occurred after two oral doses of indomethacin but not of aspirin.

Alich et al. (1992) assessed the gastric mucosal damage due to aspirin and copper aspirinate by gastric mucosal potential difference changes.

Ford et al. (1995) assessed the intestinal permeability changes induced by non-steroidal anti-inflammatory drugs in the rat. A subcutaneous air pouch was formed by injection of 10 ml sterile air prior to the experiment. Five ml of a 0.4 % carrageenan solution were injected into the pouch simultaneously with a subcutaneous injection of various doses of the non-steroidal anti-inflammatory drug and the rats allowed access to food. After various time intervals, different markers were given orally and urine collected for 5 h. The use of [^{51}Cr]-EDTA as marker was found to be the most sensitive and reproducible method. The results correlated well with data of ulcer formation.

Scarpignato et al. (1995) evaluated NSAID-induced gastric mucosal damage by continuous measurement of gastric potential difference in the rat. The method allows simultaneous measurement of gastric potential difference and intragastric pH with an automatic data analysis.

Matsui et al. (2001) reported that the gastric mucosa in rats emits fluorescence of porphyrins at the onset of gastric lesions induced by diclofenac. HPLC analysis demonstrated that the fluorescent substances were mesoporphyrin and protoporphyrin.

Rainsford (1987) published an assay for gastro-ulcerogenic activity of non-steroidal anti-inflammatory drugs in cholinomimetic-treated **mice** using visual image analysis.

Johnston et al. (1995) studied the effect of misoprostol on aspirin-induced gastrointestinal lesions in **dogs**. Lesions were induced by 35 mg/kg aspirin p.o. tid. En-

doscopy was performed on days 0, 5, 14, und 30. Five regions of the upper gastrointestinal tract were qualitatively scored from 1 to 12 based on the presence of submucosal hemorrhage, erosion, or ulceration.

Owunwanne et al. (1988) evaluated technetium-99 m mercaptoacetylglycine for the detection and localization of gastrointestinal bleeding in a **sheep** model for the detection and localization of the site of gastrointestinal bleeding.

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I.K.3 Measurement of Gastric Mucosal Damage by Intragastric Inulin

PURPOSE AND RATIONALE

Wittmers et al. (1990) used intragastric inulin as a measure of mucosal damage caused by aspirin. The movement of inulin out of the gastric contents into the stomach wall and into the vascular compartment is an estimate of mucosal damage.

PROCEDURE

Adult male Sprague-Dawley rats are anesthetized with sodium pentobarbital and functionally nephrectomized by placing a ligature around both renal pedicles in order to prevent loss of inulin by renal clearance. One cannula is inserted in the carotid artery, another into the stomach. The duodenum is ligated 1 cm distal to the gastro-duodenal junction. The stomach is rinsed with saline and then the test solutions (total volume 2 ml/100 g body weight) are given intragastrally. The control solution contains 0.01 g/100 ml inulin and 0.30 μCi/ml ^{3}H-inulin. Test solutions contain the irritant, e.g. 100, 200, or 500 mg/kg aspirin. During the exposure period of 90 min blood is withdrawn in 15 min intervals. At the end of the exposure period a sample of gastric contents is obtained, the esophagus ligated at its junction with the stomach, and the animal sacrificed. The stomach is removed, opened along the lesser curvature, rinsed with saline, and pinned across a rubber stopper. Ten full thickness tissue samples are removed with a No. 2 cork bore in a predetermined pattern. The samples of tissue, plasma, and gastric content are digested and bleached with 0.2 ml 60 % perchloric acid and 0.5 ml 30 % hydrogen peroxide at a temperature of 80 °C for 20–30 min. Ten ml of liquid scintillation cocktail is added and ^{3}H-inulin is determined by liquid scintillation counting.

EVALUATION

The ^{3}H-inulin radioactivity is expressed as disintegrations per min per ml of plasma or per gram of tissue. Plasma ^{3}H-inulin levels being dose-dependent increased after treatment are plotted versus time in comparison with control. ^{3}H-Inulin content in the gastric wall is dose-dependent increased after treatment especially in the antrum region.

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I.K.4 Determination of Blood Loss

PURPOSE AND RATIONALE

Use of non-steroidal anti-inflammatory drugs induces micro bleeding, which is not detected by observation of overt ulcers. Moreover, determination of the amount of

lost blood may give a better quantitative assessment of gastrointestinal side effects.

PROCEDURE

Male Sprague-Dawley rats with a weight of 180–200 g are used. They are fasted in wire-floor cages for 24 h with free access to water. The animals receive the test drug or the standard compound (300 mg/kg acetylsalicylic acid) by gavage in 1 ml of 1 % Arabic gum. For determination of fecal blood loss, the rats are kept in individual metabolism cages and food is given to them 5 h after treatment. Feces from each rat are collected separately every 24 h for 5 days. Heme is extracted from the feces and determined spectrophotometrically. For determination of the site of gastrointestinal bleeding the rats are sacrificed 5 h after treatment by ether inhalation. The stomach is tied on both the esophageal and pyloric ends, removed and washed free of external blood. The stomach content is released into a graduated test tube. Then the stomach is rinsed with 5 ml of saline. Similarly, the intestine is cut into 3 portions, each being tied at both ends. The contents after flushing the intestines twice with saline are collected. In the fluid of stomach and intestine content heme is determined spectrophotometrically.

EVALUATION

Time course of blood loss in the feces and dose-depended increase of blood in the content of stomach and intestine are determined.

CRITICAL ASSESSMENT OF THE METHOD

The determination of the time course of blood loss and the site of bleeding is interesting for in depth investigations but has to be regarded as a secondary screen.

MODIFICATIONS OF THE METHOD

Davies et al (1994) induced small intestinal permeability by anti-inflammatory drugs in rats. After oral administration of ^{51}Cr-EDTA urinary excretion was increased by non-steroidal anti-inflammatory drugs. Misoprostol and sulfasalazin significantly reduced the effect of indomethacin.

Chan et al. (1995), Riendeau et al. (1997) used a fecal ^{51}Cr excretion assay in rats and primates to detect gastrointestinal integrity after application of a selective cyclooxygenase inhibitor.

Rainsford et al. (2003) described gastrointestinal mucosal injury following repeated daily oral administration of conventional formulations of indomethacin and other non-steroidal anti-inflammatory drugs to Landrace **pigs** as a model for human gastrointestinal disease. Gastrointestinal bleeding was measured by determination of radioactive iron in the faeces from ^{59}Fe-pre-labeled red blood cells. After a treatment period of 10 days, the animals were sacrificed and gastric and intestinal mucosal damage was determined by counting the number of mucosal lesions and ulcers and the area of mucosal injury with a millimeter rule. Myeloperoxidase was determined in randomly selected areas adjacent to the lesions.

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I.K.5 Determination of Specific COX-1 and COX-2 Inhibition

PURPOSE AND RATIONALE

The anti-inflammatory action of non-steroidal anti-inflammatory drugs rests on their ability to inhibit the activity of cycloogenase (COX) enzyme, which in turn results in dimished proinflammatory prostaglandins (Vane 1971; Hinz and Brune 2003). Cyclooxygenase metabolizes arachidonic acid to prostaglandin H_2, which serves as precursor for the biosynthesis of various prostaglandins, thromboxane and prostacyclin (Hamberg & Samuelsson 1973). COX activity originates from two distinct and independently regulated isoenzymes, COX-1 and COX-2 (Smith et al. 1996). COX-1 is a constitutive enzyme, whereas COX-2 is inducible and short-lived. COX-2 is the product of an immediate-early gene, and its expression is stimulated by many growth factors, cytokines and mitogens (Herschman 1996). COX-1 appears to be responsible for the biosynthesis of prostaglandins in the gastric mucosa and in the kidney, whereas COX-2 appears responsible for biosynthesis in inflammatory cells and the central nervous system (Needleman and Isakson

1997). Non-steroidal anti-inflammatory drugs inhibit the two isoforms to different extents, and this feature accounts for their shared therapeutic properties and side effects (Vane et al. 1998). The differential tissue distribution of the COX isoenzymes has provided a rationale for the development of COX-2-selective inhibitors as non-ulcerogenic, anti-inflammatory, and analgesic agents (Seibert et al. 1997; Chan et al. 1999; Goldenberg 1999; Leblanc et al. 1999; Prasit et al. 1999; Warner et al. 1999; Kalgutkar et al. 2000a,b; Riendeau et al. 2001; Ulbrich et al. 2002; Chintakunta et al. 2002; Banoglu et al. 2003; Hu et al. 2003; Khanapure et al. 2003; Rao et al. 2003; Uddin et al. 2004).

Several assays were described to characterize COX-1 and COX-2-inhibitors, such as in vitro COX enzyme assay (Seibert et al. 1994), COX-2 protein extraction and analysis (Anderson et al. 1996), a human whole blood assay using LPS-induced PGE_2 production as an index for cellular COX-2 activity (Riendeau et al. 1997) or whole-cell assays with transfected Chinese hamster ovary cells expressing COX-1 and COX-2 or COX-2 specific (osteosarcoma cells) and COX-1 specific (U937 cells) making use of PGE_2 production after arachidonic challenge as an index of cellular potency and selectivity of cyclooxygenase inhibitors (Chan et al. 1999).

PROCEDURE

In vitro Cyclooxygenase Inhibition

The ability of test compounds to inhibit COX-1 and COX-2 (IC_{50} values, μM) is determined using an enzyme immunoassay kit (Cyman Chemical, Ann Arbor, MI, USA, no. 560101) (Khanapure et al. 2003; Rao et al. 2003; Uddin et al. 2004). This COX (ovine) inhibitor screening assay directly measures $PGF_{2\alpha}$ produced in the cyclooxygenase reaction. The prostanoid product is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds to all the major prostaglandin compounds. Thus, this COX assay is more accurate and reliable than an assay based on peroxidase inhibition. The COX (ovine) inhibitor screening assay includes both ovine COX-1 and COX-2 enzymes in order to screen isozyme-specific inhibitors. This assay is an excellent tool, which can be used for general inhibitor screening, or to eliminate false positive leads generated by less specific methods.

Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid to PGH_2. $PGF_{2\alpha}$ produced from PGH_2 by reduction with stannous chloride, is measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells, since the concentration of PG tracer is held constant while the concentration of PGs varies. This antibody-PG complex binds to a mouse anti-rabbit monoclonal antibody that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent, which contains the substrate to acetylcholinesterase, is added to the well. The product of this enzymatic reaction produces a distinct yellow color that absorbs at 405 nm. The intensity of this color, determined spectrophotmetrically, is proportional to the tracer bound to the well, which is inversely proportional to the amount of PGs present in the well during the incubation. Percent inhibition is calculated by the comparison of compound treated to various control incubations. The concentration of the test compound causing 50 % inhibition (IC_{50}, μM) is calculated from the concentration-inhibition response curve.

Inhibition Studies with Recombinant Human COX-1 and COX-2

Microsomal preparations of recombinant human COX-1 and COX-2 are prepared from a vaccine virus-COS-7 cell expression system (O'Neill et al. 1994). Recombinant human COX-1 and COX-2 are expressed in baculovirus-Sf9 cells, and enzymes are purified (Ouellet and Percival 1995; Cromlish and Kennedy 1996). Enzymatic activity is monitored continuously by either a fluorescence assay measuring the appearance of the oxidized form of the reducing agent cosubstrate homovanillic acid or by oxygen consumption.

Classical NSAIDs and COX-2 inhibitors are time-dependent, irreversible inhibitors of hCOX-2, which is consistent with a two-step process, involving an initial rapid equilibrium binding of enzyme and inhibitor, followed by a slow formation of a tightly bound enzyme-inhibitor complex. COX-2 inhibitors show a time-independent inhibition of hCOX-1, consistent with the formation of a reversible enzyme-inhibitor complex (Ouellet and Percival 1995; Riendeau et al. 2001).

HPLC Assay for Oxygenation of Radiolabeled Arachidonic Acid by COX-1

Purified recombinant human COX-1 (50 μl of 1 μg/ml in 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol, 1 μM hematin) is preincubated with 2 μl of

the inhibitor solution (50 fold concentrated stock in DMSO, 0–2.5 mM) for 15 min. The reaction is then initiated by the addition of 5 µl of 1 µM [^{14}C]-arachidonic acid (0.005 µCi) to obtain a final concentration of 0.1 µM. After 7 min incubation at room temperature, the reaction is stopped by the addition of 5 µl 1 M HCl and 50 µl acetonitrile. Aliquots of 50 µl of each reaction mixture are analyzed for substrate conversion by reverse phase HPLC onto a C-18 Nova-Pak column (3.9 × 150 mm) which is developed with acetonitrile/water/acetic acid (85:15:0.1) at 2 ml/min. Arachidonic acid metabolites and arachidonic acid eluted at 0.6–1 min and 2.2–2.5 min, respectively, are quantitated by a Packard radiochromatography detector. Percentages of inhibition are calculated from the difference in conversion of arachidonic acid to prostaglandin metabolites between inhibitor-treated samples and controls exposed to DMSO vehicle.

Determination of the Stoichiometry of Inhibitor Binding

Aliquots of purified COX-2 (0.25 mg/ml, concentration of subunit of 3.4 mM) are incubated in buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol) in the presence of various inhibitors (0–8 µM) for 15 or 30 min. An aliquot (20 µl) is then removed for determination of the cyclo-oxygenase activity, which is monitored continuously by oxygen consumption by a Clark-type polarographic oxygen probe. The oxygen chamber is filled with 0.6 ml of reaction buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 µM hematin,1 mM phenol, 100 µM arachidonic acid at 30°or 37 °C) and the reaction is initiated by the addition of 20 µl of a solution of 4 mM hydrogen peroxide and 0.5 mM *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD) in assay buffer. Enzyme concentration is determined by amino acid concentration following acid hydrolysis.

Determination of the Dissociation Rate Constant of the Enzyme-Inhibitor Complex

Purified COX-2 (2.0 nmol, 2 ml) in 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 2 µM hematin, 0.1 % β-octylglucoside is treated with 2.0 nmol [^{14}C]-DFU (18 Ci/mol) and incubated at 20 °C for 3 h. A control (0.7 ml) is removed and 13 nmol unlabeled DFU is added to the remaining 1.3 ml of the mixture containing COX-2 and [^{14}C]-DFU. At timed intervals, 0.1 ml (in duplicate) is transferred to a Microcon-30 micro concentration device (Amicon) and the free inhibitor is separated from enzyme-bound inhibitor by centrifugation at 14 000 g for 6 min at 4 °C. Buffer (0.1 ml) is added to the retentate and the centrifugation repeated. The filtrate and retentate are then removed and mixed with 10 ml scintillation fluid and counted in a liquid scintillation counter.

An aliquot of purified COX-2 (1.0 nmol) is treated with 1.25 mol equivalents of inhibitor or with DMSO vehicle control and incubated at 20 °C for 1 h. The enzyme-inhibitor mixture is then transferred to a Pierce Microdialyzer 100 apparatus and dialyzed continuously against 2 l of buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1 mM phenol, 0.1 % octylglucoside) for 5 h at 22 °C during which aliquots are moved and frozen at <70 °C until assayed for cyclyo-oxygenase activity by oxygen uptake as described above.

Recovery of Inhibitor from the COX-2-Inhibitor Complex

Purified COX-2 (0.79 nmol) is treated with 1.0 mol equivalent of inhibitor and the mixture is incubated for 60 min at room temperature. The remaining activity at this time is 4 % that of a vehicle-treated control. The sample is then divided in two and the protein denaturated by treatment with four volumes of ethyl acetate/methanol/1 M citric acid (30:4:1). After extraction and centrifugation (10 000 g for 5 min), the organic layer is removed and the extraction repeated. The two organic layers are combined and dried under N_2. The extract is dissolved in 10 µl of HPLC solvent mixture consisting of water/acetonitrile/acetic acid (50:41:0.1) and 50 µl are injected onto a Novapak C-18 column (3.9 × 150 mm) and developed at 1 ml/min. The inhibitor is detected by absorption at 260 nm and eluted with a retention time of 6.6 min in this system. Control experiments for inhibitor recovery are performed with incubation of the inhibitor in the absence of enzyme and processing of the samples in an identical fashion before quantitation by HPLC.

Spectrophotometric Assay of Recombinant Human COX-2

Enzymatic activity of the purified COX-2 is measured using a chromogenic assay based on the oxidation of *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2 (Copeland et al. 1994; Chan et al. 1999; Chintakunta et al. 2002). The assay mixture (180 µl) contains 100 mM sodium phosphate, pH 6.5, 1 µM hematin, 1 mg/ml gelatin, 2 to 5 µg/ml of purified COX-2, and 4 µl of the test compound in DMSO. The assay is also performed in the presence of the detergent Genapol X-100 at a final concentration of 2 mM. The mixture is preincubated at room temperature (22 °C) for 15 min before the

initiation of the enzymatic reaction by the addition of 20 µl of a solution of 1 mM arachidonic acid and 1 mM TMPD in assay buffer (without enzyme or hematin). For assays in the presence of Genapol, the arachidonic acid and TMPD solution is prepared in 50 % aqueous ethanol. The enzyme activity is measured by estimation of the initial velocity of TMPD oxidation over the first 36 s of the reaction as followed from the increase in absorbancy at 610 nm. A low rate of non-enzymatic oxidation is observed in the absence of COX-2 and is subtracted before the calculation of the percentage of inhibition.

Whole-Cell Assays with Transfected Chinese Hamster (CHO) Cells Expressing COX-1 and COX-2

Stably transfected CHO cells expressing human COX-1 and COX-2 are cultured and assayed for the production of PGE2 after stimulation with arachidonic acid (Kargman et al. 1996; Riendeau 2001). Cells (0.3×10^6 cells in 200 µl) are pre-incubated in HBSS containing 15 mM HEPES, pH 7.4, with 3 µl of the test drug or DMSO vehicle for 15 min at 37 °C before challenge with arachidonic acid. Cells are challenged for 15 min with an arachidonic acid solution (10 % ethanol in HBSS) to yield final concentrations of 10 µM arachidonic acid in the CHO[COX-2] assay and 0.5 µM arachidonic acid in the CHO[COX-1] assay. In the absence of addition of exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] are < 30 pg $PGE_2/10^6$ cells. In the presence of 0.5 µM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] cells increase to 260 to 1500 pg $PGE_2/10^6$ cells. After stimulation with 10 µM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-2] cells increase from < 120 to 700 to 1600 pg $PGE_2/10^6$ cells. Compounds are tested in eight concentrations in duplicate using 3-fold serial dilutions in DMSO. COX activity in the absence of test compounds is determined as the difference in PGE_2 levels of cells challenged with arachidonic acid versus PGE_2 levels in cells mock-challenged with ethanol vehicle.

Arachidonic acid-dependent production of PGE_2 is measured in both cell lines after addition of test drugs. Indomethacin shows similar IC_{50} values in both CHO[COX-1] and CHO[COX-2] cells, whereas specific COX-2 inhibitors show a 1000 fold specificity.

Assays with Murine Macrophages

Mitchell et al. (1994), Hu et al. (2003), Joo et al. (2004) used mouse peritoneal macrophages for evaluation of COX-2 inhibitors.

Adherent peritoneal macrophages were harvested from the peritoneal cells of male C5BL-6J mice after intraperitoneal injection of brewer thioglycollate medium (5 ml/100 g body weight for 3 days. The peritoneal cells obtained from 3–4 mice were mixed and seeded in 48 well culture cluster at a cell density of 1×10^9 cell/L in RPMI-1640 supplemented with 5 % newborn calf serum, penicillin and streptomycin. After settlement for 2–3 h, non-adherent cells were washed by D-Hanks' balanced salt solution. Then macrophages were cultured in RPMI-1640 without serum. Almost all of the adherent cells were macrophages as assessed by Giemsa staining. Cell viability was examined by trypan blue dye exclusion. All incubation procedures were performed with 5 % CO_2 in humidified air at 37 °C.

COX-1 Assay: Macrophages were incubated with test compound at different concentrations or solvent (Me_2SO) for 1 h and were stimulated with calcimycin 1 µmol/l for 1 h. The amount of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) in supernatants was measured by RIA according to manufacturer's guide. The inhibitory ratio was calculated as

$$IR = \frac{Cs - Ct}{Cs - Cc}$$

Cs, *Ct*, *Cc* refer to 6-keto-$PGF_{1\alpha}$ concentration in supernatants of calcimycin, test compound, and control groups, respectively.

COX-2 assay: Macrophages were incubated with test compound at different concentrations or solvent (Me_2SO) for 1 h and were stimulated with LPS 1 mg/L for 9 h. The amount of PGE_2 in supernatants was measured by RIA. The inhibitory ratio was calculated using the same formula as in COX-1 assay section. *Cs*, *Ct*, *Cc* refer to PGE_2 concentration in supernatants of LPS, test compound, and control groups, respectively.

Statistical analysis data were expressed as the mean ± SD of more than three independent experiments. Dose-inhibitory effect curves were fit through 'uphill dose–response curves, variable slope' using Prism, GraphPad version 3.00:

$$Y = \frac{1}{1 + 10^{\lceil (\log IC_{50} - X) \times Hillslope \rceil}}$$

Whole-Cell Assays with Osteosarcoma Cells (COX-2) and U937 Cells (COX-1)

The human osteosarcoma cell line has been shown selectively to express COX-2 by reverse transcription-polymerase chain reaction and immunoblot analysis, whereas undifferentiated human lymphoma U937 cells

selectively express COX-1. The production of PGE_2 by these cells after arachidonic acid challenge is used as an index of cellular COX-2 and COX-1 activity, respectively. (Chan et al. 1999). Test substances are preincubated for 5 to 15 min with the cells under serum-free conditions (HBSS) before a 10-min stimulation with 10 μM arachidonic acid and measurement of PGE_2 production (Wong et al. 1997). COX activity in each cell line is defined as the difference in PGE_2 concentrations in samples incubated in the presence or absence of arachidonic acid.

Human Whole Blood Assay

For the COX-2 assay, fresh heparinized human whole blood is incubated with lipopolysaccharide from *E. coli* at 100 μg/ml and with 2 μl of vehicle or a test compound for 24 h at 37 °C (Brideau et al. 1996). PGE_2 levels in the plasma are measured using radioimmunoassay after deproteination. For the COX-1 assay, an aliquot of fresh blood is mixed with either DMSO or test compound and is allowed to clot for 1 h at 37 °C. TBX_2 levels in the serum are measured using an enzyme immunoassay after deproteination.

MODIFICATIONS OF THE METHOD

Young et al. (1996), Khanapure et al. (2003), Shin et al. (2004) used a similar assay to determine COX-1 and COX-2 enzyme activity in human whole blood. Human blood from non-fasted donors, who had not taken any aspirin or NSAIDs for 14 days, was collected in sodium heparin and distributed in 1 ml aliquots per well in a 24-well tissue culture plate. The plate was placed on a gently rotating platform shaker in a 5 % CO_2 incubator at 37 °C for 15 min. Test compounds were dissolved and diluted in DMSO and 1 μl of each dilution of test compound was added per well in duplicate wells. To induce COX-2, lipopolysaccharide from *E. coli* was added at 10 μg/ml to appropriate wells 15 min after addition of the test compounds. For the stimulation of COX-1, the calcium ionophore A23187 was added to a final concentration of 25 μM to separate wells 4.5 h after the addition of the test compounds. At 30 min after addition of A23187 or 5 h after LPS addition, all incubations were terminated by cooling on ice and adding EGTA to a final concentration of 2 mM. The blood samples were then transferred to 15 ml polypropylene centrifuge tubes and centrifuged at 1200 g for 10 min at 4 °C. One hundred microliters of plasma was removed from each blood sample and added to 1 ml of methanol in a 15 ml polypropylene centrifuge tube, mixed vigorously, and stored overnight at –20 °C. The next day, the samples were centrifuged at 2000 g for 10 min at 4 °C, and the supernatants were transferred to glass tubes and evaporated to dryness. After reconstitution with EIA buffer, and appropriate dilution (2000-fold for COX-1 and 500-fold for COX-2), the samples were assayed for TBX^2 using EIA kits (Cayman Chemical Co, Ann Arbor, MI) in duplicate wells.

Similar methods were used by Patrignani et al. (1994), Puig et al. (2000), Banoglu et al. (2003), Caturia et al. (2004).

Berg et al. (1997) developed a cell assay system using the human erythroleukemic cell line HEL as a source for COX-1 and the human monocytic cell line Mono Mac 6 as a source for COX-2.

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Chapter I.L
Safety Pharmacology of Drugs with Osteoarthritis-Related Activity

Ruth Raiss

I.L.1
Cartilage Matrix Turnover in vitro

I.L.1.1
Modulation of Chondrocytic Proteoglycan Metabolism

PURPOSE AND RATIONALE

Several agents and therapeutic principles addressing musculoskeletal disorders, applied either systemically or intraarticularly, have been discussed to potentially impair the integrity of articular cartilage (Raynauld et al. 2003; Adams et al. 2000; Theiler et al. 1994). Therefore, a reliable cellular assay is of interest to detect chondrodeleterious properties of a test drug in its early developmental stages.

In primary cultures, articular chondrocytes grown in an artificial matrix after digestion of the original one, maintain their characteristic synthesis and turnover rate of cartilage matrix macromolecules for a long time. These metabolic processes can be influenced pharmacologically. In the following assay, compounds are tested for their effect upon the normal turnover of cartilage matrix by chondrocytes. The test is used to detect beneficial effects like stimulation of matrix formation, but also to check for potential impairment of cartilage function. Specific matrix staining reveals the amount of newly formed matrix remaining around the cells at the end of treatment.

PROCEDURE

Reagents

A 1 % (w/v) solution of Pronase from Boehringer in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10 % FCS.

A 0.025 % (w/v) solution of Collagenase type II, activity 242 U/mg, from Worthington, corresponding to an activity of 6 U/ml in Ham's F12 is prepared, filtered under sterile conditions, and supplemented with 10 % FCS.

Hank's balanced salt solution (HBSS) is obtained from Biochrome.

Ham's F12 is supplemented with 50 μg/ml gentamycine and 2.5 μg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

A 2 % solution of low melting agarose from Seaplaque in 0.9 % NaCl is prepared by heating in a microwave, and stored in a water bath at about 50 °C.

A buffer of 25 mM sodium-acetate (2.051 g/l) with 0.4 M magnesium-chloride 6-hydrate (81.32 g/l) is prepared, and adjusted with acetic acid to pH 5.6.

A staining solution is prepared with 0.1 g of alcian-blue, obtained from Sigma, in 67.5 ml buffer, filtered, and supplemented with 10 ml of a 25 % solution of glutaraldehyde.

An 8 M guanidinium-hydrochloride solution is prepared.

Tissue and Cell Preparation

Fetlocks of freshly slaughtered steers (age 18 to 20 months) are skinned and the metacarpo-phalangeal joint opened under semi-sterile conditions. With a sterile scalpel, articular cartilage is then carefully removed from the underlying bone from all accessible cartilaginous regions and transferred into a sterile Ham's F12 solution at +4 °C. The tissue is washed with Ham's F12 to remove adherent synovial fluid. The pieces are then transferred into a 150 ml trypsinizing flask, containing the pronase solution including the added serum, and incubated with gentle stirring for 1 h at 37 °C and 95 % humidity. The fluid is then removed, and the collagenase solution including the added serum is incubated with gentle stirring overnight. The resulting cell suspension is first filtered through a 90 μm and then a 50 μm Nylon filter and then centrifuged at 800 rpm for 10 min. Resuspension and washing is performed with HBSS and cells are counted and checked for vitality under the microscope using the Eosin staining. The vitality level should reach at least 95 %. A cell suspension is prepared of 4×10^6 cells/ml Ham's F12 supplemented with 20 % FCS.

To prepare the agarose cell cultures, 24 well plates are coated with 0.2 ml/well of a 1:1 mixture of the 2 % agarose solution with preheated Ham's F12 and left at room temperature to gel. Then 0.1 ml/well (0.2 ml/well for radiolabeling) of a 1:1 mixture of the above described cell suspension with the 2 % agarose solution is added. Care has to be taken to maintain an even cell suspension, and not to overheat the cells during this procedure. After gel formation at room temperature, the multiwell plates are placed in the incubator, and 0.5 ml/well medium is added either 4 h later or the following day. The medium consists of Ham's F12 supplemented with 5 % FCS and 25 μg/ml ascorbic acid, and is changed every second day.

Assay

The assay starts 5 days after cell preparation. Compounds are added to the medium in a final concentration of 10 μM with 6 to 8 replicae per compound, and added anew with each change of medium over a total period of 8 days. The concentration can be varied according to the expected potency of the drug studied. An untreated control group as well as standard compound groups are always included. As standard compound, pentosane polysulfate to check for matrix increase, or retinoic acid to cause matrix decrease, can be applied.

At the end of treatment, the medium is removed, the wells washed 3 × with 500 μl of medium without supplements, and 1 ml/well of the staining solution is added for 48 h. After removal of the supernatant, the following washing steps are performed for 10 min each:

3 × 500 μl/well: 3 % acetic acid,
1 × 500 μl/well: 3 % acetic acid in 25 % ethanol,
2 × 500 μl/well: 50 % ethanol,
1 × 500 μl/well: 70 % ethanol.

With 500 μl/well of 8 M guanidinium hydrochloride solution, the bound stain is then extracted for 24 h at +4 °C. After shaking the plates gently for 10 min, 100 μl/well of each supernatant is then transferred to round-bottom microtiter-plates, and the extinction photometrically assessed in the plate-reader at a wavelength of 610 nm.

EVALUATION

The extinction is expressed in percentage as staining density with the control values defined as 100 %. Values above 110 % are interpreted as stimulation of matrix formation, values lower than 80 % as inhibition of matrix formation. Experiments with 8 wells/treatment usually exhibit a standard deviation below 7 %.

CRITICAL ASSESSMENT OF THE METHOD

The described method is suitable to compare up to 50 drugs in one experiment. The price for this is the limited quantification, as the staining is not strictly stoichiometric, and does not allow the distinction between matrix synthesis and degradation. For more detailed assessment, radiolabeling is the better choice. The limitation of these primary culture assays lies in the elaborate preparation and isolation of the chondrocytes. Several attempts to immortalize this differentiated mesenchymal cell type have so far resulted in the loss of certain cartilage-specific properties.

MODIFICATIONS OF THE METHOD

The agarose culture system for chondrocytes, originally described by Benya and Schaffer (1982), has been well characterized by Aydelotte et al. (1988, 1992), and the effects of different agarose densities have been studied by Verbruggen et al. (1990).

Instead of agarose gel cultures, some authors use 3D chondrocyte clusters in suspension (Bassleer et al. 1990, 1992; Henrotin et al. 1992), or suspensions over agarose (Archer et al. 1990), or embedded in collagen gels (Malemud et al. 1994).

Alternatively, encapsulation in alginate beads, either directly after isolation (Guo et al. 1989), even as primary culture for several months (Häuselmann et al. 1994), or after expansion in monolayers (Bonaventure et al. 1994), offers the opportunity to recover the chondrocytes later by depolymerization of the alginate.

Monolayers of articular chondrocytes can be used as well, but preferentially short-term (up to three days of culture), as under this culture condition chondrocytes tend to dedifferentiate to a fibroblast-like appearance and metabolic program. Authors using this modification are e.g. Kolibas and Goldberg (1989), Lane et al. (1992), and McCollum et al. (1991). The importance of culture conditions is addressed in the study by Seid et al. (1993), and that of culture duration in the paper by van der Kraan et al. (1992).

A dot blot assay by cuprolinic blue precipitation has been described by Jortikka et al. (1993), which is restricted to serum free conditions.

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I.L.1.2 Effects on Matrix Degradation and Maintenance in Cartilage Explants

GENERAL CONSIDERATIONS

Chondrocytes vary in their metabolic activity and responses to cytokines depending on their relative location in the joint (surfacial vs deep zone, weight-bearing vs non-weight-bearing area, etc.). Therefore, cellular assays represent a blend of an originally heterogeneous cell population. Several reasons suggest a verification of the obtained cellular results in tissue culture assays: First, chondrocytes are more reactive after isolation compared to those in tissue culture, which may lead to false positive results. Second, intact cartilage matrix acts as barrier for certain compounds of high molecular size and fixed charge, so that they

may not reach their target cells. Third, explants in longterm culture over several weeks allow also to check for impairment of the collagen network, which exhibits more subtle reactions than proteoglycans, but is of equal importance for matrix stability. Forth, biomechanical forces are an important component of the chondrocytic microenvironment, although only recently included in the design of a pharmacological in vitro model. Therefore, explant assays are recommended as either an alternative or a follow-up to the cellular tests.

I.L.1.2.1 Interleukin-1-Induced Proteoglycan Loss in Articular Cartilage Explants

PURPOSE AND RATIONALE

Among the most common agents (mono-iodoacetate, retinoic acid, interleukins, tumor necrosis factor α and others), used to induce matrix degradation in cartilage, interleukin-1 is still regarded the most relevant one to induce an osteoarthritic-like tissue reaction in vitro. For considerations of drug safety, the well-characterized and standardized system of bovine articular explants is rivalled by the expectance of higher predictability using explant specimen of human origin.

PROCEDURE

Reagents

Ham's F12 medium is supplemented with 50 μg/ml gentamycin and 2.5 μg/ml amphotericin B.

A sterile stock solution of 5 mg/ml ascorbic acid in Ham's F12 is prepared, and aliquots are stored at –20 °C.

Human recombinant interleukin-1 (IL-1) α or β from Genzyme is stored in aliquots at –20 °C.

For radiolabeling, $Na_2{}^{35}SO_4$ is purchased from Amersham.

A 4 M and 8 M guanidinium-hydrochloride (GuHCl) solution is prepared.

Tissue Preparation

Fetlocks of freshly slaughtered steers (age 18 to 20 months) are skinned and the metacarpo-phalangeal joint opened under semi-sterile conditions. With a sterile punch (as used for obtaining skin biopsies) full thickness disks of cartilage are obtained from all accessible cartilaginous areas and their wet weight is assessed. In each well, 1 ml of medium is added, consisting of Ham's F12 supplemented with 10 % FCS and 25 μg/ml ascorbic acid, and approximately 30 mg wet weight of cartilage are transferred corresponding to 3 discs of 4 mm diameter.

Assay

The assay is started 1 to 2 days after tissue preparation. Except for a control group, interleukin-1α is added in a concentration of 8 U/well, and with each of the following medium changes (every second day). Except for an IL-1-control group, compounds are added to the medium in a final concentration of 10 μM with 6 to 8 replicae per compound, which are also added with each medium change. The concentration can be varied according to the expected potency of the drug studied. At the end of an 8 day treatment, the medium is replaced by a medium containing 15 μCi $Na_2{}^{35}SO_4$/well and incubated for 24 h. The supernatant is removed, mixed 1:1 with 8 M GuHCl, and separated with a PD10-Sephadex column into free versus incorporated sulfate. The explants are washed three times with Ham's F12 at +4 °C, and extracted with 1 ml/well 4 M GuHCl supplemented with inhibitors for 48 h, and then a second time with 0.5 ml/well for 24 h. Both fractions are mixed, and separated with a PD10 Sephadex column into free versus incorporated sulfate. The samples containing the incorporated sulfate derived from medium as well as explant extraction are then mixed with scintillating fluid and assessed in a β-scintillation counter.

EVALUATION

Counts per minute (cpm) from medium and explant fractions are calculated related to mg wet weight of cartilage of the respective wells. They are either added if total incorporation is measured, or left separately, in case the ratio between released versus retained matrix is of interest. The data are converted into percent incorporation in comparison with the values of the untreated control or of the IL-1 control group serving as 100 %.

CRITICAL ASSESSMENT OF THE METHOD

Punched discs of similar size standardize the surface/volume ratio, and give more reproducible results than chips of cartilage obtained by scalpel dissection. A disadvantage is the greater amount of cartilage needed, which makes it unsuitable for human tissue obtained from joint replacement surgery.

When using human tissue, IL-1β is the more effective stimulus, and, depending on the disease stage of the tissue, often lower amounts are needed. Because of the striking differences observed between specimens from

surgery or postmortem, between different joints (hip vs. knee), and different ages of the donor (young or adolescent vs. 35 years and older), as well as different stages of severity and the duration of degenerative joint diseases, in cases of surgical specimens, interpretation and comparison of results should be restricted to the same source.

MODIFICATIONS OF THE METHOD

The effect of serum concentrations on proteoglycan synthesis has been studied by McQuillan et al. (1986), serum-free conditions with CR-ITS as a substitute were administered by Steinmeyer et al. (1998), and the effect of different concentrations of DMSO and glycerol, of importance for cryopreservation, has been examined on human fetal hip cartilage by Yang and Zhang (1991).

Some authors (Nixon et al. 1991; Adcocks et al. 2002) also use bovine nasal septum as cartilage source, but the convenient homogeneity and mass of this tissue is outweighed by a matrix composition and biomechanical properties clearly distinct from articular cartilage.

Several authors use human cartilage from joint replacement surgery (e.g. Pelletier et al. 1989; Pelletier and Martel-Pelletier 1989), and some compare drug effects upon visually normal cartilage to those with fibrillated or osteoarthritic cartilage (Lafeber et al. 1992, 1993; Verbruggen et al. 1989, 1990).

A step towards organ culture represents the culture of full thickness cartilage with subchondral bone, cultured for 24 h on moist lens tissue (Chayen et al. 1994).

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I.L.1.2.2
Collagenolytic Activity in Bovine Nasal Cartilage Explants

PURPOSE AND RATIONALE

Although proteoglycan loss is the more obvious and more readily occurring event in the disease process, alterations in the collagenous network appear of much higher irreversibility, and, even when progressing slower and more subtle, leading to a final collapse of weight-bearing areas in cartilage. In the nasal cartilage culture system, the degradation and loss of these two major matrix components can be studied separately: In the presence of interleukin-1, proteoglycans are released in the first week of culture, whereas collagenolytic activity becomes apparent only in the third week, and almost complete dissolution of the collagen occurs in week 3 or 4 (Kozaci et al. 1997). A three (Pratta et al. 2003) to four (Adcocks et al. 2002) week nasal cartilage culture in the presence or absence of interleukin-1 therefore allows to check for a potential collagenolytic drug activity in cartilage.

PROCEDURE

Disks of approximately 1 mm^3 are punched out of the nasal septum cartilage of freshly slaughtered steers. Three disks per well in a 24-well plate are incubated overnight at 37 °C in control medium. Control culture medium consists of Dulbecco's modified Eagle medium (DMEM) containing 25 mM HEPES, supplemented with 2 mM glutamine, 100 μg/ml streptomycin, 2.5 μg/ml gentamycin, 100 units/ml penicillin, and 40 units/ml nystatin, all reagents available either from Sigma, BDH Chemicals (Poole, UK), Gibco (Paisley UK), or Fisher Scientific (Loughborough, UK) (Cawston et al. 1995). Fresh control medium with or without

test reagents is added at the begin of the experiment, and anew with each medium change, either every 2nd or 3rd day (Raiss unpublished), or in a weekly interval (Milner et al. 2001). At the end of the experiment, the degraded collagen is assessed photometrically as amount of hydroxyproline either deriving directly from the supernatant, or, after papain digestion, from the explant tissue. In this hydroxyproline assay (Pratta et al. 2003), 50 µl of medium or 10 µl of cartilage digest are hydrolyzed with 50 µl 12 N HCl for 18 h at 100 °C, the hydrolysate then dried over NaOH pellets. After dissolution in water and transfer to a 96 well plate, assay buffer in a ratio 3:2:10 propanol/water/pH 6 buffer is added, shaking until complete solubilization, then adding 40 µl of a solution containing 0.282 g of chloramine T in 1 ml water, 1 ml 1-propanol and 8 ml of pH 6 buffer. After shaking for 15 min at room temperature, 80 µl of DMBA reagent (2 g dimethylaminobenzaldehyde, 1.25 ml 1-propanol, 2.75 ml perchloric acid) are added, and the plate incubated for 20 min at 70 °C. After cooling, absorbances are determined at 570 nm using a microplate reader, and then converted to ng hydroxyproline based on a standard curve.

EVALUATION

Collagen release or retained denatured collagen respectively is measured as a function of drug concentration, and is related to the control experiments without drug, but with and/or without induction of increased release.

CRITICAL ASSESSMENT OF THE METHOD

Although the matrix architecture of nasal cartilage greatly differs from articular specimen, this limitation is more than compensated by the opportunity to study the drug interaction with both matrix components, the proteoglycans as well as the collagens, in one longterm culture system of a topographically uniform and standardized tissue.

MODIFICATIONS OF THE METHOD

In nasal as well as articular cartilage explants, several authors choose either retinoic acid or rTNFα to induce proteoglycan or collagen release (Gendron et al. 2003; Sugimoto et al. 2004), and Sugimoto et al. (2004) use retinoic acid to also induce hyaluronic acid release from cartilage explants, measured in the supernatant by an HA-test from Pharmacia, which utilizes an iodinated HA binding protein for detection.

A highly specific method to determine hydroxyproline by liquid chromatography (LC) and mass spectrometry (MS) has been developed by Kindt et al. (2003), and the sample preparation, instrumentation, procedure, and quality control is described there in detail.

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I.L.1.2.3 Biomechanically Induced Matrix Degradation in Cartilage Explants

PURPOSE AND RATIONALE

Whereas the importance of biomechanical load for chondrocytes and articular cartilage have been recognized and studied in many respects, e.g. cell mechanics and biophysics (Mow et al. 1994), matrix synthesis and degradation (Larsson et al. 1991; Urban 1994), the shift in balance of intracellular organelles involved in synthesis and transport (Szafranski et al. 2004), or changes in gene expression patterns (Fitzgerald et al. 2004); and although computer-controlled devices have been designed to tightly control the biophysical microenvironment (Aspden et al. 1991), only recently a computer-controlled mechanically-based culture system has been developed for longterm survival of the biopsies and pharmacological purposes specifically (Steinmeyer 1997).

PROCEDURE

In a surgical grade titanium specimen holder allowing for a 8 ml medium volume and a cartilage disc of up to 10 mm in diameter, confined cyclic intermittent load is applied electropneumatically by a displacement transducer system to a porous glass load platen, to the effect that no statical compression of the explant occurs (Steinmeyer 1997). Six individual explant chambers comprise one unit to be placed in the incubator, and several units can be subjected simultaneously to a given load protocol or an unloaded condition as control. By stepwise variation of each parameter separately, load amplitude (0.1 to 1 MPa), duration of unloading period (10 to 1000 sec), as well as total duration of loading (1 to 6 days), for each condition the viability of superficial zone chondrocytes and the amount of cartilage compression can be determined (Steinmeyer et al. 1997). In subsequent studies a defined load protocol can then be identified, by which mild osteoarthritic-like changes are induced in respect to proteoglycan metabolism (Steinmeyer et al. 1999; Sauerland et al. 2003a), fibronectin metabolism (Wolf et al. 2003), and sulfation patterns of chondroitin sulfate chains (Sauerland et al. 2003b). For bovine articular cartilage explants this protocol consists of an intermittent cyclic loading at 0.5 MPa in a frequency of 0.1 Hz for 10 sec, followed each by a loadfree period of 100 sec, for a total duration of 6 days. Protocols for cartilage explants from other sources or species have to be optimized accordingly.

Fetlocks of freshly slaughtered steers (age 18 to 20 months) are skinned and the metacarpo-phalangeal joint opened under semi-sterile conditions. With a sterile punch (as used for obtaining skin biopsies) full thickness disks of cartilage are obtained from weightbearing cartilaginous areas. After their wet weight is assessed, the thickness of explants is then determined twice in the center of the cartilage using a digital caliper with a resolution of 0.01 mm and an accuracy of $\pm$ 0.02 mm in order to determine the degree of compression during loading. Care is taken that plugs from corresponding areas are assigned pairwise to treatment and control conditions alike. In each chamber, 1 cartilage disk is transferred with the articular surface upward, and cultured in 2.5 ml Ham's F-12 nutrient media supplemented with 2.5 mM HEPES, pH 7.2, containing 30 μg/ml alpha-ketoglutarate, 300 μg/ml L-glutamine, 50 μg/ml ascorbate, 1.0 mM Na_2SO_4, 20 U/ml penicillin, 10 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 50 μg/ml gentamycin, 485 μg/ml $CaCl_2 \times 2H_2O$, and 1 % (v/v) of the serum substitute CR-ITS^{+TM} Premix (Collaborative Biomedical Products, Bedford, MD, USA). The addition of the CR-ITS^{+TM} Premix results in final concentrations of 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml albumin, and 5.35 μg/ml linoleic acid.

Mechanical loading starts on day 0 since previous studies ascertained that the main parameters measured remain constant over a 10 day culture period as compared to the level at day 0 (Steinmeyer et al. 1999). Explants are loaded perpendicular to their longer axis in radially-unconfined compression. During the period of unloading, the load plate is lifted from the cartilage surface. Unloaded cartilage discs of the same condyle are cultured in identically constructed loading chambers, and serve as controls. The degree of compression of cartilage explants during loading is measured using a displacement transducer system (Steinmeyer 1997).

Media are changed on day 3 and collected media stored frozen at –20 °C in the presence of a 10 % (v/v) protease inhibitors mixture containing 10 mM PMSF (phenylmethylsulfonyl fluoride), 200 mM EDTA (ethylenediaminetetraacetic acid), 16 mM benzamidine/HCl, 100 mM N-ethylmaleimide and 15 μM pepstatin A until analysis. During the final 18 h of the experiments, cartilage explants are radiolabeled with 10 μCi/ml [^{35}S]-SO_4 (DuPont de Nemours GmbH, Bad Homburg, Germany). At the end of the loading and radiolabeling period, media are harvested and stored frozen at –20 °C in the presence of the 10 % (v/v) protease inhibitors mixture. Cartilage explants are washed three times with GBSS (Gey's balanced salt solution) to remove unincorporated radioisotope, then frozen at –20 °C in GBSS together with 10 % (v/v) protease inhibitors mixture until further analysis. For determination of the PG synthesis and content, the load plates are extracted on a rocker for 48 h at 4 °C with 1 ml of buffer containing 4 M guanidinium chloride, 50 mM sodium acetate, pH 5.8, 100 mM 6-aminocaproic acid, 5 mM benzamidine/HCL, 10 mM EDTA, 10 mM N-ethylmaleimide and 1 mM PMSF, and the extracts stored frozen at –20 °C until analysis.

To determine proteoglycan synthesis, radiolabeled cartilage explants are thawed and washed three times with 1.0 ml cold GBSS. Excess liquid is removed by blotting the specimen on filter paper, and tissue wet weights are determined. Samples are then digested for 4 h at 65 °C with 1 ml of 0.5 mg/ml papain digestion solution at pH 6.5 containing 50 mM monosodium phosphate, 2 mM N-acetylcysteine and 10 mM EDTA. [^{35}S]-SO_4-labeled PGs within the papain-digested explants, media and load plate extracts are determined by separation of free [^{35}S]-SO_4 from macromolecular

[^{35}S]-SO_4-labeled GAGs by size exclusion chromatography on Sephadex^{R}G-25 columns (Amersham Pharmacia Biotech GmbH, Freiburg, Germany). PG synthesis is calculated from the total [^{35}S]-SO_4-labeled GAGs found in the digested explants, media and load plate extracts. PG biosynthesis is normalized with respect to DNA content of the tissue, incubation time and sulfate concentration in the media, and reported as μM of SO_4^{2-} -incorporation per μg DNA per hour.

For quantitation of DNA content and proteoglycans, papain-digested cartilage explants, culture media, and load plate extracts (25 μl aliquots) are assayed for sulfated GAGs by reaction with 0.25 ml 1,9-dimethylmethylene blue dye solution in polystyrene 96 well plates and quantified by spectrophotometry at 523 nm using an ELISA-plate reader. Chondroitin sulfate A from bovine trachea (Sigma GmbH, Deisenhofen, Germany) is used as the standard (Farndale et al. 1986). This method determines endogenous as well as newly synthesized PGs. However, the majority of PGs quantified by this procedure represent endogenous PGs. The DNA content of papain-digested cartilage explants is determined fluorometrically using the bisbenzimidazol dye Hoechst 33258 as described by Kim et al. (1988).

EVALUATION

Results of the treated specimen under load are calculated in percentage to those derived from untreated loaded specimen, and, when of interest, compared to the results obtained with or without treatment in unloaded conditions.

CRITICAL ASSESSMENT OF THE METHOD

The biomechanical assay systems are of special importance when functional impairment of joints may be a critical issue of the therapeutic principle under investigation. As chondrocytes are known to metabolically behave very distinct, even controversial in conventional culture conditions compared to those under biomechanical forces much closer to their natural environment, a mechanically well-defined assay system is expected to yield results more representative of joint biomechanics, and such even bridge the gap to an in vivo model.

MODIFICATIONS OF THE METHOD

A custom-designed bioreactor system of a different design, but equally fitting in an incubator, allows for static as well as oscillating load protocols, and can be used successfully for isolated chondrocytes embedded in an artificial matrix like e.g. fibrin gels (Hunter et al. 2004), even for as long as a 39 day time course (Kisiday et al. 2004). This could be of specific interest for the assessment of substances designed to support or facilitate tissue engineering tasks.

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I.L.2 Osteoarthritis-like Effects in vivo

GENERAL CONSIDERATIONS

For assessment of degenerative joint disorders as potential side effects of drugs under investigation, a multitude of animal models for osteoarthritis is available, and their respective suitability for the pleiotropic disease aspects have been recently described and discussed by Oegema and Visco (1999), van den Berg (2001), and Brandt (2002). However, novel therapeutic principles, like e.g. the matrix metalloproteinases, have revealed their musculoskeletal side effects as late as in clinical studies only, which then retraced the focus of drug developmental steps back to scrutinize and refine existent models for different applications (Jacobson et al. 1999; Renkiewicz et al. 2003; Peterson 2004).

As most of the osteoarthritis-like effects are progressing slowly, and as the predictability of the different models for the disease course is still limited, we propose to examine for these alterations not in separate in vivo models, but rather applying two well-established evaluation methods to longterm safety studies to be conducted anyway: this would be the determination of collagen crosslinks in the urine as non-invasive detection of an increased collagen degradation, and a histopathological scoring of the knee joints at the end of the experiment to discriminate for structural changes within the joint.

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I.L.2.1 Matrix Degradation Monitored as Urinary Levels of Collagen Crosslinks

PURPOSE AND RATIONALE

Pyridinoline (PYR) and deoxypyridinoline (dPYR) are two non-reducible collagen crosslinks which, whenever degradation of collagen fibrils occurs, are released into circulation, cleared by the kidneys, and excreted through the urine (Knott et al. 1998). Whereas dPYR mainly originates from bone, PYR derives from other connective tissues as well, such as cartilage, and also from bone (Eyre 1995). Therefore, dPYR is considered a rather selective marker of bone collagen degradation. The ratio PYR/dPYR is used to detect changes from non-bone tissue sources, otherwise difficult to discriminate from the normally high bone mass being constantly remodeled. A rise in the PYR/dPYR ratio therefore implicates a substantial degradation of soft joint tissues like cartilage (Verzijl et al. 2001). Monitoring both parameters over the time period of the drug exposition, a ranking in susceptibility within the different collagenous tissues can be revealed, as shown in an age study supported by histology at the respective timepoints (Wachsmuth et al. 2002).

PROCEDURE

The hydrolysis of urinary collagen fragments, their HPLC separation, and the fluorimetric determination of pyridinoline and deoxypyridinoline are performed as described by James et al. (1990) with the following modifications: 250 µl aliquots of the 24 h urine samples are mixed with an equal volume of 37 % (w/v) HCl and hydrolized at 110 °C for 18 h. The samples are then diluted with 2.5 ml solvent of acetic acid:acetonitrile 2:3 (v/v), and applied to poly-prep chromatography columns (2 ml bed volume, Biorad, Munich, Germany) equilibrated with that solvent. After washing twice, the crosslinks are eluted by 4 ml of aqueous 20 mM/l heptafluorobutyric acid (Aldrich, Steinheim Germany). The first 1 ml eluate is discarded, whereas a 100 µl aliquot of the sample fraction is applied directly to HPLC.

The HPLC equipment (Waters, Eschborn, Germany) consists of an isocratic pump no. 510, an injector no. 717, and a temperature control module. The pyridinoline crosslinks are separated on NovaPak^{TM} C-18 (140* 3.9 mm, 5 µm particles) isocratic by 20 mM/l heptafluorobutyric acid in 725 ml water/175 ml acetonitrile (1 ml/min) at 30 °C. Crosslinks urine standard containing 953 pM/ml PYR and 212 pM/ml dPYR (Biorad, Munich, Germany) was used for

calibrating the fluorescence detector (FP 920 Jasco, Gross-Umstadt, Germany, λ_ex=290 nm, λ_{em}=400 nm) (Weithmann et al. 1997).

EVALUATION

The experimental results are expressed as nM urinary pyridinium or desoxypyridinium per mM creatinine.

CRITICAL ASSESSMENT OF THE METHOD

This non-invasive method allows to follow an experiment longitudinally, and relates to more than one skeletal tissue. Care has to be taken however to not only normalize the values in respect to creatinine levels, in order to compensate for individual differences in urine concentration, but also to either collect the samples over a longer period than 24 h, as proposed by Smith et al. (2004), or to collect each time at the same hour to exclude the known circadian variation in excretion (Stone et al. 1998). In addition, when using female animals, the measurements should be recorded regularly over a longer period to correct for considerable peak variation during the estrous cycle (Blanque et al. 2001).

MODIFICATIONS OF THE METHOD

Bank et al. (1997) developed a sensitive single-step HPLC procedure, by which hydrolysates from a wide variety of tissue sources can be analysed without prior sample treatment down to a detection limit of 0.4 pM for the PYR/dPYR crosslinks.

Also assessing these collagen crosslinks from different sources as serum, urine, synovial fluid and synovial tissue, Kaufmann et al. (2003) even reach a detection limit of 25 fM for pyridinoline and 56 fM for deoxypyridinoline with a modified HPLC procedure.

In their methodological concept, Mueller et al. (2003) discern between free and peptide-bound collagen crosslinks from urine samples of patients displaying different skeletal diseases, and argue that this discrimination might further characterize the underlying disorder.

In a method-based systematic comparison of urinary crosslink assays Seibel et al. (1998) find that automated techniques are superior in terms of precision to manual immunoassays, but that essentially reliable data can be obtained with all the methods under investigation.

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I.L.2.2 Modulation of Structural Joint Integrity Revealed by Histological Scoring

PURPOSE AND RATIONALE

Histological examination of the knee joint provides a good opportunity to detect degenerative changes caused by a prolongued drug administration. For rats and mice, as described here, whole joints can be studied after decalcification, whereas for larger animals tissues and/or regions of special interest have to be selected. Using a semiquantitative grading system, differences to control animals can be documented in an unbiased fashion and subjected to statistical validation.

PROCEDURE

Tissue Processing: Skinned mouse knee joints are immersed in neutral buffered formaldehyde (3.7 %) directly after preparation. Fixation times are limited to one week to ensure tissue integrity. Tissue samples are rinsed overnight with tap water. Tissue samples that contain bone are decalcified (D-calcifier normal, Shandon 1779) for 2 or 3 days. Decalcified samples or cartilage samples without bone are rinsed overnight with tap water. After dehydration with alcohol in stepwise increasing concentrations, tissue samples are embedded in paraffin.

The histological analysis is performed in general in the main weight-bearing areas of the respective knee joints. Experience from previous studies suggests that the earliest changes occur in these areas. Therefore whole knee joints obtained in mouse experiments are embedded for sagittal sectioning. Slides are cut from the median tibial plateau. Mouse knee joints are cut in sections of 4 μm thickness.

Histochemical Staining: Immediately before the staining procedure the sections are deparaffinized and rehydrated. Deparaffinization is performed immersing the sections three times for 5 minutes in Histoclear. Rehydration is performed by stepwise rinsing of the sections in ethanol in decreasing concentrations. Histological sections are stained routinely with safranin O/fast green to reveal matrix proteoglycans qualitatively (Rosenberg 1971). The cationic dye safranin O binds to sulfated glycosaminoglycans and stains them intensely red. The counterstain fast green stains collagen green. Fast green staining is applied for 3 minutes, followed by 30 sec of acetic acid, then 5 min safranin O. The haematoxylin/eosin staining allows a better assessment of the matrix structure, the cellularity, and the synovial tissue (Romeis 1989). The basophilic haematoxylin binds to nucleic acids, hence cell nuclei selectively appear blue. The remaining cellular structures and the collagens of articular cartilage and bone turn red by eosin-staining. 5 min haematoxylin are followed by 20 min rinsing with tap water, followed by 5 min eosin submersion, and then for 1.5 min aqua bidest changed twice. Sections are then dehydrated with ethanol in increasing concentrations, mounted in histomount and coverslipped.

EVALUATION

The basis for histopathologic grading systems assessing osteoarthritic lesions is the so-called Mankin score, originally consisting of the categories “matrix structure”, “cells”, “Safranin O staining”, and “tidemark integrity”: an index reaching a maximum of 14 points of pathologic changes, developed for human samples, and proposed by Mankin (1971). Osteoarthritic changes observed in animals, especially mice, exhibit somewhat different pecularities in their pathology: formation of osteophytes, fibrous cartilage, or synovial villi, changes in subchondral bone architecture, or perichondrocytal staining, and appearance of chondocyte clusters separately from changes of cell density in cartilage become important features in the pathology, whereas pannus formation, or a tidemark as border between hyaline and calcified articular cartilage, is rarely seen. Therefore, a comprehensive score with 8 categories: matrix structure, fibrous cartilage, cellularity, cluster formation, matrix staining, pericellular staining, synovial tissue, and subchondral bone, has been developed for mouse studies (Wachsmuth et al. 1999). Depending on the severity of pathologic changes, each category consists of maximum 8 points, resulting in a total score sum of 56 points of maximum damage. Changes interpreted as repair like increased pericellular staining are substracted from the total score. The extended score sum allows to differentiate pathologically relevant stages more clearly, and is in good accordance to progressive changes depicted on the articular surface by scanning electronmicroscopy (Wachsmuth et al. 2002). When focussing on articular cartilage only, as necessary in larger animals, or when only subtle histochemical changes can be expected, the score should be adapted to the nature and the extent of the alterations to occur. For any score however it is important, that an unequivocal definition is available for each stage a score point is assigned to. Evaluation should be performed by an investigator blinded to the group status of the samples, and preferentially even by more than one investigator independently.

CRITICAL ASSESSMENT OF THE METHOD

Crucial for the validity of the data is the care with which the intraarticular region, the angle of the sections, and the staining procedure are standardized throughout the processing. Even when applying the drug under investigation locally into or near one joint, the contralateral joint never should be used as control or reference, because afflictions in one joint will alter the gait and thus change the biomechanics in the contralateral joint, and also because systemic effects can not be excluded.

MODIFICATION OF THE METHOD

Although the Mankin score is still widely used to assess histopathological changes semiquantitatively, the group of Salter remarked on its limitations with respect

to reproducibility and validity (Ostergaard et al. 1997), as well as the assignation to different stages of disease severity (Ostergaard et al. 1999).

Instead of a more elaborate score, Brewster et al. (1998) reduced the staging originating from the Mankin grades to a more simplified score, which still leads to statistically significant differences between the treatment groups.

Several investigators assess the proteoglycan content of SO-stained histological sections of articular cartilage also quantitatively with computer-based image analysis systems (Shimizu et al. 1997). However, although changes in the PG content can be detected by image analysis, absolute values for proteoglycan content are not reliable. Unavoidable variations in the staining procedure as e.g. differences in the concentration of the dye solution, or slightly varying staining times already result in changes of the optical densities measured. Slight variations in the section thickness between experimental groups can also influence the results. Finally the sensitivity of this staining method is limited. A proteoglycan loss of approximately 50 % results in a total absence of Safranin O staining as noted by visual examination, obscuring changes in proteoglycan contents below the detection level (van der Kraan et al. 1994). In contrast, osteophyte formation can be assessed by image analysis more reliably (van Valburg 1996).

The group of van den Berg prefers for assessment of structural changes in the mouse knee the coronar sections over the sagittal plane (van der Kraan et al. 1994), depicting both condyles, and facilitating the detection of osteophytes.

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Chapter I.M
Safety Pharmacology of Blood Constituents

Shaker A. Mousa

I.M.1 In vitro Tests

I.M.1.1 Blood Coagulation Tests

PURPOSE AND RATIONALE

The coagulation cascade consists of a complex network of interactions resulting in thrombin-mediated conversion of fibrinogen to fibrin which is one major component of a thrombus. The coagulation cascade can be initiated either by the "exogenous pathway", the release of thromboplastin (tissue factor) leading to activation of factor VII to the tissue factor/factor VIIa complex, or by the "endogenous pathway", so-called contact activation leading via factors XII, XI and IX to the assembly of the tenase complex consisting of activated factors VIII and IX and Ca^{2+} on a phospholipid surface. Both complexes can activate factor X which induces the formation of the prothrombinase complex consisting of factor X_a, factor Va and Ca^{2+} on a phospholipid surface. The latter leads to the activation of thrombin which in turn cleaves fibrinogen to fibrin. The three coagulation tests (PT, APTT and TT) allow to differentiate between effects on the exogenous or endogenous pathway or on fibrin formation. The influence of compounds on the plasmatic blood coagulation is determined by measuring the coagulation parameters prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) ex vivo.

PROCEDURE

Male Sprague-Dawley rats weighing 200–220 g receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time, they are anesthetized by intravenous injection of 60 mg/kg sodium pentobarbital. The caudal caval vein is exposed by a midline incision and 1.8 ml blood are collected into a plastic syringe containing 0.2 ml 100 mM citrate buffer pH 4.5 (Behring Werke, Marburg). The sample is immediately agitated and centrifuged in a plastic tube at 1500 × g for 10 min. Plasma is transferred to another plastic tube and the coagulation tests for the determination of thrombin time (TT), thromboplastin time (PT) and activated partial thromboplastin time (APTT) are performed within 3 h.

In general, citrated plasma is coagulated by the addition of the respective compounds (see below) and the time to clot formation is determined in the coagulometer (= coagulation time).

For detailed laboratory diagnosis of bleeding disorders and assessment of blood coagulation see Palmer (1984) and Nilsson (1987).

Prothrombin Time (PT). An aliquot of 0.1 ml of citrated plasma is incubated for 1 min at 37 °C. Then 0.2 ml of human thromboplastin (Thromborel, Behring Werke, Marburg) is added and the coagulometer (Schnittger+Gross coagulometer, Amelung, Brake) is started. The time to clot formation is determined. The PT measures effects on the exogenous pathway of coagulation.

Activated Partial Thromboplastin Time (APTT). To 0.1 ml of citrated plasma 0.1 ml of human placenta lipid extract (Pathrombin, Behring Werke, Marburg) is added and the mixture is incubated for 2 min at 37 °C. The coagulation process is initiated by the addition of 0.1 ml 25 mM calcium chloride when the coagulometer is started and the time to clot formation is determined. The APTT measures effects on the endogenous pathway of coagulation.

Thrombin Time (TT). To 0.1 ml of citrated plasma 0.1 ml of diethylbarbiturate-citrate buffer, pH 7.6 (Behring Werke, Marburg) is added and the mixture is incubated for 1 min at 37 °C. Then 0.1 ml of bovine test-thrombin (30 IU/ml, Behring Werke, Marburg) is added and the coagulometer is started. The time to clot formation is determined. The TT measures effects on fibrin formation.

EVALUATION

Mean values of TT, PT and PTT are calculated in dosage groups and vehicle controls. Statistical evaluation is performed by means of the unpaired Student's t-test.

Based on the contributions by M. Just and V. Laux in the book H.G. Vogel (ed) (2002) "Drug Discovery and Evaluation – Pharmacological Assays", Springer-Verlag, second edition.

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I.M.1.2 Thrombelastography

PURPOSE AND RATIONALE

Thrombelastography (TEG) was developed first by Hartert (1948). The thrombelastograph (Haemoscope Corp, Skokie, Illinois, USA) is a device that provides a continuous recording of the process of blood coagulation and subsequent clot retraction. The blood samples are transferred to cuvettes and maintained at 37 °C. The cuvettes are set in motion around their vertical axes. Originally, a torsion-wire suspended mirror in the plasma remains immobile as long as the plasma is fluid. The cuvette and the mirror become dynamically related as fibrin forms, resulting in transmission of cuvette motion to the mirror. The mirror then oscillates with an amplitude governed by the specific mechanical properties of the clot and reflects its light to a thermopaper. The modern thrombelastograph transfers the analogous recording to a digital signal which is evaluated by a computer program.

PROCEDURE

Thrombelastography can be performed in either whole blood, or in citrated platelet-rich or platelet-poor plasma after recalcification. Blood samples are obtained from Beagle dogs, weighing 12–20 kg, from rabbits, weighing 1.7–2.5 kg or from Wistar rats weighing 150–300 g or from humans. The test subjects receive the compound by intravenous, subcutaneous or by oral administration. Ten or 20 min post dosing (i.v, s.c. administration) or 60, 90 or 180 min post dosing (oral administration) blood is collected. The blood samples are mixed with 3.8 % trisodium citrate solution (one part citrate solution to 9 parts blood) as anticoaglant. The citrated whole blood is recalcified by adding 0.4 ml isotonic calcium chloride solution. An aliquot of 0.36 ml of the recalcified whole blood is transferred to the prewarmed cup of the thrombelastograph. After the apparatus has been correctly adjusted and the samples sealed with liquid paraffin to prevent drying, the time for the whole procedure is noted. The thrombelastogram is recorded for 2 h.

EVALUATION

The following measurements are the standard variables of TEG:

1. Reaction time (r): the time from sample placement in the cup until onset of clotting (defined as amplitude of 1 mm). This represents the rate of initial fibrin formation.
2. Clot formation time (k): the difference from the 1 mm r to 20 mm amplitude. It represents the time taken for a fixed degree of viscoelasticity achieved by the forming clot, caused by fibrin build up and cross linking.
3. Alpha angle ($\alpha°$): angle formed by the slope of the TEG tracing from the r to k value. It denotes speed at which solid clot forms.
4. Maximum amplitude (*MA*): greatest amplitude on the TEG trace. *MA* represents the absolute strength of the fibrin clot and is a direct function of the maximum dynamic strength of fibrin and platelets.
5. Clot strength (G in dynes per square centimeter): defined by G = (5000*MA*)/(96–*MA*). In a tissue factor-modified TEG (Khurana et al. 1997), clot strength is clearly a function of platelet concentration.
6. Lysis 30, Lysis 60 (*Ly30*, *Ly60*): Reduction of amplitude relative to maximum amplitude at 30 and 60 min after time of maximum amplitude. These parameters represent the influence of clot retraction and fibrinolysis.

MODIFICATIONS OF THE METHOD

Bhargava et al. (1980) compared the anticoagulant effect of a new potent heparin preparation with a commercially available heparin by TEG in vitro using citrated dog and human blood. Barabas et al. (1993) used fibrin plate assay and TEG to assess the antifibrinolytic effects of synthetic thrombin inhibitors. Scherer et al. (1995) described a short-time, endotoxin-induced rabbit model of hyper-coagulability for the study of the coagulation cascade and the therapeutic effects of coagulation inhibitors using various parameters including TEG.

Khurana et al. (1997) introduced tissue factor-modified TEG to study platelet glycoprotein IIb/IIIa function and to establish a quantitative assay of platelet function. With this modification, Mousa et al. (2000) found two classes of glycoprotein IIb/IIIa antagonists, one with high binding affinity for resting and activated platelets and slow platelet dissociation rates (class I) demonstrating potent inhibition of platelet function, in contrast to those with fast platelet dissociation rates (class II). Additionally, Mousa et al. (2005) utilized the

Table 1 Effect of various stimulus on platelet/fibrin clot dynamics as shown by Mousa et al. (2000).

TEG Parameters	TF (25 ng) Mean ± SEM	LPS (0.63 ug)	Xa (0.25 nM)	Thrombin (0.3 mU)
R (minutes)	29.7 ± 2.3	23.4 ± 1.4	15.6 ± 2.9	3.4 ± 0.6
K (minutes)	5.8 ± 1.0	7.6 ± 0.9	4.8 ± 0.5	5.5 ± 0.8
α° (Angle)	45.0 ± 2.6	47.8 ± 3.2	61.5 ± 2.1	57.8 ± 2.9
MA (mm)	58.2 ± 1.7	50.0 ± 2.0	65.0 ± 0.8	50.1 ± 2.4

Citrated human whole blood plus 2.5 mM calcium. Data represent mean for $n = 6\pm$ SEM_0

TEG in Phase II clinical trial in monitoring the efficacy of oral platelet GPIIb/IIIa antagonist on platelet/fibrin clot dynamics.

CRITICAL ASSESSMENT OF THE METHOD

Zuckerman et al. (1981) compared TEG with other common coagulation tests (fibrinogen, prothrombin time, activated thromboplastin time, platelet count and fibrin split products) and found that there is a strong relationship between the thrombelastographic variables and these common laboratory tests. Moreover, TEG has an increased sensitivity for detecting blood clotting anomalies; it contains additional information on the hemostatic process. This is due to the following: (1) the fact that most laboratory measurements end with the formation of the first fibrin strands while TEG measures the coagulation process on whole blood from initiation of clotting to the final stages of clot lysis and retraction, and (2) the possibility of TEG to use whole non-anticoagulated blood without influence of citrate or other anticoagulants.

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I.M.1.3 Chandler Loop

PURPOSE AND RATIONALE

The Chandler loop technique allows one to produce in vitro thrombi in a moving column of blood (Chandler 1958). The thrombi generated in the Chandler device show morphology very similar to human thrombi formed in vivo (Robbie et al. 1997) with platelet-rich upstream sections ("white heads") that are relatively resistant to t-PA-mediated thrombolysis in contrast to the red blood-cell-rich downstream parts ("red tails") (Stringer et al. 1994).

PROCEDURE

One millimeter of non-anticoagulated whole blood is drawn directly into a polyvinyl tube with a length of 25 cm and an internal diameter of 0.375 cm (1 mm = 9.9 cm tubing). The two ends of the tube are then brought together and closed by an outside plastic collar. The circular tube is placed and centered on a turntable, tilted to an angle of 23 degrees, and rotated at 17 rpm. At the moment the developing thrombus inside the tube becomes large enough to occlude the lumen, the blood column becomes static and moves around in the direction of rotation of the tube.

EVALUATION

Time to occlusion of the tube by the thrombus establishes a definite end point in this system.

MODIFICATIONS OF THE METHOD

Stringer et al. (1994) used this method to determine the influence of an anti-PAI-1 antibody (CLB-2C8) on the

t-PA-induced lysis of Chandler thrombi in vitro. They used citrated blood and supplemented it with 5.8 μM [^{125}I]-labelled fibrinogen prior to recalcification. After generation in the Chandler loop, the thrombi were washed with isotonic saline and then cut transversally into an upstream (head) and a downstream part (tail). The radioactivity of both parts was determined in a gamma counter (pre-value). The head and the tail were then subjected to thrombolysis by adding 300 μl phosphate-buffered saline containing plasminogen (2 μM) and t-PA (0.9 nM). During the observation time of 240 min aliquots of 10 μl were taken at 30, 60, 120, 180 and 240 min, and the radioactivity was determined. The relation of the measured radioactivity to the pre-value was expressed as percentage of clot lysis.

Van Giezen et al. (1998) used this method to differentiate the effect of an anti-PAI-1 polyclonal antibody (PRAP-1) on human or rat thrombi.

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I.M.1.4 Platelet Aggregation and Deaggregation in Platelet-Rich Plasma or Washed Platelets (Born Method)

PURPOSE AND RATIONALE

Platelets play a crucial role in primary hemostasis by forming hemostatic plugs at sites of vascular injury. Moreover, they contribute to intravascular thrombus formation mostly upon rupture of an atherosclerotic plaque. The contact of unactivated platelets to exposed subendothelial tissue leads to adhesion via two main mechanisms: binding of subendothelial von Willebrand factor (vWF) to the platelet GPIb-IX-V-complex at high shear rates and binding of collagen to two receptors, integrin α2β1 and GPVI. Platelet adhesion initiates the reactions of shape change, secretion, and activation of GPIIb-IIIa-ligand binding sites. These reactions result in the formation of platelet aggregates. Activation of GPIIb-IIIa is also achieved through signaling by a number of agonists that bind to G-protein-coupled receptors. Consequently, for the measurement of platelet aggregation platelets are activated by the addition of one of the following agonists to platelet-rich plasma (PRP) or washed platelets: ADP, arachidonic acid (forming thromboxane A2) or U 46619, collagen, thrombin or TRAP, serotonin, epinephrine, PAF. The formation of platelet aggregates with stirring leads to changes in optical density which are monitored photometrically usually for 4 min. The test has been developed originally by Born (1962a,b) and is used to evaluate quantitatively the effect of compounds on induced platelet aggregation in vitro or ex vivo. For in vitro studies, human PRP is preferred.

PROCEDURE

The test is carried out either ex vivo or in vitro.

For ex vivo assays, mice, rats, or guinea pigs from either sex receive the test compound or the vehicle (for controls) by oral, intraperitoneal or intravenous administration. At the end of the absorption time, blood is collected by caval venipuncture under pentobarbital sodium anesthesia and xylazine (8 mg/kg i.m.) premedication.

From rabbits (Chinchilla strain, weighing 3 kg), blood is withdrawn by cardiopuncture under xylazine (20 mg/kg i.m.) sedation. The first blood sample (control) is collected before administration of the test compound, the second sample at the end of the absorption time of the test agent.

For in vitro assays, human blood is collected from the antecubital vein of adult volunteers, who had not received any medication for the last two weeks.

Preparation of PRP, PPP, and WP

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Freshly collected venous blood is anticoagulated with hirudin (1 volume + 9 volumes of animal blood) or ACD solution (1 volume + 9 volumes of human blood) and centrifuged at 170 × g for 15 min to obtain platelet-rich plasma (PRP). The PRP-supernatant is carefully removed, and the rest is further centrifuged at 1500 × g for 10 min to obtain platelet-poor plasma (PPP). PRP is diluted with PPP to a platelet count of 3×10^8/ml before use in the aggregation assays. To obtain washed platelets (WP), 8.5 volumes of human blood are collected into 1.5 volumes of ACD and centrifuged as for PRP. PRP is acidified to a pH of 6.5

Table 2 Materials and solutions.

Anticoagulating substances	
Hirudin (Sigma)	200 μg/ml
Trisodium citrate	0.11 M
ACD solution	
Citric acid	38 mM
Sodium citrate	75 mM
Glucose	124 mM
Platelet aggregating substances (final concentrations in the test)	
ADP: for reversible or biphasic aggregation	0.1–5 μM
ADP: for irreversible aggregation (Sigma)	3–10 μM
Sodium arachidonate (Biodata)	0.3–1 mM
Calcium ionophore A 23187 (Calbiochem)	10 μM
Collagen (Hormonchemie)	3 μg/ml
PAF-acether (C 16-PAF, Bachem)	0.1 μM
Thrombin (Sigma)	0.02–0.05 IU/ml
TRAP (SFLLRNP, Bachem)	1–10 μM
U 46619 (ICN)	1–10 μM
Ristocetin	0.1–1 mg/ml
GPRP (fibrin antipolymerant, Bachem)	0.5 mM
4-channel aggregometer (PAP 4, Bio Data)	

by addition of approximately 1 ml ACD to 10 ml PRP. Acidified PRP is centrifuged for 20 min at 430 × g. The pellet is resuspended in the original volume with Tyrode's solution (mM: NaCl 120, KCl 2.6, $NaHCO_3$ 12, NaH_2PO_4 0.39, HEPES 10, glucose 5.5; albumin 0.35 %) and set to platelet count of 3×10^8/ml.

Studies should be completed within 3 h after blood withdrawal.

For ex vivo assays, duplicate samples of 320 μl PRP from drug-treated and vehicle control subjects (for rabbits: control samples before drug administration) are inserted into the aggregometer at 37 °C under continuous magnetic stirring at 1000 rpm. After the addition of 40 μl physiological saline and 40 μl aggregating agent, changes in optical density are monitored continuously at 697 nm.

For in vitro assays, 40 μl of the test solution are added to samples of 320 μl PRP or WP from untreated subjects. The samples are inserted into the aggregometer and incubated at 37 °C for 2 min under continuous magnetic stirring at 1000 rpm. After the addition of 40 μl aggregating agent, changes in optical density are monitored continuously at 697 nm either for 4 min or until constant values for aggregation are achieved. In cases of thrombin activation of PRP, Glycine-Proline-Aspartate-Proline (GPRP) is added in order to avoid fibrin formation. In order to measure deaggregation, experimental compounds are added to stimulated PRP at 70 or 100 % of control aggregation and monitoring is performed for further 10 min. Deaggregation is measured by the decrease of light transmission (see Haskel et al. 1989).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation (0 % = transmission of PRP, 100 % = transmission of PPP).

For in vitro assays:

1. Percent inhibition of platelet aggregation is determined in concentration groups relative to vehicle controls. Statistical significance is evaluated by means of the unpaired Student's t-test.
2. IC_{50} values are determined from the non-linear curve fitting of concentration-effect relationships. IC_{50} is defined as the concentration of test drug for half maximal inhibition of aggregation.
3. Percent deaggregation is determined at 10 min after addition of compound; IC_{50} is calculated from the concentration-effect relationship.

For ex vivo assays:

1. Mean values for aggregation in dosage groups are compared to the vehicle control groups (for rabbits: control values before drug administration). Statistical significance is evaluated by means of the Student's t-test (paired for rabbits; unpaired for others).
2. ED_{50} values are determined from the dose-response curves. ED_{50} is defined as the dose of drug leading to 50 % inhibition of aggregation in the animals.

CRITICAL ASSESSMENT OF THE METHOD

The assay, introduced by Born (1962a,b), has become a standard method in clinical diagnosis of platelet function disorders and of aspirin intake. Furthermore, the method is used in the discovery of antiplatelet drugs with the advantage of rapid measurement of a functional parameter in intact human platelets. However, processing of platelets during the preparion of PRP, washed or filtered platelets from whole blood results in platelet activation and separation of large-size platelets.

MODIFICATIONS OF THE METHOD

Several authors described modifications of the assay procedure. Breddin (1975) described spontaneous aggregation of platelets from vascular patients in a rotating cuvette. Klose et al. (1975) measured platelet aggregation under laminar flow conditions using a thermostated cone-plate streaming chamber in which shear rates are continuously augmented and platelet aggregation is measured from light transmission through a transilluminating system. Marguerie et al. (1979, 1980) developed a method measuring two phases of platelet aggregation after gel filtration of a platelet suspension (see below). Lumley and Humphrey (1981) described a method to measure platelet aggregation in whole blood (see below). Fratatoni et al. (1990) performed aggregation measurements using a microtiter plate reader with specific modification of the agitation of samples. Comparison of the 96-well microtiterplate method with conventional aggregometry showed similar dose-response curves for thrombin, ADP, and arachidonic acid.

Ammit and O'Neil (1991) used a quantitative bioassay of platelet aggregation for rapid and selective measurement of platelet-activating factor.

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I.M.1.5 Platelet Aggregation After Gel Filtration

PURPOSE AND RATIONALE

Triggering of platelet activation by low concentrations of ADP, epinephrine or serotonin – so-called weak platelet agonists – in plasma- and fibrinogen-free platelet suspensions does not result in platelet aggregation unless exogenous fibrinogen is added. As opposed to this, platelet aggregation induced by thrombin, collagen or prostaglandin-endoperoxide – so-called strong agonists – is independent of exogenous fibrinogen because these substances lead to the secretion of intracellular platelet ADP and fibrinogen. Studies of platelet aggregation in gel-filtered platelets are performed in cases where the adhesive ligand fibrinogen or vWF is needed in a defined concentration or where plasma proteins could negatively interfere with the effect of compounds. The assay is mostly used to evaluate the influence of compounds on platelet GPIIb-IIIa or other integrins or on GPIb-IX-V.

Table 3 Materials and solutions.

Acid-citrate-dextrose (ACD) solution	
Citric acid	0.8 %
Sodium citrate	2.2 %
Glucose	2.45 %
Hirudin	0.6 U/ml
Tyrode's solution	
NaCl	137 mM
KCl	2.7 mM
$MgCl_2$	5.5 mM
NaH_2PO_4	3.0 mM
HEPES	3.5 mM
Glucose	5.5 mM
Albumin	0.2 %
Hirudin	0.06 U/ml
Apyrase	40 μg/ml
pH	7.2
Epinephrine (different concentrations; μM range)	
ADP	10 μM
Thrombin	0.02–0.05 U/ml
$CaCl_2$	0.5 mM
Fibrinogen (American Diagnostica)	1 mg/ml
von Willebrand factor	10 μg/ml
Sepharose CL 2B (Pharmacia)	
Acrylic glass column (Reichelt Chemietechnik, 3 cm inner diameter, 18 cm length)	
Aggregometer (PAP 4, Biodata)	

PROCEDURE

Preparation of Gel-Filtered Platelets

The entire procedure is performed in plastic (polystyrene) tubes at room temperature according to Marguerie et al. (1979).

Blood is drawn from healthy adult volunteers, who had no medication for the last two weeks. Venous blood (8.4 ml) is collected into 1.4 ml ACD-solution and centrifuged for 10 min at 120 × g. The platelet-rich plasma (PRP) is carefully removed, the pH adjusted to 6.5 with ACD-solution and centrifuged at 285 × g for 20 min. The resulting pellet is resuspended in Tyrode's buffer (approx. 500 µl buffer/10 ml PRP). The platelet suspension is applied immediately to a Sepharose CL 2B column; equilibration and elution at 2 ml/min flow rate is done with Tyrode's buffer without hirudin and apyrase. Platelets are recovered in the void volume. Final platelet suspension is adjusted to 4×10^8/ml. Gel-filtered platelets (GFP) are kept at room temperature for 1 h until the test is started.

Experimental Course

For the aggregation studies, GFP in Tyrode's buffer is incubated with $CaCl_2$ (final concentration 0.5 mM) with or without fibrinogen (final conc. 1 mg/ml) in polystyrene tubes. After 1 min, 20 µl of the test compound or the vehicle (controls) are added and the samples are incubated for another 2 min. After the addition of 20 µl platelet agonist, changes in light transmission are recorded. The whole procedure is done under continuous magnetic stirring at 37 °C (1000 rpm) in the aggregometer. Samples with added $CaCl_2$ but without fibrinogen identify proper exclusion of plasma proteins if neither spontaneous aggregation occurs nor aggregation in the presence of weak agonists. Full aggregatory response of GFP to 10 µM ADP shows intact platelets (with only minor pre-activation with gel-filtration).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation. Each test compound is assayed with at least two different donor-GFPs; in the case of an anti-aggregating effect, the test is performed with 4–6 GFPs.

Mean values of the dosage groups are compared to the controls. Statistical significance is evaluated by means of the Student' t-test.

The percent inhibition of platelet aggregation in the dosage groups is calculated relative to the vehicle controls.

IC_{50} values (50 % inhibition of aggregation) are determined from the concentration-effect curves.

For detailed methology and evaluation of different agents see Marguerie et al. (1979, 1980) and Markell et al. (1993).

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I.M.1.6 Platelet Aggregation in Whole Blood

PURPOSE AND RATIONALE

The method uses a whole blood platelet counter which counts single platelets and does not require their separation from other blood cell types. Platelet aggregation is induced in anti-coagulated human whole blood samples by the addition of the aggregating agents arachidonic acid or collagen. The number of platelets is determined in drug-treated and vehicle control samples; the percentage of inhibition of aggregation and IC_{50} values are calculated in dosage groups. The effect of compounds on other blood cells which secondarily can influence platelet aggregation is included in this test system. The method has been described by Lumley and Humphrey (1981) and Cardinal and Flower (1980).

Table 4 Materials and solutions.

Anticoagulant: sodium citrate to induce platelet aggregation	3.8 %
Sodium arachidonate (Biodata)	3.6×10^{-4} M
Collagen (Hormonchemie)	10 µg/ml
Serono Hematology System 9000 or Sysmex Mircellcounter F 800	

PROCEDURE

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Blood is drawn from healthy adult volunteers, who had not received medication for the last 2 weeks. 9 ml venous blood are anti-coagulated with 1 ml of sodium citrate and kept in a closed tube at room temperature for 30–60 min until the start of the test.

For the aggregation studies, 10 µl test substance or vehicle (control) are added to 480 µl citrated blood. Samples in closed tubes are pre-incubated for 5 min in a 37 °C water shaker bath at 75 strokes/min. Ten µl

aggregating agent are added and samples are incubated for another 10 min. The number of platelets (platelet count) is determined in 10 µl samples immediately before and 10 min after the addition of the aggregating agent ('initial platelet count', '10-min-platelet count') in a hematology cell counter.

The following samples for the determination of the platelet count are prepared in duplicate:

1. control aggregation = spontaneous aggregation (without aggregating agent): 480 µl blood + 20 µl vehicle. Blood samples with >20 % spontaneous aggregation are not used to test for induced aggregation.
2. maximal aggregation: 480 µl blood + 10 µl vehicle + 10 µl aggregating agent. Values represent the maximal induced aggregation rate of the blood sample.
3. test substance aggregation: 480 µl blood + 10 µl test substance + 10 µl aggregating agent.

EVALUATION

From the samples for maximal aggregation (vehicle), the percentage of maximal aggregation is calculated according to the following formula:

$$\%\ \text{maximal aggregation} = 100 - \frac{\text{10-min-platelet count} \times 100}{\text{initial platelet count}}$$

This value for maximal aggregation is taken as 100 %.

From the samples for test substance induced aggregation, the percentage of aggregation in dosage groups is calculated according to the following formula:

$$\%\ \text{aggregation} = \frac{\text{10-min-platelet count} \times 100}{\text{initial platelet count}}$$

IC_{50} values (50 % inhibition of aggregation) are determined from the dose-response curves (log concentration test substance versus % inhibition of aggregation).

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I.M.1.7 Platelet Micro- and Macro-Aggregation Using Laser Scattering

PURPOSE AND RATIONALE

1. A new highly sensitive method to study platelet aggregation based on the measurement of mean radius or particle size makes it possible to record kinetics of formation of micro- and macro-aggregates in real time
2. Sensitivity in measurements of spontaneous aggregation is higher than in routine light transmittance.

A new platelet aggregometer (AG-10; Kowa, Japan) that uses a laser-light-scattering beam was introduced (Tohgi et al. 1996). Platelet aggregates, the size of which can be measured as total voltage of light-scatter intensity at 1.0-second intervals for a 10-minute period, can be divided into 3 ranges: small aggregates (diameter 9 to 25 µm), medium (26 to 50 µm), and large (> 50 µm). Using laser scatter aggregation, it was found that young smokers had an increased number of small platelet aggregates, which cannot be detected with a conventional aggregometer based on the turbidometric method (Matsuo et al. 1997). This device detects platelet aggregation in the small-aggregate size range by the addition of unfractionated heparin (UFH), and the aggregates are disaggregable in incubation with protamine sulfate. When platelet aggregation induced by UFH at a final concentration of 0.5 U/mL was observed in 36 normal subjects with no history of heparin exposure, 13 had a positive response in excess of 0.5 V of light intensity in the small-aggregate size range. In chronic hemodialysis patients in whom heparin had been used regularly for many years, a positive response with heparin-induced aggregates was noted in 37 of 59 patients, which was increased compared with that of normal subjects. The light intensity in the small-aggregate size range was enhanced during heparinized dialysis. In patients with a positive heparin response, the intensity of aggregates after heparin was significantly increased compared with that in nonresponders to heparin. Also, we obtained the same results by this system, that enhanced platelet aggregation response to heparin was not inhibited by aspirin or argatroban but was inhibited by anti-glycoprotein IIb/IIIa antibodies. The findings of enhanced platelet aggregation during heparin infusion could be directly obtained without the addition of ADP or TRAP using laser aggregometry (Xiao and Théroux 1998).

Limitations

This technique can not be applied in whole blood yet but can be used with PRP, washed platelet or GFP.

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I.M.1.8 Fibrinogen Receptor Binding

PURPOSE AND RATIONALE

The assay is used to evaluate the binding characteristics of drugs at the fibrinogen receptor. A constant concentration of the radioligand ^{125}I-fibrinogen (30–50 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of gel-filtered human platelets. If the test drug exhibits any affinity to fibrinogen receptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent the test drug. Platelets are activated with 10 mmol/l ADP to stimulate the ^{125}I-fibrinogen binding at the GPIIb/IIIa receptor.

PROCEDURE

Preparation of Gel-Filtered Platelets

From a healthy volunteer 200 ml blood is collected. An aliquot of 8.4 ml blood is mixed with 1.4 ml ACD-buffer in polystyrol tubes and centrifuged at 1000 rpm for 15 min. The resulting platelet-rich plasma (PRP) is collected and an aliquot is taken for platelet counting. Ten milliliters of PRP are mixed with 1 ml ACD-buffer (ACD-PRP, pH ~ 6.5); 5 ml portions of ACD-PRP are transferred to plastic tubes and centrifuged at 1600 rpm for 20 min. The resulting supernatant is decanted, and each pellet is resuspended in 500 µl Tyrode buffer C. An aliquot is taken for platelet counting to calculate the loss of platelets. The platelet suspension is then transferred to the Sepharose-packed column which has been eluted with approx. 100 ml degassed Tyrode buffer B (2 ml/min). The column is closed and eluated with degassed Tyrode buffer B (2 ml/min). The first platelets appear after 18–20 min and are then collected for 10 min in a closed plastic cup. Gel-filtered platelets (GFP) are set to 4×10^8 platelets/ml with Tyrode buffer B and kept at room temperature until the start of the test.

Experimental Course

For each concentration, samples are tested in triplicate (test tubes No. 72708, Sarstedt). The total volume of each incubation sample is 500 µl. The concentration of ^{125}I-fibrinogen is constant for all samples (10 µg/500 µl).

Competition Experiments

The competition reaction is characterized by one buffer value (bidistilled water) and various concentrations of non-labeled fibrinogen or test compound.

1. 100 µl ^{125}I-fibrinogen
2. 100 µl non-labeled fibrinogen or test drug (various concentrations, 10^{-10}–10^{-3} M)
3. 5 µl ADP.

Non-specific-binding: The non-specific binding of ^{125}I-fibrinogen is defined as the radioligand binding in the presence of 10^{-5} M of non-labeled fibrinogen.

The binding reaction is started by adding 250 µl GFP (4×10^8 platelets/ml). The samples are incubated for 30 min at room temperature. Subsequently, a 100 µl aliquot of the incubation sample is transferred to a microtainer tube containing 400 µl glucose solution. The tubes are centrifuged at 1500 × g for 2 min to separate ^{125}I-fibrinogen bound at the platelet glycoprotein IIb–IIIa receptor from free radioligand. The supernatant is carefully decanted and is allowed to run off for approx. 30 min. Radioactivity of the platelet pellets is counted for 1 min in a gamma counter with an efficiency of 65.3 %.

EVALUATION

The quantity of the specific ^{125}I-fibrinogen binding results from the difference between the total and the non-specific binding.

Platelet glycoprotein IIb-IIIa receptor binding is given as fmol ^{125}I-fibrinogen/10^8 platelets or ^{125}I-fibrinogen molecules bound per platelet.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of

Table 5 Materials and solutions.

Solutions for platelet preparation		
Stock solution I	Citrate	0.8 %
	Sodium citrate	2.2 %
Stock solution II	NaCl	120 mM
	KCl	2.8 mM
	NaH_2PO_4	10.0 mM
	HEPES	10.0 mM
ACD-buffer	Stock solution I	
	+ glucose	2.45 %
	+ hirudin	0.06 U/ml
Tyrode buffer A	Stock solution II	
	+ $NaHCO_3$	12 mM
Tyrode buffer B	Stock solution II	
	+ $NaHCO_3$	12 mM
	+ glucose	5.5 mM
	+ bovine albumin	0.35 %
Tyrode buffers A and B are degassed by aspiration for approx. 1 h after setting the pH to 7.2.		
Tyrode buffer C	Tyrode buffer B (degassed)	
	+ apyrase	40 μg/ml
	+ hirudin	0.06 U/ml
Chromatography column	Acryl glass column (200 × 170 mm, 30 mm diameter), closed with 3 perlon filters, pore sizes 63, 90 and 230 μm, and gauze 50 μm filled with degassed Sepharose CL2B-suspension (Pharmacia LKB); equilibrated with 500 ml degassed Tyrode buffer A (2 ml/min)	
Incubation buffer		
Stock solution	NaCl	120 mM
	KCl	2.6 mM
	NaH_2PO_4	0.39 mM
	HEPES	10.0 mM
	$CaCl_2$	0.5 mM
Incubation buffer, pH 7.2	stock solution	
	+ $NaHCO_3$	12 mM
	+ glucose	5.5 mM
	+ human albumin	0.35 %
Glucose solution (in incubation buffer)		
Radioligand	^{125}I-fibrinogen specific activity 3.7 Mbq/mg fibrinogen (100 μCi/mg fibrinogen) (Amersham), 1 mg radiolabeled fibrinogen is dissolved in 10 ml incubation buffer	
Non-labeled fibrinogen (mw 340 000, grade L, Sigma; in bidistilled water)		10^{-3}–10^{-10} M
ADP (in incubation buffer)		10 μM
Gamma-counter (1282 Compugamma CS, LKB)		

^{125}I-fibrinogen versus non-labeled drug by a computer-supported analysis of the binding data.

$$K = \frac{K_D{}^{125}\mathrm{I} \times \mathrm{IC}_{50}}{K_D{}^{125}\mathrm{I} + [^{125}\mathrm{I}]}$$

IC_{50} = concentration of the test drug, which displaces 50 % of the specifically glycoprotein IIb-IIIa receptor bound ^{125}I-fibrinogen in the competition experiment.

$[^{125}I]$ = concentration of ^{125}I-fibrinogen in the competition experiment.

$K_D{}^{125}I$ = dissociation constant of ^{125}I-fibrinogen, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50 % of the fibrinogen receptors are occupied by the test drug.

For detailed methodology and evaluations of various mechanisms and agents see the following selected references: Bennett, Vilaire (1979); Marguerie et al. (1979, 1980); and Mendelsohn et al. (1990).

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I.M.1.9 Euglobulin Clot Lysis Time

PURPOSE AND RATIONALE

The euglobulin lysis time is used as an indicator for the influence of compounds on the fibrinolytic activity in rat blood according to Gallimore et al. (1971). The euglobulin fraction of plasma is separated from inhibitors of fibrinolysis by acid precipitation and centrifugation. Euglobulin predominantly consists of plasmin, plasminogen, plasminogen activator and fibrinogen. By addition of thrombin to this fraction, fibrin clots are formed. The lysis time of these clots is determined as a measurement of the activity of activators of fibrinolysis (e.g. plasminogen activators). Thus, compounds can be detected which stimulate the release of tissue-type plasminogen activator from the vessel wall.

PROCEDURE

Rats are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium and placed on a heating pad (37 °C). At the same time, the test solution or the vehicle (controls) is administered intravenously or intraperitoneally. Twenty-five min later, the animals receive another intraperitoneal injection of 12 mg/kg sodium pentobarbital to keep them in deep narcosis for 45 min.

Plasma Preparation

After the test compound is absorbed, blood is withdrawn from the inferior caval vein exposed by a mid-line excision. Blood (1.8 ml) is removed with a plastic syringe containing 0.2 ml 3.8 % sodium citrate solution. The sample is thoroughly mixed, transferred to a plastic tube and immediately immersed in ice. Plasma is prepared by centrifugation at 2000 × g for 10 min at 2 °C.

Euglobulin Preparation

A 0.5 ml portion of plasma is added to 9.5 ml of ice-cold distilled water; the pH is brought to 5.3 by the addition of 0.13 ml of 1 % acetic acid. The diluted plasma is kept on ice for 10 min and the precipitated euglobulin fraction is collected by centrifugation at 2000 × g for 10 min at 2 °C. The supernatant is discharged and the remaining fluid is removed by drying the tube on a filter paper for 1 min. The euglobulin precipitate is dissolved in 1 ml of 0.12 M sodium acetate solution.

Euglobulin Lysis Assay

Aliquots (0.45 ml) of the euglobulin solution are transferred to test tubes, and 0.05 ml thrombin (Test Thrombin, Behring Werke) (25 U/ml) are added. The tubes are transferred to a water bath at 37 °C. The time interval between the addition of thrombin and the complete lysis of the clots is measured.

EVALUATION

The lysis time [min] is determined. The lysis time is shortened when activators of fibrinolysis are increased.

Percent lysis time is calculated in dosage groups as compared to controls.

Statistical evaluation is performed by means of the Student's t-test.

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I.M.1.10 Flow Behavior of Erythrocytes

PURPOSE AND RATIONALE

The deformation of erythrocytes is an important rheological phenomenon in blood circulation according to Teitel (1977). It allows the passage of normal red cells through capillaries with diameters smaller than that of the discoid cells and reduces the bulk viscosity of blood flowing in large vessels. In the following test, the initial flow of filtration is taken as a criterion for erythrocyte deformability. A prolonged time of filtration can be due to 2 basic pathologic phenomena: an increased rigidity of the individual red cells or an increased tendency of the cells to aggregate. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified by one (or by the combination) of the following stress factors:

1. addition of calcium ions (increase in erythrocyte rigidity)
2. addition of lactic acid (decrease in pH value)

3. addition of 350–400 mmol NaCl (hyperosmolarity)
4. storing the sample for at least 4 h (cellular ageing, depletion of ADP).

The following procedure can be used to evaluate the effect of test compounds on the flow behavior of erythrocytes.

PROCEDURE

Apparatus

Erythrocyte filtrometer MF 4 (Fa. Myrenne, 52159 Roetgen, Germany)

Membrane filter (Nuclepore Corp.) pore diameter: 5–10 μm, pore density: 4×10^5 pores/cm^2.

Ex vivo

Blood is collected from Beagle dogs, weighing 12–20 kg, or from rabbits, weighing 1.2–2.5 kg, or from Wistar rats, weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, blood is incubated at 37 °C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with K-EDTA (1 mg/ml blood) or heparin (5 IU/ml heparin sodium) and centrifuged at 3000 rpm for 7 min. The supernatant (plasma) and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in autologous plasma containing 0.25 % human albumin and the haematocrit value is fixed at 10 %. The red blood cells are altered by one or several of the stress factors mentioned above.

A sample of 2 ml of the stressed suspension is applied to the filtrometer and the initial flow rate is determined. The filtration curve is plotted automatically.

EVALUATION

The cumulative volume of the filtered suspension is recorded per time unit (10 min)

The slope of the curve is determined at different time intervals.

The initial flow rate (10 % of the cell suspension having passed the filter) is recorded.

Statistics

Data of each set are first tested for normal distribution using the Kolmogoroff/Smirnow test. The normal distribution hypothesis is eliminated if the data having a significance level of 5 % are not normally distributed. In case that both data sets to be compared are normally distributed the F-test is applied. The hypothesis of homogeneity of variance of both test series is eliminated when the significance level for homogeneity of variance is 5 %. The t-test for paired and non-paired data is performed when homogeneity of variance is present. In any case, a paired difference test (for paired data) or the U-test (for non-paired data) is likewise carried out (paired of difference test = Wilcoxon test; U-test = Wilcoxon-Mann-Whitney or Mann-Whitney test, respectively).

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I.M.1.11 Filterability of Erythrocytes

PURPOSE AND RATIONALE

The Single Erythrocyte Rigidometer (SER) allows the measurement of deformability of individual red blood cells by determining their passage time through a pore under constant shear stress. In this test, the passage times of single erythrocytes through one pore in a synthetic membrane are determined according to Kiesewetter et al. (1982a), Roggenkamp et al. (1983), and Seiffge et al. (1986a). The pore in the membrane practically represents a capillary with defined diameter and length. The driving pressure is produced by the constant shear stress. The passage of the red blood cells is measured with the help of an electrical device. A constant current of 50–200 nA is applied. When an erythrocyte passes through the pore the current is interrupted. The test is used to detect compounds that improve filterability of erythrocytes. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified either by one or by a combination of the following stress factors:

1. addition of calcium ions (increase in erythrocyte rigidity)
2. addition of lactic acid (decrease in pH value)
3. addition of 350–400 mmol NaCl (hyperosmolarity)
4. storing the sample for at least 4 h (cellular ageing, depletion of ADP).

PROCEDURE

Apparatus

Single erythrocyte rigidometer (Myrenne, 52159 Roetgen, Germany)

Data

- driving pressure: dp = 70 Pa (dog, rabbit, rat), dp = 100 Pa (man)
- wall shear stress: τ = 3 Pa

Single pore membrane:
length: 30 μm
diameter: 3.5 μm (rat); 4.0 μm (rabbit, dog); 4.5 μm (man)

Ex vivo

Blood is collected from Beagle dogs, weighing 12–20 kg, or from rabbits, weighing 1.2–2.5 kg, or from Wistar rats, weighing 150–300 g, or from man. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, the blood samples are incubated at 37 °C for 5 or 30 min.

The blood samples are mixed with K-EDTA (1 mg/ml blood) or heparin (5 IE/ml heparin sodium) to prevent clotting. The blood is centrifuged at 3000 rpm for 7 min. The plasma and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in filtrated HEPES-buffer containing 0.25 % human albumin and the haematocrit value is fixed to < 1 %. The red blood cells are altered by one or several stress factors mentioned above. A sample of 2 ml of the stressed suspension is applied to the measuring device and the passage time of a population of 250 erythrocytes (tm) is determined. Cells remaining in the pore for more than 100 ms (tm > 100 ms) lead to a rheological occlusion.

Untreated red blood cell suspensions serve as control.

EVALUATION

The mean passage time of 250 single erythrocytes and the number of rheological occlusions/250 erythrocytes is determined.

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

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Roggenkamp HG, Jung F, Kiesewetter H (1983) Ein Gerät zur elektrischen Messung der Verformbarkeit von Erythrocyten. A device for the electrical measurement of the deformability of red blood cells. Biomed Techn 28:100–104

Seiffge D, Behr S (1986) Passage time of red blood cells in the SER; their distribution and influences of various extrinsic and intrinsic factors. Clin Hemorheol 6:151–164

I.M.1.12 Erythrocyte Aggregation

PURPOSE AND RATIONALE

The aggregation of red blood cells into rouleaux and from rouleaux into 3-dimensional cell networks is a rheological parameter that decisively influences the flow behavior of blood especially in disturbed microcirculation. In the following procedure an apparatus (erythrocyte aggregometer) is used to measure erythrocyte aggregation. The transparent measuring chamber (cone/plate configuration) is transilluminated by light of a defined wave length. The intensity of the transmitted light, which is modified by the aggregation process, is recorded. The method can be used to determine the effect of test compounds on erythrocyte aggregation according to Kiesewetter et al. (1982) and Schmid-Schoenbein et al. (1973).

PROCEDURE

Apparatus

Selective Erythrocyte Rigidometer (Fa. Myrenne, 52159 Roetgen, Germany)

Ex vivo

Blood is collected from Beagle dogs, weighing 12–20 kg, or from rabbits, weighing 1.2–2.5 kg, or from Wistar rats, weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, the blood sample is incubated at 37 °C for 5 or 30 min.

Blood is obtained from the test subjects by venipuncture and mixed with K-EDTA (1 mg/ml) or heparin (5 IU/ml heparin sodium) to prevent clotting. Erythrocyte aggregation is determined in whole blood of 40 % haematocrit. A sample of 40 μl blood is transferred to the measuring device. The red cells are dispersed at a shear rate of 600/s. After 20 s, flow is switched to stasis and the extent of erythrocyte aggregation is determined photometrically.

EVALUATION

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

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Schmid-Schoenbein H, v Gosen J, Heinich L et al. (1973) A counter-rotating "Rheoscope chamber" for the study of the microrheology of blood cell aggregation by microscopic observation and microphotometry. Microvasc Res 6:366–376

I.M.1.13 Determination of Plasma Viscosity

PURPOSE AND RATIONALE

One of the principal methods for measuring viscosity is based on the rate of flow of a liquid through an orifice according to Harkness (1971). In this test, a defined volume of plasma is transferred into a capillary viscometer and the efflux time required for the plasma to flow from the upper to the lower mark is measured. Using this procedure, the effect of test compounds on the viscosity of blood plasma can be determined. The test can be carried out either ex vivo or in vitro.

PROCEDURE

Ex vivo

Beagle dogs weighing 12–20 kg, or rabbits weighing 2.0–3.0 kg or Wistar rats weighing 150–300 of either sex are used as test animals. Likewise, the test procedure can be performed in man. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In vitro

Following addition of the test compound, plasma (obtained as described below) is incubated at 37 °C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with 1 mg/ml blood K^+-EDTA or heparin sodium (5 IU/ml blood) and centrifuged at 3000 rpm for 5 min. The supernatant (plasma) is removed and a sample of 0.9 ml plasma is transferred into a capillary viscometer (Coulter Harkness, Coulter Electr. LTD, England) provided with a glass capillary of 0.5 mm inside diameter. The temperature during measurement is 37 °C. The flow time (t), required for the plasma to flow through the capillary is measured. Untreated plasma serves as control.

EVALUATION

The viscosity of each sample can be determined using the following formula:

$$\eta = K \times t \times \rho$$

η = viscosity of plasma
K = calibration constant of viscometer
t = flow time of 0.9 ml plasma
ρ = density of plasma

The change in viscosity relative to the control group is determined.

Statistical evaluation is carried out using the Student's t-test.

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I.M.2 In vitro Models of Thrombosis

PURPOSE AND RATIONALE

There is abundant evidence suggesting that platelets play a pivotal role in the pathogenesis of arterial thrombotic disorders, including unstable angina (UA), myocardial infarction (MI) and stroke. The underlying pathophysiological mechanism of these processes has been recognized as the disruption or erosion of a vulnerable atherosclerotic plaque leading to local platelet adhesion and subsequent formation of partially or completely occlusive platelet thrombi.

The specific platelet surface receptors that support these initial adhesive interactions are determined by the local fluid dynamic conditions of the vasculature and the extracellular matrix constituents exposed at the sites of vascular injury. Konstantopolous et al. (1998) and Alveriadou et al. (1993) demonstrated that under high shear conditions, the adhesion of platelets to exposed subendothelial surfaces of atherosclerotic or injured vessels presenting collagen and von Willebrand factor (vWF) is primarily mediated by the platelet glycoprotein (GP)Ib/IX/V complex. This primary adhesion to the matrix activates platelets, leading ultimately to platelet aggregation mediated

predominantly by the binding of adhesive proteins such as fibrinogen and vWF to GPIIb/IIIa. In addition, direct platelet aggregation in the bulk phase under conditions of abnormally elevated fluid shear stresses, analogous to those occurring in atherosclerotic or constricted arterial vessels as shown by Turitto (1982), may be important. Shear-induced platelet aggregation is dependent upon the availability of vWF and the presence of both GPIb/IX and GPIIb/IIIa on the platelet membrane. It has been postulated that at high shear stress conditions, the interaction of vWF with the GPIb/IX complex is the initial event leading to platelet activation, that also triggers the binding of vWF to GPIIb/IIIa to induce platelet aggregate formation.

A variety of methods has been utilized to assess the ex vivo and/or in vitro efficacy of platelet antagonists, including photometric aggregometry, whole blood electrical aggregometry and particle counter methods as described in the above segments. In photometric aggregometry, a sample is placed in a stirred cuvette in the optical light path between a light source and a light detector. Aggregate formation is monitored by a decrease in turbidity, and the extent of aggregation is measured as percent of maximal light transmission. The major disadvantage of this technique is that it cannot be applied in whole blood since the presence of erythrocytes interferes with the optical responses. Furthermore, it is insensitive to the formation of small aggregates. Particle counters are used to quantitate the size and the number of particles suspended in an electrolyte solution by monitoring the electrical current between two electrodes immersed in the solution. Aggregation in this system is quantitated by counting the platelets before and after stimulation, and is usually expressed as a percentage of the initial count as shown by Jen and McIntire (1984). However, the disadvantage of this technique is that it cannot distinguish platelets and platelet aggregates from other blood cells of the same size. Thus, one is limited to counting only a fraction of single platelets, as well as aggregates that are much larger than erythrocytes and leukocytes. The technique of electrical aggregometry allows the detection of platelet aggregates as they attach to electrodes immersed in a stirred cuvette of whole blood or platelet suspensions. Such an attachment results in a decrease in conductance between the two electrodes that can be quantitated in units of electrical resistance. However, a disadvantage of this method is that it is not sensitive in the detection of small aggregates as demonstrated by Sweeney et al. (1989).

This segment discusses two complementary in vitro flow models of thrombosis that can be used to accurately quantify platelet aggregation in anticoagulated whole blood specimens, and evaluate the inhibitory efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure direct shear-induced platelet aggregation in the bulk phase as demonstrated by Konstantopolous et al. (1995); and (2) a parallel-plate perfusion chamber coupled with a computerized videomicroscopy system to quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood flowing over an immobilized substrate (e.g. collagen I) as shown by Konstantopolous et al. (1995) and Mousa et al. (2002). Furthermore, Mousa et al. (2002) demonstrated a third in vitro flow assay in which surface-anchored platelets are pre-incubated with a GPIIb/IIIa antagonist, and unbound drug is washed away prior to the perfusion of THP-1 monocytic cells, thereby enabling us to distinguish agents with markedly distinct affinities and receptor-bound lifetimes.

PROCEDURE

Isolation of Human Platelets: The steps described in the subsequent subheading outline the procedure for isolation and purification of platelets from whole blood obtained by venipuncture from human volunteers. Obtain blood sample by venipuncture from an antecubital vein into polypropylene syringes containing either sodium citrate (0.38 % final concentration) or heparin (10 U/ml final concentration). Centrifuge anticoagulated whole blood at 160 × g for 15 min to prepare platelet-rich plasma (PRP).

Isolation of Washed Platelets (Evangelista et al. 1996): PRP specimens are subjected to a further centrifugation (1100 × g for 15 min) in the presence of 2 μM PGE_1.

The platelet pellet is resuspended in HEPES-Tyrode buffer containing 5 mM EGTA and 2 μM PGE_1.

Platelets are then washed via centrifugation (1,100 × g for 10 min) and resuspended at 2×10^8/mL in HEPES-Tyrode buffer, and kept at room temperature for no longer than 4 hours before use in aggregation/adhesion assays.

Materials

- Anticoagulant solution (sodium citrate, porcine heparin, PPACK etc.)
- Fluorescently labeled platelet-specific antibody
- Dulbecco's phosphate-buffered saline (D-PBS) (with and without Ca^{2+}/Mg^{2+}).
- Formaldehyde

- Type I collagen, from bovine Achille's tendon
- 0.5 mol/L glacial acetic acid in water
- Glass coverslips (24x50 mm; Corning; Corning, NY)
- Silicone sheeting (gasket) (0.005-in or 0.010-in thickness; Specialty Manufacturing Inc; Saginaw, MI)
- Quinacrine dihydrocholride
- Prostaglandin E_1 (PGE_1) and EGTA
- Thrombin
- Bovine serum albumin
- HEPES-Tyrode buffer (129 mM NaCl, 9.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM K_2PO_4, 0.8 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, 10 mM HEPES, 5.6 mM dextrose)
- 3-aminopropyltriethoxysilane (APES)
- Acetone
- 70 % nitric acid in water
- THP-1 monocytic cells
- Platelet antagonists such as abciximab.

EVALUATIONS

The methods described below outline three dynamic adhesion/aggregation assays used to assess the in vitro and/or ex vivo efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure shear-induced platelet-platelet aggregation in the bulk phase, (2) a perfusion chamber coupled with a computerized videomicroscopy system to visualize in real time and quantify (a) the adhesion and subsequent aggregation of platelets flowing over an immobilized substrate (e.g. extracellular matrix protein) and (b) free-flowing monocytic cell adhesion to immobilized platelets.

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Turitto VT (1982) Blood viscosity, mass transport, and thrombogenesis. Prog Hemost Thromb 6:139–177

I.M.2.1 Cone-and-Plate Viscometery under Shear-Flow Cytometry

PURPOSE AND RATIONALE

The cone-and-plate viscometer is an in vitro flow model used to investigate the effects of bulk fluid shear stress on suspended cells. Anticoagulated whole blood specimens (or isolated cell suspensions) are placed between the two platens (both of stainless steel) of the viscometer. Rotation of the upper conical platen causes a well-defined and uniform shearing stress to be applied to the entire fluid medium as described by Konstantopolous et al. (1998). The shear rate (γ) in this system can be readily calculated from the cone angle and the speed of the cone using the formula: $\gamma = \left(\frac{2\pi\omega}{60\theta_{cp}}\right)$ where γ is the shear rate in sec^{-1}, ω is the cone rotational rate in revolutions per minute (rev/min) and θ_{cp} is the cone angle in radians. The latter is typically in the range of 0.3 to 1.0°. The shear stress, τ, is proportional to shear rate, γ, as shown by: $\tau = \mu\gamma$, where μ is the viscosity of the cell suspension (the viscosity of anticoagulated whole blood is ~ 0.04 cp at 37 °C). This type of rotational viscometer is capable of generating shear stresses from ~ 2 dyn/cm^2 (venous level) to greater than 200 dyn/cm^2 (stenotic arteries).

PROCEDURE

Single platelets and platelet aggregates generated upon shear exposure of blood specimens are differentiated from other blood cells on the basis of their characteristic forward-scatter and fluorescence (by the use of fluorophore-conjugated platelet-specific antibodies) profiles by flow cytometry as desribed by Konstantopolous et al. (1995). This technique requires no washing or centrifugation steps that may induce artifactual platelet activation, and allows the study of platelet function in the presence of other blood elements. Konstantopolous et al. (1995) described the procedure used to quantify platelet aggregation induced by shear stress as follow:

1. Incubate anticoagulated whole blood with platelet antagonist or vehicle (control) at 37 °C for 10 min.

2. Place a blood specimen (typically ~ 500 μl) on the stationary platen of a cone-and-plate viscometer maintained at 37 °C.
3. Take a small aliquot (~ 3 μl) from the pre-sheared blood sample, fix it with 1 % formaldehyde in D-PBS (~ 30 μl), and process it as outlined in steps 6–8.
4. Expose the blood specimen, in the presence or absence of a platelet antagonist, to well-defined shear levels (typically 4000 sec^{-1} to induce significant platelet aggregation in the absence of a platelet antagonist) for prescribed periods of time (typically 30 to 60 sec).
5. Take a small aliquot (~ 3 μl) from the sheared blood specimen, and immediately fix it with 1 % formaldehyde in D-PBS (~ 30 μl).
6. Incubate the fixed blood samples with a saturating concentration of a fluorescently labeled platelet-specific antibody, such as anti-GPIb(6D1)-FITC, for 30 min in the dark.
7. Dilute specimens with 2 ml of 1 % formaldehyde, and analyze them by flow cytometry.
8. Flow cytometric analysis is used to distinguish platelets from other blood cells on the basis of their characteristic forward scatter and fluorescence profiles, as shown in Fig. 1. Data acquisition is then carried out on each sample for a set period (usually 100 sec), thereby allowing equal volumes for both the pre-sheared and sheared specimens to be achieved. As a result, the percent platelet aggregation can be determined by the disappearance of single platelets into the platelet aggregate region using the formula:

$$\% \text{ Platelet Aggregation} = (1 - Ns/Nc \times 100),$$

where *Ns* represents the single platelet population of the sheared specimen and *Nc* represents the single platelet population of the pre-sheared specimen. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be readily determined.

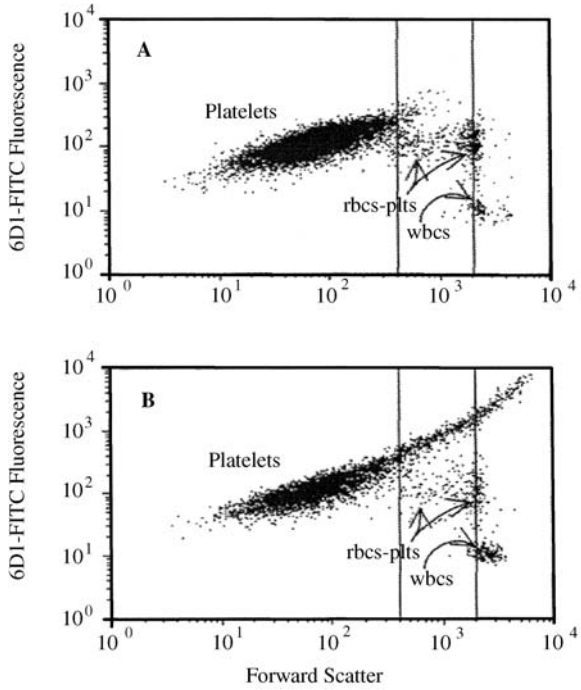

Fig. 1. Quantification of shear-induced platelet aggregation by flow cytometry. Panel A corresponds to an unsheared blood specimen. Panel B corresponds to a blood specimen that has been subjected to a pathologically high level of shear stress for 30 sec. As can be seen in the figure there are three distinct cell populations. The upper population consists of platelets and platelet aggregates. The "rbcs-plts" population corresponds to platelets associated with erythrocytes and leukocytes. The "wbcs" population consists of some leukocytes that have elevated levels of FITC autofluorescence. The *left vertical line* separates single platelets (≤4.5 μm in diameter) from platelet aggregates, whereas the *right vertical line* separates "small" from "large" platelet aggregates. The latter were defined to be larger than 10 μm in equivalent sphere diameter.

I.M.2.2 Platelet Adhesion and Aggregation under Dynamic Shear

PURPOSE AND RATIONALE

The steps described and outlined an in vitro flow model of platelet thrombus formation, which can be used to evaluate the ex vivo and/or in vitro efficacy of platelet antagonists. Thrombus formation may be initiated by platelet adhesion from rapidly flowing blood onto exposed subendothelial surfaces of injured vessels containing collagen and vWF, with subsequent platelet activation and aggregation. Konstantopolous et al. (1995) described the use of a parallel-plate flow chamber which provides a controlled and well-defined flow environment based on the chamber geometry and the flow rate through the chamber. The wall shear stress, τ_w, assuming a Newtonian and incompressible

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Konstantopolous K, Kukreti S, McIntire LV (1998) Biomechanics of cell interactions in shear fields. Adv Drug Delivery Rev 33:141–164

fluid, can be calculated using the formula:

$$\tau_w = \frac{6\mu Q}{wh^2}$$

where Q is the volumetric flow rate, μ is the viscosity of the flowing fluid, h is the channel height, b is the channel width. A flow chamber typically consists of a transparent polycarbonate block, a gasket whose thickness determines the channel depth, and a glass coverslip coated with an extracellular matrix protein such as type I fibrillar collagen. The apparatus is held together by vacuum. Shear stress is generated by flowing fluid (e.g. anticoagulated whole blood or isolated cell suspensions) through the chamber over the immobilized substrate under controlled kinematic conditions using a syringe pump. Mousa et al. (2002) combined the parallel-plate flow chamber with a computerized epi-fluorescence videomicroscopy system, which enables us to visualize in real time and separately quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood (or isolated platelet suspensions) flowing over an immobilized substrate.

PROCEDURE

1. Preparation of Collagen-Coated Surfaces (Folie et al. 1988)
2. Dissolve 500 mg collagen type I from bovine Achille's tendon into 200 ml of 0.5 mol/L acetic acid in water, pH 2.8
3. Homogenize for 3 hours.
4. Centrifuge the homogenate at 200 × g for 10 min, collect supernatant, and measure collagen concentration by a modified Lowry analysis.
5. Coat glass coverslips with 200 µl of fibrillar collagen I suspension on all but first 10 mm of the slide length (coated area = 12.7 × 23), and place in a humid environment at 37 °C for 45 min.
6. Rinse excess collagen with 10 ml of D-PBS maintained at 37 °C before assembly into the flow chamber.
7. Platelet Perfusion Studies
8. Add the fluorescent dye quinacrine dihydrochloride to anticoagulated whole blood samples at a final concentration of 10 µM immediately after blood collection.
9. Prior to the perfusion experiment, incubate blood with either a platelet antagonist or vehicle (control) at 37 °C for 10 min.
10. Perfuse anticoagulated whole blood through the flow chamber for 1 min at wall shear rates ranging from 100 sec^{-1} (typical of venous circulation) to 1500 sec^{-1} (mimicking partially constricted arteries) for prescribed periods of time (e.g. 1 min). Platelet-substrate interactions are monitored in real time using an inverted microscope equipped with an epifluorescent illumination attachment and silicon-intensified target video camera, and recorded on videotape. The microscope stage and flow chamber are maintained at 37 °C by an incubator heating module and incubator enclosure during the experiment.

EVALUATION

Videotaped images are digitized and computer analyzed at 5, 15 and 60 sec for each perfusion experiment. The number of adherent individual platelets in the microscopic field of view during the initial 15 sec of flow is determined by image processing and used as the measurement of platelet adhesion that initiates platelet thrombus formation. The number of platelets in each individual thrombus is calculated as the total thrombus intensity (area × fluorescence intensity) divided by the average intensity of single platelets determined in the 5-sec images. By comparing the extent of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be determined (Fig. 2). Along these lines, any potential inhibitory effects of a platelet antagonist on platelet adhesion can be readily assessed.

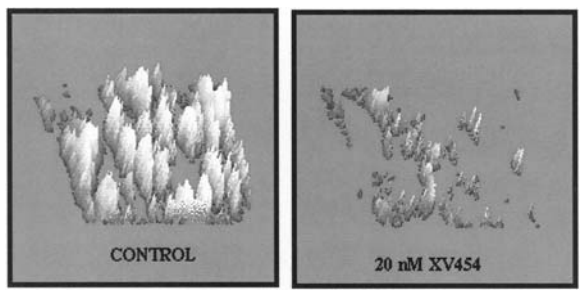

Fig. 2. Three-dimensional computer-generated representation of platelet adhesion and subsequent aggregation on collagen I/von Willebrand factor from normal heparinized blood perfused in the absence (control) or presence of a GPIIb/IIIa antagonist (XV454) at 37 °C for 1 min at 1500 sec^{-1}

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I.M.2.3 Cell Adhesion to Immobilized Platelets: Parallel-Plate Flow Chamber

PURPOSE AND RATIONALE

In this assay, immobilized platelets are pretreated with a GPIIb/IIIa antagonist, and any unbound drug is washed away before the perfusion of monocytic THP-1 cells. McCarty et al. (2004) demonstrated that agents with slow platelet off-rates such as XV454 ($t_{1/2}$ of dissociation = 110 min; K_d = 1 nM) and abciximab ($t_{1/2}$ of dissociation = 40 min; K_d = 9.0 nM) that are distributed predominantly as receptor-bound entities with little unbound in the plasma, can effectively block these heterotypic interactions as shown by Abulencia et al. (2001) and by Mousa et al. (2002). In contrast, agents with relatively fast platelet dissociation rates such as orbofiban ($t_{1/2}$ of dissociation = 0.2 min; K_d > 110 nM), whose antiplatelet efficacy depend on the plasma concentration of the active drug, do not exhibit any inhibitory effects as described by Mousa et al. (2002).

PROCEDURE

Preparation of 3-Aminopropyltriethoxysilane-Treated Glass Slides

1. Soak glass coverslips overnight in 70 % nitric acid.
2. Wash coverslips with tap water for 4 hours.
3. Dry coverslips by washing once with acetone, followed by immersion in a 4 % solution of APES in acetone for 2 min.
4. Repeat Step 3, followed by a final rinse of the glass coverslips with acetone.
5. Wash coverslips three times with water, and allow them to dry overnight.

Immobilization of Platelets on 3-Aminopropyltriethoxysilane-Treated Glass Slides

1. Layer washed platelets or PRP (2×10^8 cells/ml) on the surface of a coverslip at ~ 30 μl/cm^2.
2. Allow platelets to bind to APES-treated coverslip in a humid environment at 37 °C for 30 min.

Monocytic THP-1 Cell-Platelet Adhesion Assay

1. Assemble the platelet-coated coverslip on a parallel-plate flow chamber which is then mounted on the stage of an inverted microscope equipped with a CCD camera connected to a VCR and TV monitor.
2. Perfuse the antiplatelet antagonist at the desirable concentration or vehicle (control) over surface-bound platelets, and incubate for 10 min. The extent of platelet activation can be further modulated by the presence of chemical agonists such as thrombin (0.02–2 U/ml) during the 10 min incubation. The microscope stage and flow chamber are maintained at 37 °C by an incubator heating module and incubator enclosure during the experiment.
3. In some experiments, unbound platelet antagonist is removed by a brief washing step (4 min) prior to the perfusion of the cells of interest over the platelet layer. In others, the desirable concentration of the platelet antagonist is continuously maintained in the perfusion buffer during the entire course of the experiment.
4. Perfuse cells (e.g. THP-1 monocytic cells, leukocytes, tumor cells, protein-coated beads, etc.) over surface-bound platelets, either in the presence or absence of a platelet antagonist (see above), at the desirable flow rate for prescribed periods of time, THP-1 cell binding to immobilized platelets is monitored in real time, and recorded on videotape.
5. Determine the extent of THP-1 cell tethering, rolling and stationary adhesion to immobilized platelets as well as the average velocity of rolling THP-1 cells. By comparing the corresponding extents of THP-1 cell tethering, rolling and stationary adhesion to immobilized platelets in the presence and absence of a platelet antagonist (Figure 3), its antiplatelet efficacy can be determined as shown by McCarty et al. (2004).

EVALUATION

Low-speed centrifugation results in the separation of platelets (top layer) from larger and more dense cells such as leukocytes and erythrocytes (bottom layer). To minimize leukocyte contamination in PRP specimens, slowly aspirate the uppermost 2/3 of the platelet layer. Furthermore, certain rare platelet disorders, such as Bernard-Soulier Syndrome (BSS), are characterized by larger than normal platelets which must therefore be isolated by allowing whole blood to gravity separate for 2 hours post-venipuncture.

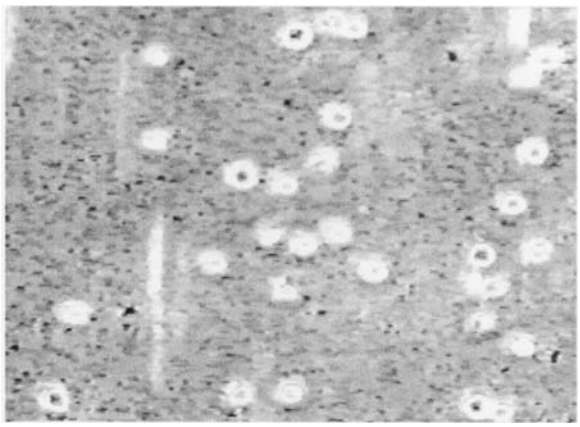

Fig. 3. Phase-contrast photomicrograph of THP-1 cells (phase bright objects) attached to a layer of thrombin-treated platelets (phase dark objects) after THP-1 cell perfusion for 3 min at a shear stress level of 1.5 dyn/cm^2.

The mechanical force most relevant to platelet-mediated thrombosis is shear stress. The normal time-averaged levels of venous and arterial shear stresses range between 1–5 dyn/cm^2 and 6–40 dyn/cm^2, respectively. However, fluid shear stress may reach levels well over 200 dyn/cm^2 in small arteries and arterioles partially obstructed by atherosclerosis or vascular spasm. The cone-and-plate viscometer and parallel-plate flow chamber are two of the most common devices used to simulate fluid mechanical shearing stress conditions in blood vessels.

Due to the large concentration of platelets and erythrocytes in whole blood, small aliquots (~3 μl) of pre-sheared and post-sheared specimens must be obtained and processed prior to the flow cytometric analysis. This will minimize an artifact produced as a platelet and an erythrocyte pass through the light beam of a flow cytometer at the same time.

The "rbcs-plts" population represents 3–5 % of the displayed cells. A small fraction (~ 5 %) of this population seems to be leukocyte-platelet aggregates as evidenced by the use of an anti-CD45 monoclonal antibody. The remaining events correspond to erythrocytes associated with platelets. However, it appears that the majority of the latter population is an artifact generated by the simultaneous passage of a platelet and an erythrocyte through the beam of a flow cytometer. This concept is corroborated by the fact that further dilution of pre-sheared and sheared blood specimens and/or reduction of the sample flow rate during the flow cytometric analysis results in a dramatic relative decrease of the "rbcs-plts" population.

The collagen density remaining on glass coverslips after D-PBS rinsing can be measured by the difference in weight of 20 clean uncoated slides versus 20 collagen-treated slides.

Experiments are optimally monitored ~ 100–200 μm downstream from the collagen/glass interface using a 60 × FLUOR objective and 1 × projection lens, which gives a 3.2×10^4 μm^2 field of view. A field of view closer to the interface may lead to non-reproducible results due to variations in the collagen layering in that region. In contrast, positions farther downstream are avoided in order to minimize the effects of upstream platelet adhesion and subsequent aggregation on both the fluid dynamic environment as well as bulk platelet concentration.

The digitization of a background image (at the onset of perfusion prior to platelet adhesion to the collagen I surface) and its subtraction from a subsequent image acquired 5-sec after an initial platelet adhesion event allows the determination of the fluorescence intensity emitted by a single platelet. The intensity level of each single platelet is measured as a mean gray level between 0 (black) and 255 (white) through the use of an image processing software (e.g. OPTIMAS; Agris-Schoen Vision Systems, Alexandria, VA), and is multiplied by its corresponding area (total number of pixels covered by each single platelet). The aforementioned products are then averaged for all single platelet events detected at the 5-sec time point, thus enabling us to calculate the average intensity of single platelets.

A single field of view (10×0.55 mm^2) is monitored during the 3 min period of the experiment, and at the end five additional fields of view (0.55 mm^2) are monitored for 15 sec each. The following parameters can be quantified: (a) the number of total interacting cells per mm^2 during the entire 3-min perfusion experiment; (b) the number of stationary interacting cells per mm^2 after 3 min of shear flow; (c) the percentage of total interacting cells that are stationary after 3 min of shear flow; and (d) the average rolling velocity (μm/sec) of interacting cells. The number of interacting cells per mm^2 is determined manually by reviewing the videotapes. Stationary interacting cells per mm^2 are considered as those that move < 1-cell radius within 10 sec at the end of the 3 min attachment assay. To quantify their number, images can be digitized from a videotape recorder using an imaging software package (e.g. OPTIMAS). Rolling velocities can be computed as the distance traveled by the centroid of the rolling THP-1 cell divided by the time interval using image processing.

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I.M.3 In vivo or ex vivo Models

PURPOSE AND RATIONALE

The general understanding of the pathophysiology of thrombosis is based on the observations of Virchow in 1856. He proposed three factors responsible for thrombogenesis: obstruction of blood flow, changes in the properties of blood constituents (hypercoagulability), and vessel wall injury. Experimental models of thrombosis focus on one, two or all three factors of Virchow's triad. Therefore, they differ with respect to the prothrombotic challenge – either stenosis, stasis, vessel wall injury (mechanical, electrical, chemical, photochemical, Laser-light), insertion of foreign surface, or injection of a prothrombotic factor – with respect to the vessel type, and with respect to the animal species.

Roughly, two types of models can be differentiated (Didisheim 1972): (1) models in which thrombi are produced in veins by stasis and/or injection of a procoagulant factor resulting in fibrin rich "red" venous type thrombi. (2) models in which thrombi are produced in arteries by vessel wall injury and/or stenosis resulting in platelet rich "white" mural thrombi. But the differentiation is not strict because platelets and the coagulation system influence each other. Drugs preventing fibrin formation may well act in arterial models and vice versa. Thrombosis models are usually performed in healthy animals. The underlying chronic diseases in humans, namely atherosclerosis or thrombophilias are not included in the models. Thus, any model is limited regarding its clinical relevance. The pharmacological effectiveness of a new antithrombotic drug should be studied in more than one animal model. In spite of these limitations animal models predict clinical effectiveness of drugs for the treatment and prevention of thrombotic diseases fairly well. A list of such drugs is presented in a recent review by Leadley et al. (2000). Furthermore, the clinical usefulness of an antithrombotic drug is determined by its safety/efficacy ratio regarding the bleeding risk. Assessment of a parameter of the hemostatic system should therefore be included in the models if possible.

The development of antithrombotic agents requires pre-clinical assessment of the biochemical and pharmacologic effects of these drugs. It is important to note that the second- and third-generation antithrombotic drugs are devoid of in vitro anticoagulant effects, yet in vivo, by virtue of endogenous interactions, these drugs produce potent antithrombotic actions. The initial belief that an antithrombotic drug must exhibit in vitro anticoagulant activity is no longer valid. This important scientific observation has been possible only because of the availability of animal models.

Several animal models utilizing species such as rats, rabbits, dogs, pigs and monkeys have been made available for routine use. Other animal species such as the hamster, mouse, cat and guinea pig have also been utilized. Species variations are an important consideration in selecting a model and interpreting the results as these variations can result in different antithrombotic effects. Rats and rabbits are the most commonly used species in which both arterial and venous thrombosis has been investigated. Both pharmacologic and mechanical means have been used to produce a thrombogenic effect in these models. Both rat and rabbit models for studying bleeding effects of drugs have also been developed. The rabbit ear blood loss model is most commonly used to test the hemorrhagic effect of drugs. The rat tail bleeding models have also been utilized for the study of several antithrombotic drugs.

These animal models have been well established and can be used for the development of antithrombotic drugs. It is also possible to use the standardized bleeding and thrombosis models to predict the safety and efficacy of drugs. Thus, in addition to the evaluation of in vitro potency, the endogenous effect of antithrombotic drugs can also be investigated. Such standardized methods can be recommended for inclusion in pharmacopoeial screening procedures. Numerous models have now been developed to mimic a variety of clinical conditions where antiplatelet and antithrombotic drugs are used, including myocardial infarction, stroke, cardiopulmonary bypass, trauma, peripheral vascular diseases and restenosis. While dog and primate models are relatively expensive, they have also provided useful information on the pharmacokinetics and pharmacodynamics of antithrombotic drugs. The primate models in particular have been extremely useful, as the hemostatic pathways in these species are comparable to those in humans. The development of such agents as the specific glycoprotein IIb/IIIa inhibitor antibodies relies largely on these models. These models are, however, of pivotal value in the

development of antithrombotic drugs and provide extremely useful data on the safety and efficacy of new drugs developed for human usage.

PROCEDURE

Animal Models of Thrombosis

In most animal models of thrombosis, healthy animals are challenged with thrombogenic (pathophysiologic) stimuli and/or physical stimuli to produce thrombotic or occlusive conditions. These models are useful for the screening of antithrombotic drugs.

I. Stasis-thrombosis Model: Since its introduction by Wessler (1959), the rabbit model of jugular stasis thrombosis has been extensively used for the pharmacologic screening of antithrombotic agents. This model has also been adapted for use in rats (Meuleman 1991). In the stasis thrombosis model, a hypercoagulable state is mimicked by administration of one of a number of thrombogenic challenges including human serum (Carrie et al. 1994), thromboplastin (Walenga et al. 1987), activated prothrombin complex concentrates (Vlasuk et al. 1991), factor Xa (Millet et al. 1994) and recombinant relipidated tissue factor (Callas et al. 1995). This administration serves to produce a hypercoagulable state. Diminution of blood flow achieved by ligating the ends of the vessel segments serves to augment the prothrombotic environment. The thrombogenic environment produced in this model simulates venous thrombosis where both blood flow and the activation of coagulation play a role in the development of a thrombus.

II. Models based on vessel wall damage: The formation of a thrombus is not solely induced by a plasmatic hypercoagulable state. In the normal vasculature, the intact endothelium provides a non-thrombogenic surface over which the blood flows. Disruption of the endothelium not only limits the beneficial effects enumerated above, but also exposes subendothelial tissue factor and collagen that serve to activate the coagulation and platelet aggregation processes, respectively. Endothelial damage can be induced experimentally by physical means (clamping, catheter), chemical means (FITC, Rose Bengal, ferrous chloride), thermal injury or electrolytic injury.

EVALUATION

Each setting in the design of an animal model can answer specific question in relation to certain thrombotic disorders in human. However, the ultimate model of human thrombosis is in humans.

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I.M.3.1 Stenosis- and Mechanical Injury-Induced Coronary Thrombosis (Folts-Model)

PURPOSE AND RATIONALE

Thrombosis in stenosed human coronary arteries is one of the most common thrombotic diseases leading to unstable angina, acute myocardial infarction or sudden death. Treatment with angioplasty, thrombolysis, or bypass grafts can expose new thrombogenic surfaces and re-thrombosis may occur. The mechanisms responsible for this process include interactions of platelets with the damaged arterial wall and platelet aggregation.

In order to study new drugs for their antithrombotic potential in coronary arteries, Folts and Rowe (1974) developed the model of periodic acute platelet thrombosis and cyclic flow reductions (CFRs) in stenosed

canine coronary arteries. Uchida described a similar model in 1975. The model includes various aspects of unstable angina pectoris, i.e. critical stenosis, vascular damage, downstream vasospasm induced by vasoconstrictors released or generated by platelets. The cyclic variations in coronary blood flow are a result of acute platelet thrombi which may occlude the vessel but which either embolize spontaneously or can easily be embolized by shaking the constricting plastic cylinder. They are not a result of vasospasm (Folts et al. 1982). Clinically, aspirin can reduce the morbidity and mortality of coronary thrombotic diseases but its effect is limited. Similarly, CFRs in the Folts model are abolished by aspirin but the effect can be reversed by increases in catecholamines and shear forces (Folts and Rowe 1988). As part of an expert meeting on animal models of thrombosis, a review of the Folts model has been published (Folts 1991).

Five different protocols are described in the following section for the induction of coronary thrombosis.

Coronary Thrombosis Induced by Stenosis

The described preparations are characterized by episodic, spontaneous decreases in coronary blood flow interrupted by restorations of blood flow. CFRs are associated with transient platelet aggregation at the site of the coronary constriction and abrupt increase in blood flow after embolization of platelet-rich thrombi.

Damage of the vessel wall is produced by placing a hemostatic clamp on the coronary artery; a fixed amount of stenosis is produced by an externally applied obstructive plastic cylinder upon the damaged part of the vessel. In dogs, the stenosis is critical, i.e. the reactive hyperemic response to a 10-second occlusion is abolished (protocol 1); in pigs, the stenosis is subcritical, i.e. there is a partial reactive hyperemia left (Just et al. 1991a; protocol 2).

For some animals, especially for young dogs, damage of the vessel wall and stenosis are not sufficient to induce thrombotic cyclic flow variations. In these cases, an additional activation of platelets by infusion of epinephrine (protocol 3) is required leading to the formation of measurable thrombi. In another preparation (protocol 4), thrombus formation is induced by subcritical stenosis without prior clamping of the artery and infusion of platelet activating factor (PAF) according to the model described by Apprill et al. (1985). In addition to these protocols, coronary spasms induced by released platelet components can influence coronary blood flow. Therefore, this model includes the main pathological factors of unstable angina pectoris.

Coronary Thrombosis Induced by Electrical Stimulation

In this preparation, coronary thrombosis is induced by delivery of low amperage electrical current to the intimal surface of the artery according to the method described by Romson et al. (1980a). In contrast to the stenosis protocols, an occluding thrombosis is formed gradually without embolism after some hours (protocol 5). As a consequence of this time course, the thrombi formed are of the mixed type and contain more fibrin than the platelet thrombi with critical stenosis.

PROCEDURE

Coronary Thrombosis Induced by Stenosis

Protocol 1: Critical Stenosis

Dogs of either sex weighing 15–40 kg, at least 8 months of age, are anesthetized with pentobarbital sodium (bolus of 30–40 mg/kg and continuous infusion of approx. 0.1 mg/kg/min); respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth or fifth intercostal space, the pericard is opened and the left circumflex coronary artery (LCX) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LCX to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 2 mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2–4 mm in length and with an internal diameter of 1.2 to 1.8 mm (depending on the size of the LCX) is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times (2–5 times) until the appropriate narrowing of the vessel is achieved, and cyclic flow variations are observed. In case of an occlusion of the artery without spontaneous embolization of the formed thrombus, reflow is induced by shortly lifting the vessel with a thread placed beneath the stenotic site.

Only dogs with regularly repeated CFRs of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2 to 4 times over 60 min and compared to pre-treatment values.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

Protocol 2: Subcritical Stenosis
Male castrated pigs (German landrace, weighing 20–40 kg) are anesthetized with ketamine (2 mg/kg i.m.), metomidate (10 mg/kg i.p.) and xylazine (1–2 mg/kg i.m.). In order to maintain the stage of surgical anesthesia, animals receive a continuous i.v. infusion of 0.1–0.2 mg/kg/min pentobarbital sodium. Respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth and fifth intercostal space, the pericard is opened and the left descending coronary artery (LAD) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LAD to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 1 mm hemostatic clamp for 5 s. A small cylindrical plastic constrictor, 2 mm in length, is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times until the appropriate narrowing of the vessel is achieved, which produces CFRs. CFRs are similar to those in dogs; pigs, however, show a reactive hyperemic response. If embolization does not occur spontaneously, the formed thrombus is released at reduction of blood flow by shortly lifting the vessel with forceps.

Only pigs with regularly repeated CFRs of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2 times over 60 min and compared to pre-treatment values.

Protocol 3: Stenosis+Epinephrine Infusion
If protocol 1 does not lead to CFRs, additionally epinephrine (0.2 μg/kg/min) is infused into a peripheral vein for 2 times over 60 min (60 min before and 60 min following drug administration). CFRs are registered and compared in the 60 min post-drug phase to the 60 min pre-drug phase.

Protocol 4: Stenosis+PAF Infusion
The LCX is stenosed without prior mechanical wall injury. This preparation does not lead to thrombus formation (subcritical stenosis). For the induction of CFRs, in addition PAF (C 16-PAF, Bachem) (0.2 nmol/kg/min) is infused into one cannulated lateral branch of the coronary artery.

After 30 min, PAF infusion is terminated and blood flow returns to its normal, continuous course. Thirty minutes later, concomitantly the test substance is administered and a second PAF infusion is started for 30 min.

CFRs are registered and compared in the drug-treated, second PAF phase to the pre-drug, first PAF phase.

Coronary Thrombosis Induced by Electrical Stimulation

Protocol 5
The LCX is punctuated distal to the flow probe with a chrome-vanadium-steel electrode (3 mm length, 1 mm diameter). The electrode (anode) is placed in the vessel in contact with the intimal lining and connected over a Teflon coated wire to a 9 Volt battery, a potentiometer and an amperemeter. A disc electrode (cathode) is secured to a subcutaneous thoracal muscle layer to complete the electrical circuit. The intima is stimulated with 150 μA for 6 h. During this time, gradually an occluding thrombosis is formed.

The test substance or the vehicle (control) is administered either at the start of the electrical stimulation or 30 min following the start.

The time interval until the thrombotic occlusion of the vessel occurs and the thrombus size (wet weight measured immediately after removal at the end of the experiment) are determined.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

For all protocols the following preparations and measurements are performed:

1. To measure peripheral arterial blood pressure (BP) (mm Hg), the right femoral artery is cannulated and connected to a Statham pressure transducer.
2. Left ventricular pressure (LVP) (mm Hg) is determined by inserting a microtip-catheter via the carotid artery retrogradely.
3. Left ventricular enddiastolic pressure (LVEDP) (mm Hg) is evaluated through sensitive amplification of the LVP.
4. Contractility (LV *dp/dt* max) (mm Hg/s) is determined from the initial slope of the LVP curve.
5. Heart rate (min^{-1}) is determined from the pulsatile blood pressure curve.
6. The ECG is recorded in lead II.
7. Arterial pH and concentrations of blood gases are kept at physiological levels by adjusting respiration and infusion of sodium bicarbonate.
8. Blood hematocrit values (37–40 %) and number of erythrocytes are kept constant by infusion of oxypolygelatine in dogs and electrolyte solution in pigs.

9. Body temperature is monitored with a rectal thermistor probe and kept constant by placing the animals on a heated metal pad with automatic regulation of temperature.
10. Template buccal mucosal bleeding time is carried out using the Simplate device.

At the end of the test, animals are sacrificed by an overdose of pentobarbital sodium (Figure 4).

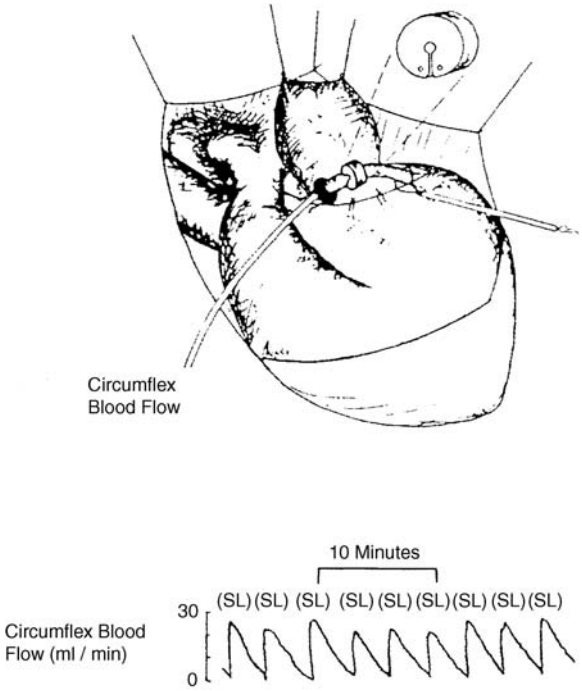

Fig. 4. Technique for monitoring platelet aggregation in the partially obstructed left circumflex coronary artery of the dog. Electromagnetic flow probes measure blood flow. Partial obstruction of the coronary artery with a plastic Lexan cylinder results in episodic cyclical reductions in coronary blood flow that are due to platelet-dependent thrombus formation. Every 2–3 mm the thrombus must be mechanically shaken loose to restore blood. For detailed application of the Folts model, see Sullivan et al. (1992); Folts, Rowe (1974, 1988); and Folts et al. (1976, 1982).

EVALUATION

For all protocols, the mean maximal reduction of blood pressure (systolic/diastolic) (mm Hg) is determined.

Protocol 1–4

The following parameters are determined to quantify stenosis-induced coronary thrombosis:

1. Frequency of cyclic flow reductions = cycle number per time
2. Magnitude of cyclic flow reductions = cycle area (mm^2) (total area of all CVRs per time, measured by planimetry).

Percent change in cycle number and cycle area after drug treatment is calculated compared to pre-treatment controls.

Statistical significance is assessed by the paired Student's t-test.

Protocol 5

The following parameters are determined to quantify electrically induced coronary thrombosis:

1. Occlusion time (min) = time to zero blood flow
2. Thrombus size (mg) = wet weight of the thrombus immediately after removal.

Percent change in mean values for occlusion time and thrombus size in drug-treated groups is compared to the control group.

Statistical significance is assessed by the non-paired Student's t-test.

CRITICAL ASSESSMENT

Both, the stenosis (Folts) and the electrical (Romson/Lucchesi) model of coronary thrombosis are widely used to study the role of mediators in the thrombotic process and the effect of new antithrombotic drugs. Bush and Patrick (1986) reviewed the role of the endothelium in arterial thrombosis and the effect of some inhibitors and mediators in the Folts model, e.g. thromboxane, prostacyclin, cyclooxygenase, serotonin, NO-donors and other vasodilators. The effect of an NO-donor could be reversed by the NO-scavenger oxyhemoglobin indicating that indeed NO was responsible for the anti-thrombotic action (Just and Schönafinger 1991a). Recent mechanisms of antithrombotic drug action which have been studied in either of the two coronary thrombosis models are: the oral GP IIb/IIIa antagonist DMP 728 (Mousa et al. 1996); the LMWH enoxaparin (Leadley et al. 1998) which inhibited CFRs in contrast to unfractionated heparin; the thrombin-inhibitors PEG-hirudin (Ruebsamen and Kirchengast 1998) and melagatran (Mehta et al. 1998); an anti-P-selectin antibody (Ikeda et al. 1999); and activated protein C (Jackson et al. 2000).

The clinical relevance of studies in the Folts-model has been questioned because the model is very sensitive to antithrombotic compounds. However, the lack of a reversal of the effect by epinephrine or increase in degree of stenosis differentiates any new drug from aspirin. Electrical coronary thrombosis is less sensitive: e.g. aspirin has no effect and with some drugs higher dose levels are required; but in principle, most drug mechanisms act in both models if at all.

Table 6 Standard data.

Standard	Dose	% decrease in CFVs		Protocol	Species
		Number	Area		
Acetyl salicylic acid	1 mg/kg, i.v.	56	80	1	dog
	20 mg/kg, i.d.	87	95	1	dog
	30 mg/kg, i.v.	56	77	2	pig

MODIFICATIONS OF THE METHOD

Romson et al. (1980b) described a simple technique for the induction of coronary artery thrombosis in the conscious dog by delivery of low amperage electric current to the intimal surface of the artery.

Benedict et al. (1986) modified the electrical induction of thrombosis by use of two Doppler flow probes proximal and distal to the needle electrode in order to measure changes in blood flow velocity. The electrical current was stopped at 50 % increase in flow velocity and thrombosis then occurred spontaneously. The important role of serotonin was demonstrated by increases in coronary sinus serotonin levels just prior to occlusion.

Warltier et al. (1987) described a canine model of thrombin-induced coronary artery thrombosis and the effects of intracoronary streptokinase on regional myocardial blood flow, contractile function, and infarct size.

Al-Wathiqui et al. (1988) described the induction of cyclic flow reduction in the coronary, carotid, and femoral arteries of conscious chronically instrumented dogs.

The method of Folts-thrombosis has also been applied to carotid arteries in monkeys. Coller et al. (1989) induced CFRs in carotid arteries of anesthetized cynomologus monkeys and showed abolition by the GP IIb/IIIa antibody abciximab.

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I.M.3.2 Stenosis- and Mechanical Injury-Induced Arterial and Venous Thrombosis: Harbauer-Model

PURPOSE AND RATIONALE

Harbauer (1984) first described a venous model of thrombosis induced by mechanical injury and stenosis of the jugular vein. In a modification, both arterial and venous thrombosis is produced in rabbits by stenosis of the carotid artery and the jugular vein with simultaneous mechanical damage of the endothelium. This activates platelets and the coagulation system and leads to changes in the bloodstream pattern. As a consequence, occluding thrombi are formed as detected by blood flow measurement. The dominant role of platelets in this model is shown by the inhibitory effect of an antiplatelet serum in both types of vessels (Just 1986). The test is used to evaluate the antithrombotic capacity of compounds in an in vivo model of arterial and venous thrombosis where thrombus formation is highly dependent on platelet activation.

PROCEDURE

Male Chinchilla rabbits weighing 3–4 kg receive the test compound or the vehicle (controls) by oral, intravenous or intraperitoneal administration. The first ligature (vein, preparation see below) is performed at the end of absorption (i.p. approx. 30 min, p.o. approx. 60 min, i.v. variable).

Sixty-five minutes before stenosis, the animals are sedated by intramuscular injection of 8 mg/kg xylazine (Rompun) and anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium 5 min later. During the course of the test, anesthesia is maintained by continuous infusion of pentobarbital sodium (30–40 mg/kg/h) into one femoral vein.

A Statham pressure transducer is placed into the right femoral artery for continuous measurement of blood pressure. Spontaneous respiration is maintained through a tracheal tube.

One jugular vein and one carotid artery are exposed on opposite sides. Small branches of the vein are clamped to avoid blood flow in spite of vessel occlusion.

Electromagnetic or Doppler flow probes are placed on the vein (directly central to the vein branching) and on the artery (as far central as possible). Blood flow (ml/min) is measured continuously.

After reaching steady state (approx. 15–30 min), a metal rod with a diameter of 1.3 mm is placed on the jugular vein (2 cm central to the vein branching) and a ligature is tightened. After 1 min, the rod is removed from the ligature. Immediately thereafter (approx. 1.5 min), the carotid artery is damaged by briefly squeezing it with forceps. Then a small plastic constricting cylinder with 1.2 mm diameter and 2 mm length is placed around the site of the endothelial damage.

Registration of parameters is terminated after 30 min.

In addition, the template bleeding time is measured at various time intervals before and after drug treatment (depending on the route of administration) in the shaved inner ear using the Simplate device. Care is taken to select parts of the skin without larger vessels.

EVALUATION

Percent thrombus formation (= thrombosis incidence) is judged by determination of the number of occluded vessels (blood flow = 0).

Percent inhibition of thrombosis incidence is calculated in dosed groups as compared to vehicle controls.

Thrombosis incidence is always 100 % in vehicle controls.

Statistical significance is assessed by means of the Fisher-exact-test.

If initial values for blood flow do not significantly differ in dosage and control groups, the area below the blood flow curves is measured by planimetry in addition and mean values in dosed groups are compared to controls by means of the unpaired Student's t-test.

Mean values of occlusion times (min) in dosage and control groups are calculated and compared by means of the t-test.

The maximal change in systolic and diastolic blood pressure during the time period of stenosis as compared to the initial values before drug administration is determined. There is no standardized assessment score. As an example, a reduction of systolic blood pressure by 30 mm Hg and of diastolic blood pressure by 20 mm Hg is quoted as a strong reduction in blood pressure.

CRITICAL ASSESSMENT OF THE METHOD

Two main factors of arterial thrombosis in men are essential in this model: high-grade stenosis and vessel wall damage. In the absence of either no thrombus is found. The occlusive thrombus is formed fast and in a highly reproducible manner. In both vessels thrombus formation is equally dependent on platelet function as shown by antiplatelet serum. Therefore, the jugular vein thrombosis in this model differs from stasis-induced deep vein thrombosis with predominant fibrin formation. On the other hand, these occlusive thrombi are more stable than the pure platelet thrombi in the Folts model since carotid blood flow cannot be restored by shaking the constrictor. The following antithrombotic drugs are effective: i) antiplatelet drugs like ticlopidine, prostacyclin/iloprost, NO-donors (SNP, molsidomine) but not aspirin, thromboxane-synthase-inhibitors; ii) anticoagulants like hirudin, high-dose heparin, warfarin; iii) streptokinase/t-PA (Bevilacqua et al. 1991; Just 1986 and unpublished). In contrast, drugs which only lower blood pressure such as hydralazine, clonidine, prazosin have no effect on thrombus formation in this model.

MODIFICATIONS OF THE METHOD

Bevilacqua et al. (1991) performed the same model in rabbit carotid arteries but compared the procedure in one artery before drug treatment with the contralateral artery after drug treatment. Heparin, the synthetic thrombin inhibitor FPRCH2Cl, iloprost and t-PA inhibited carotid occlusion in this model but not aspirin.

Spokas and Wun (1992) produced venous thrombosis in the vena cava of rabbits by vascular damage and stasis. The vascular wall was damaged by crushing with hemostat clamps. A segment of the vena cava was looped with two ligatures, 2.5 cm apart. At 2 h after ligation, the isolated venous sac was dissected and the clot removed for determination of dry weight.

Lyle et al. (1995) searched for an animal model mimicking the thrombotic reocclusion and restenosis occurring in several cases after successful coronary angioplasty in man. The authors developed a model of angioplasty-induced injury in atherosclerotic rabbit femoral arteries. Acute ^{111}indium-labelled platelet deposition and thrombosis were assessed 4 hours after balloon-injury in arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month). The effects of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition were studied.

Thrombosis Induced by Supercooling

Meng (1975), Meng and Seuter (1977) and Seuter et al. (1979) described a method to induce arterial thrombosis in rats by chilling of the carotid artery. Rats were anesthetized, the left carotid artery was exposed and occluded proximal by means of a small clamp. The artery was placed for 2 min into a metal groove which was cooled to –15 °C. The vessel was compressed by a weight of 200 g. In addition, a silver clip was fixed to the vessel distally from the injured area to produce a disturbed and slow blood flow. After 4 min, the proximal clamp was removed and the blood flow reestablished in the injured artery. In the rabbit, slightly different conditions were used: the chilling temperature was –12 °C for a period of 5 min, and the compressing weight was 500 g. The wound was closed and the animal allowed to recover from anesthesia. Antithrombotic compounds were administered in various doses at different time intervals before surgery. After 4 h, the animals received heparin and were reanesthetized. The lesioned carotid artery was removed and thrombus wet-weight was immediately measured.

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I.M.3.3 Electrical-Induced Thrombosis

PURPOSE AND RATIONALE

The use of electrical current to induce thrombosis in hamster and dog has been described in the early 1950s by Lutz et al. (1951) and Sawyer and Pate (1953a,b). In general, two different approaches exist. One method produces electrical damage by means of two externally applied hook-like electrodes (Hladovec 1973; Philp et al. 1978). The other method uses a needle electrode which is advanced through the walls of the blood vessels and positioned in their lumen; the second electrode is placed into a subcutaneous site completing the circuit (Salazar 1961; Romson et al. 1980; Benedict et al. 1986).

PROCEDURE

Anaesthetized rats weighing 200–300 g are intubated and a femoral artery is cannulated for administration of drugs. One carotid artery is isolated from surrounding tissues over a distance of 10–15 mm.

A pair of rigid stainless-steel wire hook-like electrodes with a distance of 4 mm are adjusted to the artery by means of a rack and pinion gear manipulator. The artery is raised slightly away from the surrounding tissue. Isolation of the electrodes is achieved by the insertion of a small piece of parafilm under the artery. Blood flow is measured with an ultrasonic Doppler flowmeter (Transonic, Ithaca NY, USA); the flow probe (1RB) is placed proximal to the damaged area.

Thrombus formation is induced in the carotid arteries by the application of an electrical current (350 V, DC, 2 mA) delivered by an electrical stimulator (Stoelting Co, Chicago, Cat. No 58040) for 5 min to the exterior surface of the artery (see Figure 5).

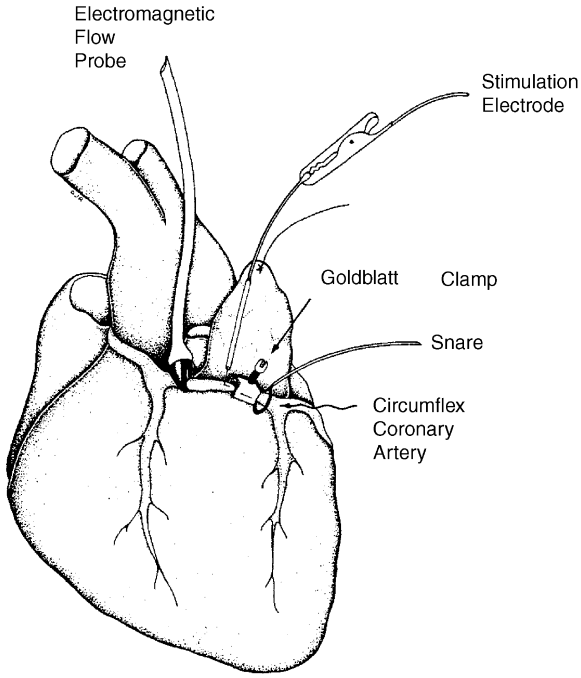

Fig. 5. Model of coronary artery thrombosis in the dog. Electrical injury to the intimal surface of the artery leads to occlusive thrombus formation. The thrombus is formed in the presence of a flow-limiting stenosis induced by a Goldblatt clamp. Upon spontaneous occlusion, heparin is administered, and the clot is aged for 1 h before initiating the t-PA infusion.

EVALUATION

1. Blood flow before and after induction of thrombus for 60 min
2. Time to occlusion (min): the time between onset of the electrical current and the time at which blood flow decreases under 0.3 ml/min
3. Patency of the blood vessel over 30 min.

CRITICAL ASSESSMENT OF THE METHOD

The electrical-induced thrombus is composed of densely packed platelets with some red cells. Moreover, the electrical injury causes extensive damage to intimal and subintimal layers. The endothelium is completely destroyed and this damage extends to subendothelial structures including smooth muscle cells. The deep damage could reduce the possibility of discrimination between drugs on the basis of their antithrombotic activity. However, Philp et al. (1978) could show that unfractionated heparin completely blocked thrombus formation, whereas other antiplatelet agents displayed differentiated antithrombotic action. They concluded that this relatively simple model of arterial thrombosis might prove a useful screening test for drugs with antithrombotic potential.

MODIFICATIONS OF THE METHOD

The technique described by Salazar et al. (1961) uses a stainless steel electrode which is inserted into a coronary artery in the dog and which delivers anodal current to the intravascular lumen. The electrode is positioned under fluoroscopic control which complicates the method. The technique was modified by Romson et al. (1980). They placed the electrode directly into the coronary artery of open-chest anaesthetized dogs.

Rote et al. (1993, 1994) used a carotid thrombosis model in dogs. A calibrated electromagnetic flow meter was placed on each common carotid artery proximal to both the point of insertion of an intravascular electrode and a mechanical constrictor. The external constrictor was adjusted with a screw until the pulsatile flow pattern decreased by 25 % without altering the mean blood flow. Electrolytic injury to the intimal surface was accomplished with the use of an intravascular electrode composed of a Teflon-insulated silver-coated copper wire connected to the positive pole of a 9-V nickel-cadmium battery in series with a 250 000 ohm variable resistor. The cathode was connected to a subcutaneous site. Injury was initiated in the right carotid artery by application of a 150 μA continuous pulse anodal direct current to the intimal surface of the vessel for a maximum duration of 3 h or for 30 min beyond the time of complete vessel occlusion as determined by the blood flow recording. Upon completion of the study on the right carotid, the procedure for induction of vessel wall injury was repeated on the left carotid artery after administration of the test drug.

Benedict et al. (1986) introduced a procedure in which anodal current is discontinued when mean distal coronary flow velocity increased by approximately 50 %, reflecting disruption of normal flow by the growing thrombus. Occlusive thrombosis occurred within 1 h after stopping the electrical current. It was observed that the final phase of thrombosis occurred independently of electrical injury.

A ferret model of acute arterial thrombosis was developed by Schumacher et al. (1996a,b). A 10-min anodal electrical stimulation of 1 mA was delivered to the external surface of the carotid artery while measuring carotid blood flow. This produced an occlusive thrombus in all vehicle treated ferrets within 41 ± 3 min with an average weight of 8 ± 1 mg. Thrombus weight was reduced by aspirin or a thromboxan receptor antagonist.

Guarini (1996) produced a completely occlusive thrombus in the common carotid artery of rats by applying an electrical current to the arterial wall (2 mA for 5 min) while simultaneously constricting the artery with a hemostatic clamp placed immediately downstream from the electrodes.

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I.M.3.4 $FeCl_3$-Induced Thrombosis

PURPOSE AND RATIONALE

A variety of chemical agents has been used to induce thrombosis in animals. Topical $FeCl_3$ was described by Reimann-Hunziger (1944) as thrombogenic stimulus in veins. Kurz et al. (1990) showed that the thrombus produced with this method in the carotid arteries of rats is composed of platelets and red blood cells enmeshed in a fibrin network. This model is used as a simple and reproducible test for evalua-

tion of antithrombotic (Broersma et al. 1991) and profibrinolytic test compounds (van Giezen et al. 1997).

PROCEDURE

Rats weighing between 250 and 300 g are anaesthetized with Inactin (100 mg/kg) and a polyethylene catheter (PE-205) is inserted into the trachea via a tracheotomy to facilitate breathing. Catheters are also placed in the femoral artery for blood samples and measurement of arterial blood pressure and in the jugular vein for administration of test agents. The right carotid artery is isolated and an ultrasonic Doppler flowprobe (probe 1RB, Transonic, Ithaca NY, USA) is placed on the vessel to measure blood flow. A small piece of Parafilm "M" (American Can Co, Greenwich, CT) is placed under the vessel to isolate it from surrounding tissues throughout the experiment.

The test agent is administered by gavage or as an intravenous injection at a defined time prior to initiation of thrombus formation. Thrombus formation is induced by the application of filter paper (2 × 5 mm), saturated with 25 % $FeCl_3$ solution, to the carotid artery. The paper is allowed to remain on the vessel 10 min before removal. The experiment is continued for 60 min after the induction of thrombosis. At that time, the thrombus is removed and weighed.

EVALUATION

1. Blood flow before and after induction of thrombus for 60 min
2. Time to occlusion (min): the time between $FeCl_3$ application and the time at which blood flow decreases under 0.3 ml/min
3. Thrombus weight after blotting the thrombus on filter paper

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I.M.3.5 Thrombin-Induced Clot Formation in Canine Coronary Artery

PURPOSE AND RATIONALE

A canine model of thrombin-induced clot formation was developed by Gold et al. (1984) in which localized coronary thrombosis was produced in the LAD. This is a variation of the technique described by Collen et al. (1983) who used radioactively labeled fibrinogen to monitor the occurrence and extent of thrombolysis of rabbit jugular vein clots. The vessel was intentionally de-endothelialized by external compression with blunt forceps. Snare occluders were then placed proximal and distal to the damaged site, and thrombin (10 U) was injected into the isolated LAD segment in a small volume via a previously isolated side branch. Autologous blood (0.3–0.4 ml) mixed with calcium chloride (0.05 M) also was injected into the isolated LAD segment, producing a stasis-type red clot superimposed on an injured blood vessel. The snares were released 2–5 minutes later and total occlusion was confirmed by selective coronary angiography. This model of coronary artery thrombosis relies on the conversion of fibrinogen to fibrin by thrombin. The fibrin-rich thrombus contains platelets, but at no greater concentration than in a similar volume of whole blood. Once the thrombus is formed, it is allowed to age for 1–2 h, after which a thrombolytic agent can be administered to lyse the thrombus and restore blood flow.

PROCEDURE

In the initial study described by Gold et al. (1984), recombinant t-PA was characterized for its ability to lyse 2-hour-old thrombi. Tissue plasminogen activator was infused at doses of 4.3, 10, and 25 μg/kg/min, i.v, and resulted in reperfusion times of 40, 31, and 13 minutes, respectively. Thus, in this model of canine coronary thrombosis, t-PA exhibited dose-dependent coronary thrombolysis. Furthermore, it is possible to study the effect of different doses of t-PA on parameters of systemic fibrinolytic activation, such as fibrinogen, plasminogen, and a_2-antiplasmin, as well as to assess myocardial infarct size. For example, Kopia et al. (1988) demonstrated that SK elicited dose-dependent thrombolysis in this model.

Subsequently, Gold et al. (1986, 1988) modified the model to study not only reperfusion, but also acute reocclusion. Clinically, reocclusion is a persistent problem after effective coronary thrombolysis, which is reported to occur in 15–45 % of patients (Goldberg

et al. 1985). Thus, an animal model of coronary reperfusion and reocclusion would be important from the standpoint of evaluating adjunctive therapies to t-PA to hasten and/or increase the response rate to thrombolysis as well as prevent acute reocclusion.

Thrombin-Induced Rabbit Femoral Artery Thrombosis: Localized thrombosis can also be produced in rabbit peripheral blood vessels such as the femoral artery by injection of thrombin, calcium chloride, and fresh blood via a side branch (Shebuski et al. 1988).

Either femoral artery is isolated distal to the inguinal ligament and traumatized distally from the lateral circumflex artery by rubbing the artery with the jaws of forceps. An electromagnetic flow probe is placed distal to the lateral circumflex artery to monitor femoral artery blood flow (FABF). The superficial epigastric artery is cannulated for induction of the thrombus and subsequent infusion of thrombolytic agents. Localized thrombi distal to the lateral circumflex artery with snares approximately 1 cm apart are induced by the sequential injection of thrombin, $CaCl_2$ (1.25 mmol), and a volume of blood sufficient to distend the artery. After 30 minutes, the snares are released and FABF is monitored for 30 mm to confirm total obstruction of flow by the thrombus (see Figure 6).

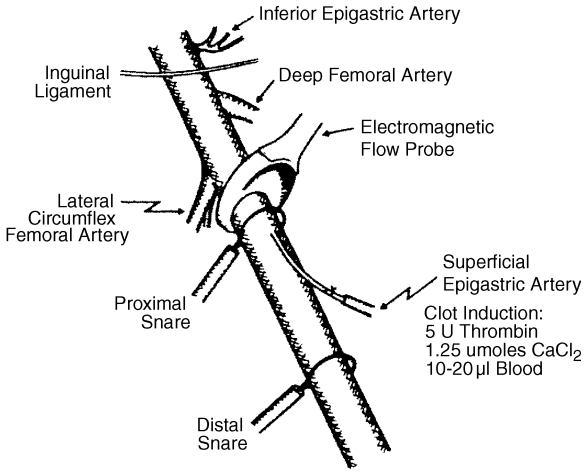

Fig. 6. Rabbit model of femoral arterial thrombosis. A clot is introduced into an isolated segment of femoral artery by injection of thrombin, $CaC1_2$, and whole blood. After aging for 1 h, t-PA is infused. Reperfusion is assessed by restoration of blood flow.

EVALUATION

The model of thrombin-induced clot formation in the canine coronary artery was modified such that a controlled high-grade stenosis was produced with an external constrictor. Blood flow was monitored with an electromagnetic flow probe. In this model of clot formation with superimposed stenosis, reperfusion in response to t-PA occurs with subsequent reocclusion. The monoclonal antibody against the human GPIIb/IIIa receptor developed by Coller et al. (1983) and tested in combination with t-PA in the canine thrombosis model hastened t-PA-induced thrombolysis and prevented acute reocclusion (Yasuda et al. 1988). These actions in vivo were accompanied by abolition of ADP-induced platelet aggregation and markedly prolonged bleeding time.

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I.M.3.6 Laser-Induced Thrombosis

PURPOSE AND RATIONALE

Thrombus formation in rat or rabbit mesenteric arterioles or venules is induced by laser beams. The test can be performed in normal or pretreated (induction

of arteriosclerosis or adjuvant arthritis) animals. The mediators for thrombus formation in this method are platelet adhesion to the injured endothelial vessel wall on one hand and ADP-induced platelet aggregation on the other. Most probably, ADP is primarily released by laser beam lysed erythrocytes, due to the fact that erythrocyte hemoglobin exerts strong adsorbability to frequencies emitted by laser beams. There is a further, secondary, aggregation stimulus following the release reaction induced by the platelets themselves.

PROCEDURE

Apparatus

4 W Argon laser (Spectra Physics, Darmstadt, FRG) wave length: 514 nm, energy below the objective: 15 mW, duration of exposure: 1/30 or 1/15 s

1. Microscope ICM 405, LD-Epipland 40/0.60 (Zeiss, Oberkochen, FRG)
2. Video camera (Sony, Trinicon tube)
3. Recorder (Sony, U-matic 3/4″)
4. Videoanalyzer and correlator to determine blood flow velocity.

In vivo Experiment

Male Sprague Dawley or spontaneously hypertensive stroke prone Wistar or Lewis rats with adjuvant induced arthritis weighing 150–300 g or New Zealand rabbits with arteriosclerosis induced by cholesterol feeding for 3 months are used. The animals receive the test compound by oral, intravenous, intraperitoneal, or subcutaneous administration. Control animals are treated with vehicle alone. Prior to thrombus induction, the animals are pretreated by s.c. injection of 0.1 mg/kg atropine sulfate solution and anaesthetized by intraperitoneal administration of 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine.

Thrombus formation is induced 15, 30, 60 or 90 min post dosing. Investigations are performed in arterioles or venules of 13 ± 1 μm in diameter of the fat-free ileocaecal portion of the mesentery. During the test procedure, the mesenterium is superfused with physiological saline solution or degassed paraffin liquid (37 °C). The ray of the argon laser is led into the inverted ray path of the microscope by means of a ray adaptation and adjusting device. The frequency of injuries is 1 per 2 min. The exposure time for a single laser shot is 1/30 or 1/15 s. The number of injuries necessary to induce a defined thrombus is determined. All thrombi formed during the observation period with a minimum length of 13 μm or an area of at least 25 μm^2 are evaluated. All measuring procedures are photographed by a video system.

Standard compounds:

1. acetylsalicylic acid (10 mg/kg, per os)
2. pentoxifylline (10 mg/kg, per os).

For detailed description and evaution of various agents and mechanisms see the following references: Arfors et al. (1968), Herrmann (1983), Seiffge, Kremer (1984 and 1986), Seiffge, Weithmann (1987), and Weichert and Breddin (1987).

EVALUATION

The number of laser shots required to produce a defined thrombus is determined. Mean values and SEM are calculated. Results are shown graphically.

For statistical evaluation the χ^2-test is used.

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I.M.3.7 Photochemical-Induced Thrombosis

PURPOSE AND RATIONALE

In 1977, Rosenblum and Sabban reported that ultraviolet light can produce platelet aggregation in cerebral microvessels of the mouse after intravascular administration of sodium fluorescein. They found that in contrast to heparin, both aspirin and indomethacin prolonged the time to first platelet aggregate. Herrmann (1983) provided a detailed study in which he showed that scavengers of singlet oxygen, not of hydroxyl radicals, inhibited platelet aggregation induced by the

photochemical reaction. He postulated that by exciting the intravascularly administrated fluorescein, singlet oxygen damages endothelial cells, which subsequently leads to platelet adhesion and aggregation.

PROCEDURE

Studies are performed in mesenteric arteries of 15–30 μm diameter in anesthetized rats. After intravenous injection of fluorescein isothiocyanate-dextran 70 (FITC-dextran, Sigma, 10 %, 0.3 ml), the FITC-dextran in arterioles is exposed to ultraviolet light (wavelength of excitation 490 nm, wavelength of emission 510 nm).

EVALUATION

Thrombus formation is quantitated by determining the time between onset of excitation and appearance of the first platelet aggregate adhering to the vessel wall.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to other thrombosis induction methods, photochemically induced thrombosis can be easily used in smaller animals. Thrombi are composed primarily of platelets; however the primary target of the photochemical insult is the endothelial cells by means of oxygen radical damage.

MODIFICATIONS OF THE METHOD

Matsuno et al. (1991) reports a method to induce thrombosis in the rat femoral artery by means of a photochemical reaction after injection of a fluorescent dye (rose Bengal, 10 mg/kg i.v.) and transillumination with a filtered xenon lamp (wave length: 540 nm). Blood flow is monitored by a pulsed Doppler flow meter. Occlusion is achieved after approximately 5–6 min. Pretreatment with heparin dose-dependently prolongs the time required to interrupt the blood flow. The model also enables the study of thrombolytic mechanisms, which had been evaluated with t-PA. A comparative data for hirudin in various models was carried out by Just et al. (1991).

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I.M.3.8 Foreign-Surface-Induced Thrombosis

The presence of foreign materials in the circulation produces activation of the coagulation and the platelet system. Various prothrombotic surfaces have been used to develop experimental animal models. In contrast to many other thrombosis models, the thrombosis induced by foreign surfaces does not presuppose endothelial damage.

I.M.3.8.1 Wire-Coil Induced Thrombosis

PURPOSE AND RATIONALE

A classical method to produce thrombosis is based on the insertion of wire coils into the lumen of blood vessels. The model was first described by Stone and Lord (1951) in an aorta of a dog and was further modified to be used in arterial coronary vessels of opened-chest dogs. The use in venous vessels was described by Kumada et al. (1980).

The formation of thrombotic material around the coil is reproducible and can be easily standardized to study pharmacological agents (Just and Schönafinger 1991a; Mellott et al. 1993; Rübsamen and Hornberger 1996).

Venous thrombosis is produced in rats by insertion of a stainless steel wire coil into the inferior caval vein. Platelets as well as plasmatic coagulation are activated on the wire coil. Thrombus formation onto the wire is quantitated by measuring the protein content of the thrombotic material isolated. The kinetics of thrombus formation show an increase in weight and protein content within the first 30 min followed by a steady state between thrombus formation and endogenous thrombolysis leading to a constant protein content of thrombi between 1 and up to 48 h following implantation of the wire coil. Thrombosis incidence in untreated control animals in this model is 100 %. The test is used to evaluate antithrombotic and thrombolytic properties of compounds in an in vivo-model of venous thrombosis in rats.

PROCEDURE

Male Sprague-Dawley rats weighing 260–300 g receive the test compound or the vehicle (controls) by oral, intravenous or intraperitoneal administration. At the end of absorption (i.v. 1 min, i.p. 30 min, p.o. 60 min), the animals are anesthetized by in-

traperitoneal injection of 1.3 g/kg urethane. Through a midline incision the caudal caval vein is exposed and a stainless steel wire coil (Zipperer size 40(st), Zdarsky Erler KG, München) is inserted into the lumen of the vein just below the left renal vein branching by gently twisting of the wire towards the iliac vein. The handle of the carrier is cut off so as to hold the back end of the wire at the vein wall. The incision is sutured and the animal is placed on its back on a heating pad (37 °C). The wound is reopened after 2 h, the wire coil is carefully removed together with the thrombus on it and rinsed with 0.9 % saline. The thrombotic material is dissolved in 2 ml alkaline sodium carbonate solution (2 % Na_2CO_3 in 0.1 N NaOH) in a boiling water bath for 3 min. The protein content is determined in 100 µl aliquots by the colorimetric method of Lowry (see Figure 7).

Thrombolysis

In addition to the described preparation, for continuous infusion of a thrombolytic test solution a polyethylene catheter is inserted in the jugular vein. One and a half hours after implantation of the wire coil, the test compound or the vehicle (controls) is infused for up to 2.5 h. The wire coil is then removed and the protein content of thrombi is determined (see above). Bernat et al. (1986) demonstrated the fibrinolytic activity of urokinase and streptokinase-human plasminogen complex in this model.

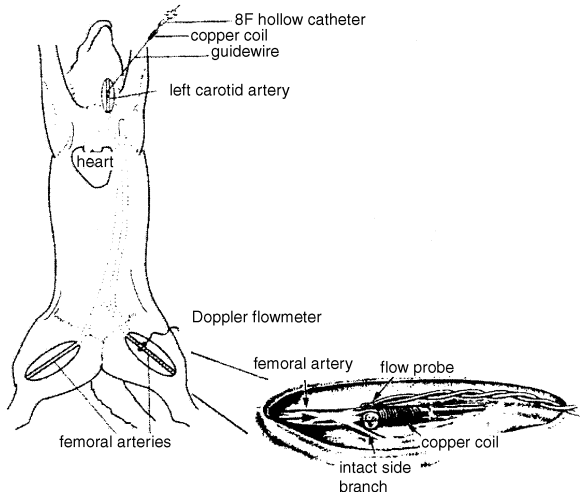

Fig. 7. Schematic diagram of the canine femoral artery copper coil model of thrombolysis. A thrombogenic copper coil is advanced to either femoral artery via the left carotid artery. By virtue of the favorable anatomical angles of attachment, a hollow polyurethane catheter advanced down the left carotid artery nearly always enters the descending aorta, and with further advancement, into either femoral artery without fluoroscopic guidance. A flexible, Teflon-coated guidewire is then inserted through the hollow catheter and the latter is removed. A copper coil is then slipped over the guidewire and advanced to the femoral artery (*see inset*). Femoral artery flow velocity is measured directly and continuously with a Doppler flow probe placed just proximal to the thrombogenic coil and distal to a prominent sidebranch, which is left patent to dissipate any dead space between the coil and the next proximal sidebranch. Femoral artery blood flow declines progressively to total occlusion over the next 10–12 mm after coil insertion.

EVALUATION

Thrombosis incidence (= number of animals with thrombi in dosage groups as compared to vehicle controls) is assessed.

The mean protein content (mg) of the thrombotic material in dosage groups and vehicle controls is determined. Percent change in protein content is calculated in dosage groups as compared to controls.

Statistical significance is assessed by means of the unpaired Student's t-test.

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I.M.3.8.2
Eversion-Graft Induced Thrombosis

PURPOSE AND RATIONALE

The eversion graft model for producing thrombosis in the rabbit artery was first described by Hergrueter et al. (1988) and later modified by Jang et al. (1989, 1990) and Gold et al. (1991). A 4- to 6-mm segment of the rabbit femoral or the dog left circumflex artery is excised, everted and then reimplanted into the vessel by end-to-end anastomoses. After restoration of the blood flow, a platelet-rich occlusive thrombus forms rapidly leading to complete occlusion of the vessel.

This model mimics a deep arterial injury since the adventitial surface is a non-endothelial tissue containing tissue factor and collagen. The rabbit model described here uses a carotid graft inserted into the femoral graft to avoid vasoconstriction often occurring in the inverted femoral segments.

PROCEDURE

In anaesthetized New Zealand White rabbits, the right A. carotis is exposed. After double ligation, a 3 mm segment of the artery is excised, everted and immersed in pre-warmed (37 °C) isotonic saline. Thereafter, the right femoral artery is exposed and occluded by means of a double-occluder (2 cm distance). The femoral artery is transected and the everted graft from the carotid artery is inserted by end-to-end anastomosis using 12 sutures with 9–0 nylon (Prolene, Ethicon, Norderstedt, Germany) under a surgical microscope (Wild M650, Leitz, Heerbrugg, Switzerland). Perfusion of the graft is measured by means of an ultrasonic flowmeter (Model T106, Transonic, Ithaca, NY, USA), The flow probe is positioned 2 cm distal from the graft. After a stabilization period of 15 min, the test substance is given intravenously through the catheterized right V. jugularis. Ten minutes after substance administration, the vessel clamps are released and the blood flow is monitored by the flowmeter for 120 min.

Arterial blood is collected from the left carotid artery at baseline (immediately before substance administration), 10 min, 60 min and 120 min after substance administration.

EVALUATION

1. Time until occlusion (time after restoring of vessel blood flow until occlusion of the vessel indicated by a flow less than 3.0 ml/min)
2. Patency (time during which perfusion of graft is measured related to an observation period of 120 min after administration of test compounds).

Statistical analysis

Time until occlusion and patency are expressed as median and the interquartile range/2 (IQR/2). Significant differences ($p < 0.05$) are calculated by the non-parametric Kruskal-Wallis test.

CRITICAL ASSESSMENT OF THE METHOD

The eversion graft is very thrombogenic, although technically difficult and time consuming. The deep occlusive thrombi can be prevented only by intra-arterially administered thrombolytics or aggressive antithrombotic treatments such as recombinant hirudin at high dosages, or PEG-hirudin. The adventitial surface is a non-endothelial tissue containing tissue-factor and collagen. Thus, both the coagulation system and blood platelets are activated.

MODIFICATIONS OF THE METHOD

Gold et al. (1991) modified the model to be used in thoracotomized dogs in partial obstructed left circumflex coronary arteries. The combination of reduced blood flow due to the constrictor, along with an abnormal non-endothelial surface, produces total thrombotic occlusion within 5 min.

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I.M.3.8.3
Arteriovenous Shunt Thrombosis

PURPOSE AND RATIONALE

A method for the direct observation of extracorporal thrombus formation has been introduced by Rowntree and Shionoya (1927). These first studies could provide evidence that anticoagulants like heparin and hirudin do inhibit thrombus development in arteriovenous shunts. Since today, the A-V-shunt thrombosis models have been often used to evaluate the antithrombotic potential of new compounds in different species including rabbits (Knabb et al. 1992), rats (Hara et al. 1995), pigs (Scott et al. 1994), dogs and cats (Best et al. 1938), and non-human primates (Yokoyama et al. 1995).

PROCEDURE

Rats are anaesthetized and fixed in supine position on a temperature-controlled heating plate to maintain body temperature. The left carotid artery and the right jugular vein are catheterized with short polyethylene catheters. The catheters are filled with isotonic saline solution and clamped. The two ends of the catheters are

connected with a 2 cm glass capillary with an internal diameter of 1 mm. This glass capillary provides the thrombogenic surface. At a defined time after administration of the test compound, the clamps which are occluding the A-V-shunt are opened.

The measurement of the patency of the shunt is performed indirectly with a NiCrNi-thermocouple which is fixed distal to the glass capillary. If blood is flowing the temperature rises from room temperature to body temperature. In contrast, decreases of temperature indicates the formation of an occluding thrombus. The temperature is measured continuously over 30 min after opening of the shunt.

CRITICAL ASSESSMENT OF THE METHOD

It has been shown by Best et al. (1938) that the thrombi formed in the AV-shunt are to a greater part white arterial thrombi. This might be due to the high pressure and shear rate inside the shunts; the thrombi tend to be more arterial in character (Chi et al. 1999).

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I.M.3.8.4
Thread-Induced Venous Thrombosis

PURPOSE AND RATIONALE

Compared to the arterial system, it seems to be more difficult to develop a thrombosis model in venous blood vessels with respect to reproducibility and variability (Chi et al. 1999). Complete stasis together with a thrombogenic stimulus (Wessler-type) is used by numerous investigators to evaluate the effect of compounds on venous thrombosis. Hollenbach et al. (1994) developed a rabbit model of venous thrombosis by inducing cotton threads into the abdominal vena cava of rabbits. The cotton threads serve as a thrombogenic surface, and a thrombus forms around it growing to a maximum mass after 2–3 h. The prolonged non-occlusive character of thrombogenesis in this model focuses on progression of thrombus formation rather than initiation. Therefore, the conditions more closely resemble pathophysiology in humans because blood continues to flow throughout the experiment (Chi et al. 1999).

PROCEDURE

Rabbits weighing between 2.5 and 3.5 kg are anaesthetized with isofluorane inhalation anesthesia and a polyethylene catheter is inserted into the left carotid artery. A polyethylene tube (PE 240, inner diameter 1.67 mm) of 14 cm length is filled with isotonic saline and a copper wire with 5 fixed cotton threads (length 6 cm) is inserted into the tube (after determination of the net weight of the cotton threads). A laparotomy is performed and the V. cava and V. iliaca are dissected free from surrounding tissue. The test agent is administered by a rabbit intragastric tube 60 min (depending on the ex vivo study) prior to initiation of thrombus formation. Blood samples will be measured at 60, 90, 120, 150 and 210 min after oral administration of the test compound.

Thrombus formation is induced by inserting the thrombosis catheter into the caval vein via the V. iliaca (7 cm). Then the copper wire is pushed forward 3 cm to liberate the cotton threads into the vessel lumen. At 150 min after thrombus initiation, the caval segment containing the cotton threads and the developed thrombus will be removed, longitudinally opened and the content blotted on filter paper. After weighing the cotton thread with the thrombus, the net thread weight will be subtracted to determine the corrected thrombus weight.

EVALUATION

1. Corrected thrombus weight after blotting the thrombus on filter paper and subtraction of the net weight of the cotton thread
2. Mean arterial blood pressure (MAP)
3. APTT, HepTest, antiFIIa- and antiFXa-activity.

CRITICAL ASSESSMENT OF THE METHOD

The composition of the cotton threaded thrombus shows a composition of fibrin together with tightly aggregated and distorted erythrocytes, thus being in accordance with human deep vein thrombosis structure. Non-occlusive thrombus formation has been successfully inhibited by heparins, prothrombinase complex inhibitors and thrombin inhibitors (Hollenbach et al. 1994, 1995).

MODIFICATIONS OF THE METHOD

In addition to the originally described method, it is possible to measure blood flow by means of an ultrasonic flow probe, attached distally to the position of the cotton threads on the vein.

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I.M.3.8.5
Thrombus Formation on Superfused Tendon

PURPOSE AND RATIONALE

In all models which include vessel wall damage, blood gets in contact with adhesive proteins of the subendothelial matrix, i.e. von Willebrand factor, collagens, fibronectin, laminin and others. Gryglewski et al. (1978) described an in vivo method where blood of an unanesthetized animal is in contact ex vivo with a foreign surface consisting mainly of collagen. The foreign surface is produced out of the tendon of another animal species. After superfusion of the tendon, blood is recirculated to the unanesthetized animal. The method aims at the quantitation of the antiplatelet potency of drugs based on the formation of platelet thrombi onto the surface of the tendons or of aortic strips from atherosclerotic rabbits.

PROCEDURE

Blood was withdrawn from the carotid artery of anesthetized and heparinized cats by a roller pump at a speed of 6 ml/min. After a passage through a warmed jacket (37 °C), blood was separated into 2 streams, each flowing at a speed of 3 ml/min superfusing in parallel 2 twin strips of the central part of longitudinally cut rabbit Achilles tendon (30 × 3 mm). The blood superfusing the strips dripped into collectors and by its gravity was returned to the venous system of the animals through the left jugular vein. The tissue strips were freely suspended in air and the upper end was tied to an auxotonic lever of a smooth muscle/heart Harvard transducer, while the lower end was loaded with a weight (1–2 g) to keep the lever with its counterweight in a neutral position. When superfused with blood, the strips were successively covered with clots changing the weight of the strips. The weight changes were continuously recorded. After a control period of 30 min, the formed thrombi were gently removed and fixed in formalin for histological examination. Then, the strips were superfused with Tyrode solution and the animals injected with the antithrombotic drug. After 10 min, blood superfusion was renewed for another 30 min.

EVALUATION

The ratio of an increase in weight of the strips after the drug treatment to the increase in weight before drug treatment was considered as an index of anti-aggregatory activity.

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I.M.3.9
Stasis-Induced Thrombosis (Wessler Model)

PURPOSE AND RATIONALE

The "Wessler model" is a classical method of inducing venous thrombosis in animals. Wessler (1952, 1953, 1955a,b, 1957, 1959) combined local venous stasis with hypercoagulability produced by injection of human or dog serum into the systemic circulation of dogs or rabbits. The jugular vein of these animals is occluded by clamps 1 min after the injection of the procoagulatory stimulus into the circulation. Within a few minutes after clamping, a red clot is formed in the isolated venous segment. Fareed et al. (1985) summarized a variety of substances which can be used as pro-coagulatory stimuli. Aronson and Thomas (1985) found an inverse correlation between the duration of stasis and the amount of the hypercoagulating agents to produce the clot.

PROCEDURE

Anaesthetized rabbits are fixed in supine position on a temperature-controlled (37 °C) heating-table. Following cannulation of both carotid arteries (the left in cranial direction) and the right V. femoralis, segments of 2 cm length of the two external jugular veins are exposed and isolated between two loose sutures. Then, 0.3 ml/kg calcium thromboplastin (SIGMA, Deisenhofen, Germany, FRG) is administered via the left carotid artery. Meticulous care is taken to maintain a standard injection time of 30 s followed by injection of 0.5 ml physiological saline within 15 s. Then, 45 s later, both jugular vein segments are occluded by distal and proximal sutures. Stasis is maintained for 30 min. Blood samples are taken immediately before occlusion and 30 s before end of stasis. After excision, the occluded vessel segments are placed on a soaked sponge and opened by a longitudinal incision.

EVALUATION

The size of the clots is assessed using a score system: (0: blood only; 1: very small clot piece[s], filling out at most 1/4 of the vessel; 2: larger clot piece[s], filling out at most 1/2 of the vessel; 3: very large clot[s], filling out at most 3/4 of the vessel; 4: one large clot, filling out the whole vessel). The scores of the left and the right jugular vein are added forming the thrombus size value of one animal. Additionally, the thrombus weight is measured after blotting the thrombus on filter paper.

Thrombus score is expressed as median (minimum–maximum). Thrombus weight is given as mean ± SEM. For the statistical evaluation of the antithrombotic effect, the nonparametric U-Test of Mann and Whitney (thrombus score) or Student's t-test for unpaired samples (thrombus weight) is used. Significance is expressed as $p < 0.05$.

CRITICAL ASSESSMENT OF THE METHOD

Breddin (1989) described the Wessler model because of its static character as the retransformation of an in vitro experiment into a very artificial test situation. One of the major drawbacks is the relative independence of platelet function and hemodynamic changes that largely influence thrombus formation in vivo. However, the model has been shown to be very useful for evaluation of the antithrombotic effect of compounds like heparin and hirudin.

MODIFICATIONS OF THE METHOD

There are a number of different procoagulant agents which had been used to induce thrombosis in this model, such as human serum, Russel viper venom, thromboplastin, thrombin, activated prothrombin complex concentrates and factor X_a (Aronson and Thomas 1985; Fareed et al. 1985). The sensitivity and accuracy of the model can be improved by injecting iodinated fibrinogen into the animals before injecting the thrombogenic agent and then measuring the specific radioactivity in the clot.

The general drawback of the Wessler model is the static nature of the venous thrombus development. To overcome this problem some investigators have developed more dynamic models with reperfusion of the occluded vessel segments after clot development. Depending on the time of test compound administration (pre- or post-thrombus initiation), the effect on thrombus growth and fibrinolysis can be evaluated. Levi et al. (1992) have used this model to assess the effects of a murine monoclonal anti-human PAI-1 antibody and Biemond et al. (1996) compared the effect of thrombin and factor X_a inhibitors with a low molecular weight heparin.

Venous reperfusion model: New Zealand white rabbits weighing 2.5 kg are anesthetized with 0.1 ml atropine, 1.0 mg/kg diazepam, and 0.3 ml Hypnorm (Duphar, 10 mg/ml fluanisone and 0.2 ml fentanyl). Further anesthesia is maintained with 4 mg/kg i.v. thiopental. The carotid artery is cannulated after exposition through an incision in the neck. The jugular vein is dissected free from tissue and small side branches are ligated over a distance of 2 cm. The vein is clamped proximally and distally to isolate the vein segment. Citrated rabbit blood (from another rabbit) is mixed with ^{131}I-radiolabeled fibrinogen (final radioactivity, approximately 25 mCi/ml). Then, 150 µl of this blood is aspirated in a 1-ml syringe containing 25 µl thrombin (3.75 IU) and 45 µl 0.25 mol $CaCl_2$, and 200 µl of the clotting blood is immediately injected into the isolated segment. Thirty minutes after clot injection the vessel clamps are removed and blood flow is restored. ^{125}I-radio-labeled fibrinogen (approximately 5 µCi) is injected through the cannula in the carotid artery (in case of the fibrinolysis studies immediately followed by 0.5 mg/kg recombinant tissue-type plasminogen activator). For each dosage group, four thrombi are analyzed. The extent of thrombolysis is assessed by measurement of the remaining ^{131}I-fibrinogen in the clot and compared with the initial clot radioactivity. The comparison between blood and thrombus ^{125}I-radioactivity reveals the extent of thrombus growth (blood volume accreted to the blood). The thrombus lysis and extension are monitored 60 or 120 min after thrombus formation and are expressed as percentage of the initial thrombus volume. Statistics is performed

as variance analysis and the Newman-Keuls test. Statistical significance is expressed at the level of $p < 0.05$.

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I.M.3.10 Disseminated Intravascular Coagulation (DIC) Model

PURPOSE AND RATIONALE

Another model that is also used widely in rats and mice is a model of systemic thrombosis or disseminated intravascular coagulation (DIC), which is induced by tissue factor, endotoxin (lipopolysaccharide), or FXa (Herbert et al. 1996; Yamazaki et al. 1994; Sato et al. 1998). After systemic administration of the thrombogenic stimulus, this model can be performed with or without mechanical vena caval stasis. When stasis is used, the major parameter is the thrombus mass, but when stasis is not used, the readouts are fibrin degradation products, fibrinogen, platelet count, PT, and APTT, among others. As shown by the many and varied parameters, when used without stenosis, the post-experimental analysis can be time-consuming and technically demanding. Although rodents are useful as a primary efficacy model, limitations such as the ability to withdraw multiple blood samples over the course of the experiment and the difference in activity of at least some FXa inhibitors in human compared to rat plasma in vitro require that compounds be characterized further in more advanced in vivo models of thrombosis.

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I.M.3.11 Microvascular Thrombosis in Trauma Models

PURPOSE AND RATIONALE

Successful replantation of amputated extremities is dependent in large degree on maintaining the microcirculation. A number of models have been developed in which blood vessels are subjected to crush injury with or without vascular avulsion and subsequent anastomosis (Fu et al. 1997; Korompilias et al. 1997; Stockmans et al. 1997). In the model of Stockmans (1997), both femoral veins are dissected from the surrounding tissue. A trauma clamp, which has been adjusted to produce a pressure of 1500 g/mm^2, is positioned parallel to the long axis of the vein. The anterior wall of the vessel is grasped between the walls of the trauma clamp and the two endothelial surfaces are rubbed together for a period of 30 seconds as the clamp is rotated. Formation and dissolution of platelet-rich mural thrombi are monitored over a period of 35 minutes by transillumination of the vessel. By using both femoral veins, the effect of drug therapy can be compared to control in the same animal, minimizing intra-animal variations.

The models of Korompilias (1997) and Fu (1997) examine the formation of arterial thrombosis in rats and rabbits, respectively. In these models, either the

rat femoral artery or the rabbit central ear artery is subjected to a standardized crush injury. The vessels are subsequently divided at the midpoint of the crushed area and then anastomosed. Vessel patency is evaluated by milking the vessel at various time points post-anastomosis. These models have been used to demonstrate the effectiveness of topical administration of LMWH in preventing thrombotic occlusion of the vessels. Such models, while effectively mimicking the clinical situation, are limited by the necessity of a high degree of surgical skill to effectively anastomose the crushed arteries.

I.M.3.12 Cardiopulmonary Bypass Models

PURPOSE AND RATIONALE

Cardiopulmonary bypass (CPB) models have been described in baboons (Van Wyk et al. 1998), swine (Dewanjee et al. 1996) and dogs (Henny et al. 1985). In each model, the variables that can affect the hemostatic system such as anesthesia, shear stresses caused by the CPB pumps and the exposure of plasma components and blood cells to foreign surfaces (catheters, oxygenators, etc) are comparable to that observed with human patients. With these models, it is possible to examine the potential usefulness of novel anticoagulants in preventing thrombosis under relatively harsh conditions where both coagulation and platelet function are altered. The effectiveness of direct thrombin inhibitors (Van Wyk et al. 1998), LMWHs (Murray 1985) and heparinoids (Henny et al. 1985) has been compared to standard heparin. Endpoints have included the measurement of plasmatic anticoagulant levels, the histological determination of microthrombi deposition in various organs, the formation of blood clots in the components of the extracorporeal circuit and the deposition of radiolabeled platelets in various organs and on the components of the extracorporeal circuit. These models, therefore, can be used to assess the antithrombotic potential of new agents for use in CPB surgery and also to assess the biocompatability of components used to maintain extracorporeal circulation. For detailed protocols and evaluations see Callas et al. (1995); Carrie et al. (1994); Fu et al. (1997); Korompilias et al. (1997); Meuleman et al. (1991); Millet et al. (1994); Stockmans et al. (1997); Vlasuk et al. (1991); Walenga et al. (1987); and Wessler et al. (1959).

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I.M.3.13 Extracorporeal Thrombosis Models

PURPOSE AND RATIONALE

These models employ passing blood over a section of damaged vessel (or other selected substrates) and

recording the thrombus accumulation on the damaged vessel histologically or by scintigraphic detection of radiolabeled platelets or fibrin (Badimon and Badimon 1989). This model is interesting because the results can be directly compared to the in vivo deep arterial injury model (Wysokinski et al. 1996) results and to results from a similar extracorporeal model used in humans (Dangas et al. 1998; Ørvim et al. 1995). Dangas et al. (1998) used this model to characterize the antithrombotic efficacy of abciximab, a monoclonal antibody-based platelet glycoprotein IIb/IIIa inhibitor, after administration to patients undergoing percutaneous coronary intervention. They demonstrated that abciximab reduces both the platelet and fibrin components of the thrombus, thereby providing further insight into the unique long-term effectiveness of short-term administration of this drug. Ørvim et al. (1995) also used this model in humans to evaluate the antithrombotic efficacy of rTAP, but instead of evaluating the compound after administration of rTAP to the patient, the drug was mixed with the blood immediately as it flowed into the extracorporeal circuit prior to flowing over the thrombogenic surface. By changing the thrombogenic surface, they were able to determine that rTAP was more effective at inhibiting thrombus formation on a tissue-factor coated surface compared to a collagen-coated surface. These results suggest that optimal antithrombotic efficacy requires an antiplatelet approach along with an anticoagulant. Although this model does not completely represent pathological intravascular thrombus formation, the use of this "human model" of thrombosis may be very useful in developing new drugs because it directly evaluates the ex vivo antithrombotic effect of a drug in flowing human blood.

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I.M.3.14 Experimental Thrombocytopenia or Leucocytopenia

PURPOSE AND RATIONALE

Intravenous administration of collagen, arachidonic acid, ADP, PAF (platelet activating factor) or thrombin activates thrombocytes leading to a maximal thrombocytopenia within a few minutes. The effect is reinforced by additional injections of epinephrine. Activation of platelets leads to intravasal aggregation and temporary sequestration of aggregates in the lungs and other organs. Depending on the dose of agonist, this experimentally induced reduction of the number of circulating platelets is reversible within 60 min after induction. Following administration of PAF, a leucocytopenia is induced in addition. The assay is used to test the inhibitory capacity of drugs against thrombocytopenia or leucocytopenia as a consequence of in vivo platelet or leukocyte stimulation.

Table 7 Materials and solutions.

Substances used to induce thrombocytopenia/ leucocytopenia (intravenous administration)	
In rabbits:	
arachidonic acid (Sigma)	1 mg/kg
collagen (Hormonchemie)	30 μg/ml
In mice:	
collagen	90 μg/kg
+ adrenaline (Hormonchemie)	20 μg/kg
In hamsters:	
collagen	50 μg/kg
+ adrenaline	10 μg/kg
In guinea pigs:	
PAF (Paf-acether, Bachem)	0.03–0.04 μg/kg
thrombin (Hoffman-LaRoche)	60 U/kg
Anesthetics:	
pentobarbital sodium (i.p.)	30 mg/kg
xylazine (i.m.)	8 mg/kg
urethane (i.p.)	1.5 g/kg
Platelet analyzer: Sysmex microcellcounter F-800	

PROCEDURE

Male guinea pigs (Pirbright White) weighing 300–600 g, or male NMRI mice (25–36 g), or Chinchilla rabbits of either sex weighing 2–3 kg are used. Animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (p.o. 60 min, i.p. 30 min, i.v. variable), the marginal vein of the ear of rabbits is cannulated and the thrombocytopenia-inducing substances collagen or arachidonic acid are injected slowly. Blood is collected from the ear artery.

Guinea pigs, hamsters, or mice are anesthetized with pentobarbital sodium (i.p.) and Rompun (i.m.) and placed on an electrically warmed table at 37 °C. The carotid artery is cannulated for blood withdrawal and the jugular vein is cannulated to administer the thrombocytopenia-inducing substances collagen + adrenaline (injection of the mixture of both within 10 s) or PAF or thrombin. In mice collagen + adrenaline are injected into a tail vein.

Approx. 50–100 µl blood are collected into potassium-EDTA-coated tubes at times –1, 1 and 2 min (guinea pigs and mice) or 5, 10 and 15 min (rabbits) following the injection of the inducer. The number of platelets and leukocytes is determined within 1 h after withdrawal in 10 µl samples of whole blood using a microcellcounter suitable for blood of various animal species.

EVALUATION

The percentage of thrombocytes (or leukocytes) is determined in vehicle control and dosage groups at the different times following injection of the inducer relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are taken as 100 %.

Percent inhibition of thrombocytopenia (or leucocytopenia) is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's t-test.

CRITICAL ASSESSMENT OF THE METHOD

The method of collagen + epinephrine induced thrombocytopenia is presently widely used to study the phenotype of mice knocked out for a specific gene with suspected role in hemostasis/thrombosis. A recent example is the Gas 6 –/– mouse (Angelillo-Scherrer et al. 2001) and mice lacking the gene for the G protein G(z) (Yang et al. 2000). The advantage of the method for this purpose is the simple experimental procedure and the small volume of blood necessary. In general, application of the method in small animals (mice, hamsters) needs only small amounts of drug substance. The model is a useful first step of in vivo antithrombotic efficacy of antiplatelet drugs.

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I.M.3.15 Collagenase-Induced Thrombocytopenia

PURPOSE AND RATIONALE

Intravenous administration of the proteolytic enzyme collagenase leads to formation of endothelial gaps and to exposure of deeper layers of the vessel wall. This vascular endothelial injury is mainly involved in triggering thrombus formation by activation of platelets through contact with the basal lamina. As a consequence, thrombocytopenia is induced, which is maximal within 5–10 min following collagenase injection and reversible within 30 min after induction. The model is used to test the inhibitory capacity of compounds against thrombocytopenia in a model of collagenase-induced thrombocytopenia in rats as an alternative to the model described before.

Table 8 Materials and solutions.

Anesthetic: pentobarbital sodium (i.p.)	60 mg/kg
Heparin (Liquemin) (i.v.)	500 U/kg
To induce thrombocytopenia (intravenous administration): collagenase (E.C. 3.4.24.3), (Boehringer, Mannheim)	10 mg/ml/kg
Platelet analyzer: Sysmex microcellcounter F-800	

PROCEDURE

Male Sprague-Dawley rats weighing 260–300 g are used. The animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), rats are anesthetized with pentobarbital sodium (i.p.). One carotid artery is cannulated for blood withdrawal and one jugular vein is cannulated for inducer injection. The animals receive an intravenous injection of heparin and 20 min later, approx. 100 µl blood are collected (initial value). Ten min later, the thrombocytopenia-inducing substance collagenase is administered intravenously.

At times 5, 10, 20 and 30 min following the injection of collagenase, samples of approx. 100 µl blood are collected into potassium-EDTA-coated tubes. The number of platelets is determined in 10 µl samples of whole blood within 1 h after blood withdrawal using a microcellcounter. See Völkl and Dierichs (1986) for details.

EVALUATION

The percentage of platelets is determined in vehicle control and dosage groups at the different times following injection of collagenase relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are set at 100 %.

Percent inhibition of thrombocytopenia is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's t-test.

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I.M.3.16 Reversible Intravital Aggregation of Platelets

PURPOSE AND RATIONALE

Isotopic labeling of platelets can be employed to monitor platelet aggregation and desegregation in vivo. ADP, (adenosine diphosphate), PAF (platelet activating factor), arachidonic acid, thrombin and collagen are known to induce platelet aggregation. In the following procedure, labelled platelets are continuously monitored in the thoracic (A) and abdominal (B) region of test animals. Administration of aggregation promoting agents produces an increase in counts in A and a fall in counts in B. This observation implies that platelets are being aggregated within the vascular system and accumulate in the pulmonary microvasculature. The in-vivo method can be used to evaluate platelet anti-aggregatory properties of test compounds.

PROCEDURE

Preparation of Labelled Platelets

Blood is obtained from rats by cardiopuncture. After centrifugation at 240 × g for 10 min, the platelet rich plasma (PRP) is transferred into a tube and suspended in calcium free Tyrode solution containing 250 ng/ml PGE_1. The suspension is centrifuged at 640 × g for 10 min. The supernatant is discarded and the sediment is suspended by gentle shaking with calcium free Tyrode solution containing 250 ng/ml PGE_1. ^{51}Cr is added to 1 ml of the platelet suspension. Following a 20 min incubation period at 37 °C, the suspension is again centrifuged at 640 × g for 10 min. The supernatant is removed and the labelled platelets are finally resuspended in 1 ml calcium free Tyrode solution containing 250 ng/ml PGE_1.

In vivo Experiment

Male Sprague-Dawley or stroke-prone spontaneously hypertensive rats weighing 150–300 g are used. The animals are anaesthetized with pentobarbital sodium (30 mg/kg, i.p.). Following tracheotomy, the vena femoralis is exposed and cannulated. The labelled platelets are administered via the cannula. The circulating platelets are monitored continuously in the thoracic (A) and abdominal (B) region. The counts are collected using a dual channel gamma spectrometer (Nuclear Enterprise 4681) incorporating a microcomputer (AM 9080A). One hour after administration of labelled platelets (when counts in A and B have stabilized), the aggregation promoting agent (ADP, PAF, arachidonic acid, thrombin or collagen) is administered twice by intravenous injection. One hour is allowed to elapse between each i.v. injection.

The test compound is administered 2 h after platelet injection concurrently with the fourth administration of the aggregating agent. Thirty minutes (ADP, PAF, arachidonic acid, thrombin), or 1 h (collagen) after compound administration another control injection of the aggregating agent is given. This injection is either used as an additional control or it may reveal long-term efficacy of a test compound.

Standard compound: PGI_2 (prostacyclin).

EVALUATION

The microcomputer continuously reveals information about aggregation and desegregation of labelled platelets.

The following parameters are recorded:

A = counts over thorax

B = counts over abdomen

Difference: A–B

Ratio: A/B.

The time course of response is shown in a curve. The area under the curve is calculated by a computer program.

Statistical significance is calculated using the Student's t-test.

MODIFICATION OF THE METHOD

Oyekan and Botting (1986) described a method for monitoring platelet aggregation in vivo in rats, using platelets labeled with indium^{3+} oxine and recording the increase in radioactivity count in the lung after injection of adenosine diphosphate or collagen.

Smith et al. (1989) monitored continuously the intrathoracic content of intravenously injected ^{111}indium labeled platelets in anesthetized guinea pigs using a microcomputer-based system.

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I.M.4 Bleeding Models

I.M.4.1 Subaqueous Tail Bleeding Time in Rodents

PURPOSE AND RATIONALE

The damage of a blood vessel results in the formation of a hemostatic plug, which is achieved by several different mechanisms including vascular spasm, formation of a platelet plug, blood coagulation, and growth of fibrous tissue into the blood clot.

A diagnostic parameter for specific defects of the hemostatic system and for the influence of drugs affecting hemostasis is the length of time that it takes for bleeding to stop from a standard incision, the so-called bleeding time.

Bleeding-time measurements in animals are used to evaluate the hemorrhagic properties of antithrombotic drugs. The transection of the tail of a rodent was first established by Döttl and Ripke (1936) and is commonly used in experimental pharmacology.

PROCEDURE

Anaesthetized rats are fixed in supine position on a temperature-controlled (37 °C) heating-table. Following catheterization of a carotid artery (for measurement of blood pressure) and a jugular vein, the test compound is administered. After a defined latency period, the tail of the rat is transected with a razor-blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail is immersed into a bath filled with isotonic saline solution (37 °C).

EVALUATION

The time until bleeding stops is determined within a maximum observation time of 600 s.

CRITICAL ASSESSMENT OF THE METHOD

There are numerous variables which can influence rodent's bleeding time measurements as discussed by Dejana et al. (1979): position of the tail (horizontal or vertical), the environment (air or saline), temperature, anesthesia, procedure of injury (Simplate method, transsection). All these variables are responsible for the different results reported in literature on compounds like aspirin and heparin under different assay condition (Stella et al. 1975; Minsker and Kling 1977).

Furthermore, it is impossible to transect exactly one blood vessel, because the transected tail region consists of a few major arteries and veins with mutual interaction between one another.

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I.M.4.2 Arterial Bleeding Time in Mesentery

PURPOSE AND RATIONALE

Arterial bleeding is induced by micropuncture of small arteries in the area supplied by the mesenteric artery. Bleeding is arrested in living blood vessels by the formation of a hemostatic plug due to the aggregation of platelets and to fibrin formation. In this test, compounds are evaluated which inhibit thrombus formation, thus prolonging arterial bleeding time. The test is used to detect agents which interfere with primary hemostasis in small arteries.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), the animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. Rats are placed on an electrically warmed table at 37 °C.

The abdomen is opened by a mid-line incision and the mesentery is lifted to display the mesenteric arteries. The mesentery is draped over a plastic plate and superfused continuously with Tyrode's solution maintained at 37 °C. Bleeding times are determined with small mesenteric arteries (125–250 μm external diameter) at the junction of mesentery with intestines.

Adipose tissue surrounding the vessels is carefully cut with a surgical blade.

Arteries are punctured with a hypodermic needle (25 gauge: 16 × 5/10 mm). The bleeding time of the mesenteric blood vessels is observed through a microscope at a magnification of 40 ×. The time in seconds is determined from the puncturing until the bleeding is arrested by a hemostatic plug.

EVALUATION

1. Mean values of bleeding times (s) are determined for each dosage group (4–6 animals, 4–6 punctures each) and compared to the controls.
2. The significance of the results is assessed with the unpaired Student's t-test.
3. The percent prolongation of bleeding time in dosage groups relative to the vehicle controls is calculated.

For further details on methods and evaluations of various mechanisms or agents see the following: Butler et al. (1982); Dejana et al. (1979); and Zawilska et al. (1982).

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I.M.4.3 Template Bleeding Time Method

PURPOSE AND RATIONALE

The template bleeding time method is used to produce a standardized linear incision into the skin of humans to detect abnormalities of primary hemostasis due to deficiencies in the platelet or coagulation system. The method has been modified with the development of a spring-loaded cassette with two disposable blades (Simplate II, Organon Teknika, Durham, NC). These template devices ensure reproducibility of length and depth of dermal incisions. Forsythe and Willis (1989) described a method which enables the Simplate technique as a method to analyze the bleeding time in the oral mucosa of dogs.

PROCEDURE

The dog is positioned in sternal or lateral recumbency. A strip of gauze is tied around both the mandible and maxilla as a muzzle. The template device is placed evenly against the buccal mucosa, parallel to the lip margin, and triggered. Simultaneously, a stopwatch is started. Blood flow from the incision is blotted using circular filter paper (Whatman No. 1, Fisher Scientific Co, Clifton, NJ) held directly below, but not touching the wounds. The position of the filter paper is changed every 15 s. The end point for each bleeding is determined when the filter paper no longer develops a red crescent.

EVALUATION

The time from triggering the device until blood no longer appears on the paper is recorded as the bleeding time. The normal range lies between 2 to 4 min.

CRITICAL ASSESSMENT OF THE METHOD

The template bleeding time varies considerably between laboratories as well as between species and strains. Therefore, it is important to perform the incisions and the blotting in an identical fashion. Prolonged bleeding times in dogs have been recognized with thrombocytopenia, von Willebrand's disease, uremia, treatment with aspirin, anticoagulants, and dextran (Forsythe and Willis 1989; Klement et al. 1998). Brassard and Meyers (1991) describe the buccal mucosa bleeding time as a test which is sensitive to platelet adhesion and aggregation deficits. Generally, results of antithrombotic drugs in bleeding time models in animals do not exactly predict bleeding risks in clinical situations. But the models allow comparison between drugs with different actions (Dejana et al. 1979; Lind et al. 1991).

MODIFICATIONS OF THE METHOD

The Simplate device can also be used to perform incisions at the shaved inner ear of rabbits taking care to avoid major vessels. The normal range of bleeding time in anaesthetized rabbits is approximately 100 s (77 ± 4 s, $n = 20$).

Klement et al. (1998) described another ear bleeding model in anaesthetized rabbits. The shaved ear was immersed in a beaker containing saline at 37 °C. Five full-thickness cuts were made with a no. 11 Bard-Parker scalpel blade avoiding major vessels and the ear was immediately re-immersed in saline. At different times thereafter (5 to 30 min) aliquots of the saline solution were removed, red cells were sedimented and lysed, and cyanohemoglobin was determined as

a measure of blood loss. In this study, hirudin produced more bleeding than standard heparin.

A cuticle bleeding time (toenail bleeding time) measurement in dogs has been described by Giles et al. (1982). A guillotine type toenail clipper is used to sever the apex of the nail cuticle. A clean transection of the nail is made just into the quick, to produce a free flow of blood. The nail is left to bleed freely. The time until bleeding stops is recorded as the bleeding time. Several nails can be cut at one time to ensure appropriate technique. The normal range lies between 2 to 8 min.

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I.M.5 Genetic Models of Hemostasis and Thrombosis

PURPOSE AND RATIONALE

Recent advances in genetic molecular biology have provided tools allowing scientists to design genetically altered animals that are deficient in certain proteins involved in thrombosis and hemostasis (so-called "knock-outs", or "nulls") (Carmeliet and Collen 1999; Pearson and Ginsburg 1999)**.** These animals have been extremely useful for identifying and validating novel targets for therapeutic intervention. That is, by examining the phenotype (e.g, spontaneous bleeding, platelet defect, prolonged bleeding after surgical incision, etc.) of a specific knock-out strain, scientists can identify the role of the knocked-out protein. Then if the phenotype is favorable (e.g, not lethal), pharmacological agents can be designed to mimic the knock-out. More recently, novel gene medicine approaches have also benefited greatly from the availability of these models, as discussed below. The following section briefly summarizes some of the major findings in thrombosis and hemostasis using genetically altered mice and concludes with an example of how these models have been used in the drug discovery process.

The majority of these gene knock-outs result in mice that develop normally, are born in the expected Mendelian ratios, and are viable as defined by the ability to survive to adulthood. Although seemingly normal, these knock-out mice display alterations in hemostatic regulation, especially when challenged. Deletion of FVIII, FIX, vWF, and the β_3-integrin (Bi et al. 1996; Denis et al. 1998; Hodivala-Dilke et al. 1999; Wang et al. 1997) all result in mice that bleed upon surgical challenge, and despite some minor differences in bleeding susceptibility, these mouse knock-out models mirror the human disease states quite well (hemophilia A, hemophilia B, von Willebrand disease, and Glanzmann's thrombasthenia, respectively). In addition, deletion of some hemostatic factors results in fragile mice with severe deficiencies in their ability to regulate blood loss. Prenatally, these mice appear to develop normally, but are unable to survive the perinatal period due to severe hemorrhage, in most cases due to the trauma of birth.

Genetic knock-outs have also been useful in dissecting the role of individual signaling proteins in platelet activation. Deletion of the ß$_3$-integrin (Hodivala-Dilke et al. 1999) or of $G_{\alpha q}$ (Offermanns et al. 1997) results in dramatic impairment of agonist-induced platelet aggregation. Alteration of the protein coding region in the ß$_3$-integrin carboxy-tail, ß$_3$-DiY, at sites that are thought to be phosphorylated upon platelet activation, also results in unstable platelet aggregation (Law et al. 1999). Deletion of various receptors such as thromboxane A_2, P-selectin, P2Y1, and PAR-3 demonstrate diminished responses to some agonists while other platelet responses are intact (Thomas et al. 1998; Subramaniam et al. 1996; Leon et al. 1999; Kahn et al. 1998). Deletion of PAR-3, another thrombin receptor in mice, has little effect on hemostasis. This indicated the presence of yet another thrombin receptor in platelets and led to the identification of PAR-4 (Kahn et al. 1998).

Given that knock-outs of prothrombotic factors yield mice with bleeding tendencies, it follows that deletion of factors in the fibrinolytic pathway results in increased thrombotic susceptibility in mice. Plasminogen (Bugge et al. 1995; Ploplis et al. 1995), tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and the combined t-PA/u-PA knock-out (Carmeliet et al. 1994) result in mice that

demonstrate impaired fibrinolysis, susceptibility for thrombosis, vascular occlusion, and tissue damage due to fibrin deposition. Interestingly, due to fibrin formation in the heart, these mice may provide a good model of myocardial infarction and heart failure caused by thrombosis (Christie et al. 1999). Intriguingly, mice deficient in PAI-1, the primary inhibitor of plasminogen activator, demonstrate no spontaneous bleeding and a greater resistance to venous thrombosis due to a mild fibrinolytic state (Carmeliet et al. 1993), suggesting that inhibition of PAI-1 might be a promising approach for novel antithrombotic agents.

In addition to their role in the regulation of hemostasis, several of these genes are important in embryonic development. For example, deletion of tissue factor (Bugge et al. 1996; Toomey et al. 1996; Carmeliet et al. 1996), tissue factor pathway inhibitor (Huang et al. 1997), or thrombomodulin (Healy et al. 1995) results in an embryonic lethal phenotype. These and other (Connolly et al. 1996; Cui et al. 1996), hemostatic factors also appear to contribute to vascular integrity in the developing embryo. These data suggest that initiation of coagulation and generation of thrombin is important at a critical stage of embryonic development, yet other factors must contribute since some of these embryos are able to progress and survive to birth.

Clearly, genetically altered mice have provided valuable insight into the roles of specific hemostatic factors in physiology and pathophysiology. Results of these studies have provided rationale and impetus for attacking certain targets pharmacologically. These types of models have also provided excellent model systems for studying novel treatments for human diseases. For example, these models provided exceptional systems for studying gene therapy for hemophilia. Specifically, deletion of FIX, generated by specific deletions in the FIX gene and its promoter, results in mice which mimic the human phenotype of hemophilia B (Lin et al. 1997). When these mice are treated with adenoviral mediated transfer of human FIX, the bleeding diathesis is fully corrected (Kung et al. 1998). Similarly, selectively bred dogs that have a characteristic point mutation in the sequence encoding the catalytic domain of FIX, also have a severe hemophilia B that is phenotypically similar to the human disease (Evans et al. 1989). When adeno-associated virus-mediated canine FIX gene was administered to these dogs intramuscularly, therapeutic levels of FIX were measured for up to 17 months (Herzog et al. 1999). Clinically relevant partial recovery of whole blood clotting time and APTT was also observed over this prolonged period. These data provided support for initiating the first study of adeno-associated virus-mediated FIX gene transfer in humans (Kay et al. 2000). Preliminary results from this clinical study provided evidence for expression of FIX in the three hemophilia patients studied and also provided favorable safety data to substantiate studying this therapy at higher doses. Although it is likely that there are differences between the human disease and animal models of hemophilia (or other diseases), it is clear that these experiments have provided pharmacological, pharmacokinetic, and safety data that were extremely useful in developing this approach and designing safe clinical trials.

Gene therapy approaches to rescuing patients with bleeding diatheses are further advanced than gene therapy for thrombotic indications. However, promising preclinical data indicates that local overexpression of thrombomodulin (Waugh et al. 1999a) or tissue plasminogen activator (Waugh et al. 1999b) inhibits thrombus formation in a rabbit model of arterial thrombosis. Similarly, local gene transfer of tissue factor pathway inhibitor prevented thrombus formation in balloon-injured porcine carotid arteries (Zoldhelyi et al. 2000). These and other studies (Vassalli et al. 1997) suggest that novel gene therapy approaches will also be effective for thrombotic indications, but these treatments will need to be carefully optimized for pharmacokinetics, safety and efficacy in laboratory animal studies prior to administration to humans.

Genetically Modified Animals

Development and application of animal models of thrombosis has played a crucial role in discovering and validating novel drug targets, selecting new agents for clinical evaluation, and providing dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. The development and application of small and large animal models of thrombosis to the discovery and development of novel antithrombotic agents is described in this review. The methods and major issues regarding the use of animal models of thrombosis, such as positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species-specificity, and pharmacokinetics, are highlighted. Finally, the use of genetic models of thrombosis/hemostasis is presented using gene-therapy for hemophilia as an example of how animal models have aided in the development of therapies that are presently being evaluated clinically.

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I.M.5.1 Knock Out Mice

PURPOSE AND RATIONALE

Genetically modified animals, in particular knock-out mice, help to understand the role of various factors in blood clotting, thrombolysis, and platelet function.

They are useful to verify the mode of action of new drugs.

Factor I (Fibrinogen)

Phenotype

Born in normal appearance, ~ 10 % die shortly after birth and another 40 % around 1–2 months after birth due to bleeding, failure of pregnancy, blood samples fail to clot or support platelet aggregation in vitro (Suh et al. 1995).

REFERENCES

Suh TT, Holmback K, Jensen NJ et al. (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 9:2020–2033

Factor II (Prothrombin)

Phenotype

Partial embryonic lethality: 50 % between embryonic day (E) 9.5–11.5; at least 1/4 survive to term, but fatal hemorrhage few days after birth; factor II important in maintaining vascular integrity during development as well as postnatal life (Sun et al. 1998; Xu et al. 1998).

REFERENCES

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Xu J, Wu Q, Westfield L et al. (1998) Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 95:7603–7607

Factor V

Phenotype

Half of the embryos die at E9–10, possibly as a result of abnormal yolk sac vasculature, remaining 50 % progress normally to term, but die from massive hemorrhage within 2 h of birth, more severe in mouse than in human (Cui et al. 1996l; Yang et al. 2000).

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Yang TL, Cui J, Taylor JM et al. (2000) Rescue of fatal neonatal hemorrhage in factor V deficient mice by low level transgene expression. Thromb Haemost 83:70–77

Factor VII

Phenotype

Develop normally but suffer fatal perinatal bleeding (Rosen et al. 1997).

REFERENCES

Rosen ED, Chan JCY, Idusogie E et al. (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 390:290–294

Factor VIII

Phenotype

Mild phenotype compared with severe hemophilia A in humans; no spontaneous bleeding, illness or reduced activity during the first year of life; have residual clotting activity (APTT) as shown by Bi et al. (1995).

REFERENCES

Bi L, Lawler AM, Antonarakis SE et al. (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 10:119–121

Factor IX

Phenotype

Factor IX coagulant activities (APTT): +/+ 92 %, +/– 53 %, –/– < 5 %; bleeding disorder (extensive bleeding after clipping a portion of the tail, bleeding to death if not cauterized (Kundu et al. 1998; Wang et al. 1997).

REFERENCES

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Wang L, Zoppè M, Hackeng TM et al. (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

Factor X

Phenotype

Partial embryonic lethality (1/3 died on E11.5–12.5); fatal neonatal bleeding between postnatal day (P) 5-20 as shown by Dewerchin et al. (2000).

REFERENCES

Dewerchin M, Liang Z, Moons L et al. (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

Factor XI

Phenotype

APTT prolonged in –/– (158–200 s) compared with +/+ (25–34 s) and +/– (40–61 s); no factor XI activity and antigen, did not result in intrauterine death, –/– similar bleeding as +/+ with a tendency to prolongation (Gailani et al. 1997).

REFERENCES

Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

TF (Tissue Factor)

Phenotype

Abnormal circulation from yolk sac to embryo ~ E8.5 leading to embryo wasting and death; TF has a role in blood vessel development (Bugge et al. 1996; Carmeliet et al. 1996; Toomey et al. 1996, 1997).

REFERENCES

Bugge TH, Xiao Q, Kombrinck KW et al. (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 93:6258–6263

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Toomey JR, Kratzer KE, Lasky NM et al. (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587

Toomey JR, Kratzer KE, Lasky NM, Broze GJ (1997) Effect of tissue factor deficiency on mouse and tumor development. Proc Natl Acad Sci USA 94:6922–6926

TFPI (Tissue Factor Pathway Inhibitor)

Phenotype

None survive the neonatal period; 60 % die between E9.5–11.5 with signs of yolk sac hemorrhage (Huang et al. 1997).

REFERENCES

Huang Z-F, Higuchi D, Lasky N, Broze GJ Jr. (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

Thrombin Receptor

Phenotype

50 % die at E9–10; 50 % survive and become grossly normal adult mice with no bleeding diathesis; –/– platelets strongly respond to thrombin; –/– fibroblast lose their ability to respond to thrombin → second TR must exist as shown by Connolly et al. (1996) and by Darrow et al. (1996).

REFERENCES

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Darrow AL, Fung-Leung WP, YE RD et al. (1996) Biological consequences of thrombin receptor deficiency in mice. Thromb Haemost 76:860–866

Thrombomodulin

Phenotype

Embryonic lethality before development of a functional cardiovascular system; die before E9.5 due to retardation of growth; TM+/– mice develop normal without thrombotic complications (Christie et al. 1999; Healy et al. 1995, 1998; Weiler-Guettler et al. 1998).

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Weiler-Guettler H, Christie PD, Beeler DL et al. (1998) A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 101:1983–1991

Protein C

Phenotype

KO mice appeared to develop normally macroscopically, but possessed obvious signs of bleeding and thrombosis; did not survive beyond 24 h after delivery; microvascular thrombosis in the brain and necrosis in the liver; plasma clottable fibrinogen was not detectable suggesting fibrinogen depletion and secondary consumptive coagulopathy (Jalbert et al. 1998).

REFERENCES

Jalbert LR, Rosen ED, Moons L et al. (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

Plasminogen

Phenotype

Severe spontaneous thrombosis; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; impaired skin healing; reduced macrophage and keratinocyte migration (Bugge et al. 1995; Ploplis et al. 1995).

REFERENCES

Bugge TH, Flick MJ, Daugherty CC, Degen JL (1995) Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 9:794–807

Ploplis VA, Carmeliet P, Vazirzadeh S et al. (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593

Alpha$_2$-Antiplasmin

Phenotype

Normal fertility, viability and development; no bleeding disorder; spontaneous lysis of injected clots → enhanced fibrinolytic potential; significant reduction of renal fibrin deposition after LPS (Lijnen et al. 1999).

REFERENCES

Lijnen HR, Okada K, Matsuo O et al. (1999) Alpha$_2$-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 93:2274–2281

t-PA (Tissue-Type Plasminogen Activator)

Phenotype

Extensive spontaneous fibrin deposition; severe spontaneous thrombosis; impaired neointima formation; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; abnormal tissue remodelling (Carmeliet et al. 1994; Christie et al. 1999).

REFERENCES

Carmeliet P, Schoonjans L, Kieckens L et al. (1994) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Christie PD, Edelberg JM, Picard MH et al. (1999) A murine model of myocardial thrombosis. J Clin Invest 104:533–539

PAI-1 (Plasminogen Activator Inhibitor 1)

Phenotype

Reduced thrombotic incidence; no bleeding; accelerated neo-intima formation; reduced lung inflammation; reduced atherosclerosis. Detailed studies on PAI-1 are reported by Carmeliet et al. (1993); Eitzman et al. (1996); Erickson et al. (1990); Kawasaki et al. (2000); and Pinsky et al. (1998).

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Pinsky DJ, Liao H, Lawson CA et al. (1998) Coordinated induction of plasminogen activator inhibitor-1 (PAI-1) and inhibition of plasminogen activator gene expression by hypoxia promotes pulmonary vascular fibrin deposition. J Clin Invest 102:919–928

TAFI (Thrombin Activatable Fibrinolysis Inhibitor)

Not described.

Vitronectin

Phenotype

Normal development, fertility, and survival; serum is completely deficient in "serum spreading factor" and plasminogen activator inhibitor 1 binding activities; delayed arterial and venous thrombus formation (Eitzman et al. 2000; Zheng et al. 1995).

REFERENCES

Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D (2000) Plasminogen activator inhibitor-1 and vitronectin promote vascular thrombosis in mice. Blood 95:577–580

Zheng X, Sunders TL, Camper SA et al. (1995) Vitronectin is not essential for normal mammalian development and fertility. Proc Natl Acad Sci USA 92:12426–12430

Urokinase, u-PA (Urinary-Type Plasminogen Activator)

Phenotype

Single u-PA deficiency: viable, fertile, normal life span; occasionally spontaneous fibrin deposits in normal and inflamed tissue; higher incidence of endotoxin-induced thrombosis. Combined t-PA and u-PA deficiency: mice survive embryonic development; retarded growth, reduced fertility, shortened life span; spontaneous fibrin deposits more extensively and in more organs (Carmeliet et al. 1994; Heckel et al. 1990).

Transgenic mice carrying the u-PA gene linked to the albumin enhancer/promoter exhibit spontaneous intestinal and intraabdominal bleeding directly related to transgene expression in the liver and elevated plasma u-PA levels; 50 % die between 3 and 84 h after birth; severe hypofibrinogenemia, loss of clotting function.

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UPAR (Urinary-Type Plasminogen Activator Receptor)

Phenotype

Phenotype normal; attenuated thrombocytopenia and mortality associated with severe malaria (Bugge et al. 1995, 1996; Dewerchin et al. 1996; Piguet et al. 2000).

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Gas 6 (Growth Arrest-Specific Gene 6 Product)

Phenotype

Mice are viable, fertile, appear normal; do not suffer spontaneous bleeding or thrombosis; have normal tail bleeding time. Platelets fail to aggregate irreversibly to ADP, collagen, or U 46619. Arterial and venous thrombosis is inhibited and mice are protected from fatal thromboembolism after injection of collagen plus epinephrine (Angelillo-Scherrer et al. 2001).

REFERENCES

Angelillo-Scherrer A, DeFrutos PG, Aparicio C et al. (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

GPIbalpha (Glycoprotein Ib Alpha, Part of the GP Ib-V–IX Complex)

Phenotype

Bleeding, thrombocytopenia and giant platelets (similar to human Bernard Soulier syndrome). See Ware et al. (2000) for details.

REFERENCES

Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci USA 97:2803–2808

GPV (Glycoprotein V, Part of the GP Ib-V–IX Complex)

Phenotype

Increased thrombin responsiveness, GpV–/– platelets are normal in size, normal amounts in GpIb-IX, functional in vWF-binding; platelets are hyperresponsive to thrombin → increased aggregation response; shorter bleeding time; → GpV = negative modulator of platelet function (Poujol et al. 2000).

REFERENCES

Poujol C, Ramakrishnan V, DeGuzman F et al. (2000) Ultrastructural analysis of megakaryocytes in GPV knockout mice. Thromb Haemost 84:312–318

Ramakrishnan V, Reeves PS, DeGuzman F et al. (1999) Increased thrombin responsiveness in platelets from mice lacking glycoprotein V. Proc Natl Acad Sci USA 96:13336–13341

GPIIb (Integrin Alpha IIb, Glycoprotein IIb, Part of the GP IIb–IIIa Complex

Phenotype

Bleeding disorder similar to Glanzmann thrombasthenia in man; platelets failed to bind fibrinogen, to aggregate and to retract a fibrinogen clot; α-granules do not contain fibrinogen (Tronik-Le Roux et al. 2000).

REFERENCES

Tronik-Le Roux D, Roullot V, Poujol C et al. (2000) Thrombasthenic mice generated by replacement of the integrin alpha$_{IIb}$gene: demonstration that transcriptional activation of this megakaryocytic locus precedes lineage commitment. Blood 96:1399–1408

GP IIIa (Integrin Beta3, Glycoprotein IIIa, Part of the GP IIb–IIIa Complex

Phenotype

Viable, fertile, increased fetal mortality; features of Glanzmann thrombasthenia in man, e.g. defective platelet aggregation, clot retraction, spontaneous bleeding, prolonged bleeding times; dysfunctional osteoclasts, development of osteosclerosis with age (Hodivala-Dilke et al. 1999; McHugh et al. 2000).

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basthenia showing placental defects and reduced survival. J Clin Invest 103:229–238
McHugh KP, Hodivala-Dilke K, Zheng MH et al. (2000) Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 105:433–440

GP IIa (Glycoprotein IIa, Integrin Beta 1, Part of the GP Ia–IIa Complex)

Phenotype

Integrin beta1 null platelets from conditional knockout mice develop normally, platelet count is normal. Collagen induced platelet aggregation is delayed but otherwise normal; tyrosine phosphorylation pattern is normal but phosphorylation is delayed. Bleeding time in bone marrow chimeric mice is normal; no major in vivo defects (Nieswandt et al. 2001).

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VWF (von Willebrand Factor)

Phenotype

Factor VIII levels strongly reduced due to defective protection by vWF; highly prolonged bleeding time, hemorrhage, spontaneous bleeding; mice useful for investigating the role of vWF; delayed platelet adhesion in ferric-chloride-induced arteriolar injury (Denis et al. 1998; Ni et al. 2000).

REFERENCES

Denis C, Methia N, Frenette PS et al. (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:9524–9529
Ni H, Denis CV, Subbarao S et al. (2000) Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J Clin Invest 106:385–392

Thromboxane A2 Receptor (TXA2r)

Phenotype

Mild bleeding disorder and altered vascular responses to TXA2 and arachidonic acid (Thomas et al. 1998).

REFERENCES

Thomas DW, Mannon RB, Mannon PJ et al. (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. J Clin Invest 102:1994–2001

Prostacyclin Receptor (PGI2r)

Phenotype

Viable, fertile, normotensive; increased susceptibility to thrombosis; reduced inflammatory and pain responses (Murata et al. 1997).

REFERENCES

Murata T, Ushikubi F, Matsuoka T et al. (1997) Altered pain reception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

PECAM (Platelet: Endothelial Cell Adhesion Molecule)

Phenotype

Normal platelet aggregation; prolonged bleeding time as described by Duncan et al. (1999) and by Mahooti et al. (2000).

REFERENCES

Duncan GS, Andrew DP, Takimoto H et al. (1999) Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1) CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J Immunol 162:3022–3030
Mahooti S, Graesser D, Patil S et al. (2000) PECAM-1 (CD 31) expression modulates bleeding time in vivo. Am J Pathol 157:75–81

Pallid (Pa)

Phenotype

Among 13 hypopigment mouse mutants with storage pool deficiency, the pallid mouse is a model of the human Hermansky Pudlak syndrome (the beige mouse is a model of the Chediak Higashi syndrome). Pallid mice exhibit prolonged bleeding time, pigment dilution, kidney lysosomal enzyme elevation serum alpha 1 antitrypsin deficiency and abnormal otolith formation. The gene defective in pallid mice encodes the highly charged 172-amino acid protein pallidin which interacts with syntaxin 13, a protein mediating vesicle docking and fusion (Huang et al. 1999).

REFERENCES

Huang L, Kuo YM, Gitschier J (1999) The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. Nat Genet 23:329–332

G alpha(q) (Guanyl Nucleotide Binding Protein G Alpha q)

Phenotype

Defective aggregation in response ADP, TXA2, thrombin, collagen; shape change normal (Offermans et al. 1997; Ohlmann et al. 2000).

REFERENCES

Offermanns S, Toombs CF, Hu Y-H, Simon MI (1997) Defective platelet activation in $G\alpha_q$-deficient mice. Nature 389:183–186

Ohlmann P, Eckly A, Freund M et al. (2000) ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. Blood 96:2134–2139

G z (Member of the Gi Family of G Proteins)

Phenotype

Impaired platelet aggregation to epinephrine; resistance to fatal thromboembolism; exaggerated response to cocaine, reduced effect of morphine and antidepressant drugs (Yang et al. 2000).

REFERENCES

Yang J, Wu J, Kowalska MA et al. (2000) Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97:9984–9989

Phospholipase C Gamma

Phenotype

Viable, fertile, decreased mature B cells; defective B cell and mast cell function; defective Fc_{gamma} receptor signaling, therefore, loss of collagen induced platelet aggregation (Wang et al. 2000).

REFERENCES

Wang D, Feng J, Wen R et al. (2000) Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity 13:25–35

CD39 (Vascular Adenosine Triphosphate Diphosphohydrolase)

Phenotype

Viable, fertile; prolonged bleeding times but minimally perturbed coagulation parameters; reduced platelet interaction with injured mesenteric vasculature in vivo. Platelets fail to aggregate to standard agonists in vitro associated with purinergic P2Y1 receptor desensitization; fibrin deposition at multiple organ sites (Enjyoji et al. 1999).

REFERENCES

Enjyoji K, Sevigny J, Lin Y et al. (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 5:1010–1017

Protein Kinase, cGMP-Dependent, Type 1

Phenotype

Viable, fertile; unresponsive to cGMP and NO; defective VASP-phosphorylation; increased adhesion and aggregation of platelets in vivo in ischemic/reperfused mesenteric microcirculation; no compensation by cAMP kinase system (Massberg et al. 1999).

REFERENCES

Massberg S, Sausbier M, Klatt P et al. (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3′,5′-monophosphate kinase I. J Exp Med 189:1255–1264

Vasodilator-Stimulated Phosphoprotein (VASP)

Phenotype

Viable, fertile; mild platelet dysfunction with megakaryocyte hyperplasia, increased collagen/thrombin activation, impaired cyclic nucleotide mediated inhibition of platelet activation (Aszodi et al. 1999; Hauser et al. 1999).

REFERENCES

Aszodi A, Pfeifer A, Ahmad M et al. (1999) The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMVBO J 18:37–48

Hauser W, Knobeloch KP, Eigenthaler M et al. (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci USA 96:8120–8125

Arachidonate 12-Lipoxygenase (P-12LO)

Phenotype

Platelets exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in ex vivo assays and increased mortality in an ADP-induced mouse model of thromboembolism (Chen et al. 1994; Johnson et al. 1998).

REFERENCES

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

Arachidonate 5-Lipoxygenase (P-5LO)

Phenotype

Develop normally and are healthy. No difference in their reaction to endotoxin shock, however resist the lethal effects of shock induced by platelet-activating factor. Inflammation induced by arachidonic acid is markedly reduced (Chen et al. 1994; Argentieri et al. 1994).

REFERENCES

Argentieri DC, Ritchie DM, Ferro MP et al. (1994) Tepoxalin: a dual cyclooxygenase/5-lipoxygenase inhibitor of arachidonic and metabolism with potent anti-inflammatory activity and a favorable gastrointestinal profile. J Pharmacol Exp Ther 271:1399–1408

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

Thrombopoietin

Phenotype

TPO –/– and c-mpl –/–: both exhibit a 90 % reduction in megakaryocyte and platelet levels; but even with these small platelet levels the mice do not have excessive bleeding; all platelets which are present are morphologically normal; in vivo TPO is required for control of megakaryocyte and platelet number but not for their maturation (Lawler et al. 1998).

REFERENCES

Lawler J, Sunday M, Thibert V et al. (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Thrombospondin-1

Phenotype

Normal thrombin-induced platelet aggregation; increase in circulating number of white blood cells; TSP-1 is involved in normal lung homeostasis (Lawler et al. 1998).

Mouse knock-out models of virtually all of the known hemostatic factors have been reported, as shown in Table 9.

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Lawler J, Sunday M, Thibert V et al. (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Bi L, Sarkar R, Naas T et al. (1996) Further characterization of Factor VIII-deficient mice created by gene targeting: RNA and protein studies. Blood 88:3446–3450

Bugge TH, Suh TT, Flick MJ et al. (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Bio Chem 270:16886–16894

Bugge TH, Xiao Q, Kombrinck KW et al. (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci USA 93:6258–6263

Carmeliet P, Schoonjans L, Kieckens L et al. (1994) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Carmeliet P, Stassen JM, Schoonjans L et al. (1993) Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, thrombolysis. J Clin Invest 92:2756–2760

Connolly AJ, Ishihara H, Kahn ML et al. (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519

Cui J, O'Shea KS, Purkayastha A et al. (1996) Fatal haemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 384:66–68

Denis C, Methia N, Frenette PS et al. (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:9524–9529

Dewerchin M, Liang Z, Moons L et al. (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854

Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al. (1999) β_3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

Huang Z-F, Higuchi D, Lasky N, Broze GJ Jr. (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

Jalbert LR, Rosen ED, Moons L et al. (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

Kahn ML, Zheng Y-W, Huang W et al. (1998) A dual thrombin receptor system for platelet activation. Nature 394:690–694

Law DA, DeGuzman FR, Heiser P et al. (1999) Integrin cytoplasmic tyrosine motif is required for outside-in αIIbβ3 signalling and platelet function. Nature 401:808–811

Leon C, Hechler B, Freund M et al. (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. J Clin Invest 104:1731–1737

Offermanns S, Toombs CF, Hu Y-H, Simon MI (1997) Defective platelet activation in $G\alpha_q$-deficient mice. Nature 389:183–186

Ploplis VA, Carmeliet P, Vazirzadeh S et al. (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593

Rosen ED, Chan JCY, Idusogie E et al. (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 390:290–294

Subramaniam M, Frenette PS, Saffaripour S et al. (1996) Defects in hemostasis in P-selectin-deficient mice. Blood 87:1238–1242

Suh TT, Holmback K, Jensen NJ et al. (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 9:2020–2033

Sun WY, Witte DP, Degen JL et al. (1998) Prothrombin deficiency results in embryonic and neona-

Table 9 Genetic models of thrombosis and hemostasis.

Knock-out	Viable	Embryonic Development Survival	References
Coagulation			
Protein C	NO	normal perinatal death	Jalbert et al. 1998
Fibrinogen	YES	normal perinatal death	Suh et al. 1995
Fibrinogen-QAGVD	YES	normal	Suh et al. 1995
fV	NO	partial embyronic loss perinatal death	Cui et al. 1996
FVII	YES	normal perinatal death	Rosen et al. 1997
fVIII	YES	normal	Bi et al. 1996
fIX	YES	normal	Wang et al. 1997
fXI	YES	normal	Gailani et al. 1997
Tissue Factor	NO	lethal	Toomey et al. 1996 Bugge et al. 1996
TFPI	NO	lethal	Huang et al. 1997
vWF	YES	normal	Denis et al. 1998
Prothrombin	NO	partial embryonic loss perinatal death	Xu et al. 1998 Sun et al. 1998
Fibrinolytic			
u-Pa & t-PA	YES	normal growth retardation	Carmeliet et al. 1994
uPAR	YES	normal	Dewerchin et al. 1996 Bugge et al. 1995
Plasminogen	YES	normal growth retardation	Bugge et al. 1995 Ploplis et al. 1995
PA-I	YES	normal	Carmeliet et al. 1993
thrombomodulin	NO	lethal	Healy et al. 1995
Platelet			
β_3	YES	normal partial embryonic loss	Hodivala-Dilke et al. 1999
β_3-DiYF	YES	normal	Law et al. 1999
P-Selectin	YES	normal	Subramaniam et al. 1996
PAR-1	YES	normal	Connolly et al. 1996
PAR-3	YES	normal	Kahn et al. 1998
$G_{\alpha q}$	YES	normal perinatal death	Offermans et al. 1997
TXA_2 receptor	YES	normal	Thomas et al. 1998
P2Y1	YES	normal	Leon et al. 1999

tal lethality in mice. Proc Natl Acad Sci USA 95:7597–7602

Thomas DW, Mannon RB, Mannon PJ et al. (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. J Clin Invest 102:1994–2001

Toomey JR, Kratzer KE, Lasky NM et al. (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587

Xu J, Wu Q, Westfield L et al. (1998) Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 95:7603–7607

Wang L, Zoppè M, Hackeng TM et al. (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

I.M.6 Critical Issues in Experimental Models

I.M.6.1 The Use of Positive Control

Clearly, there are many antithrombotic agents that can be used to compare and contrast the antithrombotic efficacy and safety of novel agents. The classic antithrombotic agents are heparin, warfarin, and aspirin. However, new, more selective agents such as hirudin, low molecular weight heparins, and clopidogrel are commercially available that will either replace or augment these older treatments. Novel

antithrombotic agents should certainly be demanded to demonstrate better efficacy than currently available therapy in animal models of thrombosis. This should be demonstrated by performing dose-response experiments that include maximally effective doses of each compound in the model. At the maximally effective dose, parameters such as APTT, PT, template bleeding time, or other, more sensitive measurements of systemic hypocoagulability or bleeding should be compared. A good example of this approach is a study by Schumacher et al. (1996a,b), who compared the antithrombotic efficacy of argatroban and dalteparin in arterial and venous models of thrombosis. Consideration of potency and safety compared to other agents should be taken into account when advancing a drug through the testing funnel.

The early in vivo evaluation of compounds that demonstrate acceptable in vitro potency and selectivity requires evaluation of each compound alone in order to demonstrate antithrombotic efficacy. The antithrombotic landscape is becoming complicated by so many agents from which to choose that it will become increasingly difficult to design preclinical experiments that mimic the clinical setting in which poly-antithrombotic therapy is required for optimal efficacy and safety. Consequently, secondary and tertiary preclinical experiments will need to be carefully designed in order to answer these specific, important questions.

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Schumacher WA, Heran CL, Steinbacher TE (1996a) Low-molecular-weight heparin (Fragmin) and thrombin active-site inhibitor (argatroban) compared in experimental arterial and venous thrombosis and bleeding time. J Card Pharmacol 28:19–25

I.M.6.2 Evaluation of Bleeding Tendency

Although the clinical relevance of animal models of thrombosis has been well-established in terms of efficacy, the preclinical tests for evaluating safety, i.e, bleeding tendency, have not been as predictable. The difficulty in predicting major bleeding, such as intracranial hemorrhage, resulting from antithrombotic or thrombolytic therapy stems from the complexity and lack of understanding of the mechanisms involved in this disorder. Predictors of anticoagulant-related intracranial hemorrhages are advanced age, hypertension, intensity and duration of treatment, head trauma, and prior neurologic disease (Stieg and Kase 1998; Sloan and Gore 1992). These risk factors are clearly difficult, if not impossible, to simulate in laboratory animals. Consequently, more general tests of anticoagulation and primary hemostasis have been employed.

Coagulation assays provide an index of the systemic hypocoagulability of the blood after administration of antithrombotic agents; however, as indicated earlier, the sensitivity and specificity of these assays varies from compound to compound so these assays do not provide a consistent safety measure across all mechanisms of inhibition. Consequently, many laboratories have attempted to develop procedures that provide an indication of bleeding risk by evaluating primary hemostasis after generating controlled incisions in anesthetized animals. Some of the tests used in evaluating FXa inhibitors include template bleeding time, tail transection bleeding time, cuticle bleeding time, and evaluation of clinical parameters such as hemoglobin and hematocrit. Unfortunately, template bleeding tests, even when performed in humans, have not been good predictors of major bleeding events in clinical trials (Bernardi et al. 1993; Bick 1995; Rodgers and Levin 1990). However, these tests have been able to demonstrate relative advantages of certain mechanisms and agents over others. For example, hirudin, a direct thrombin inhibitor, appears to have a narrow therapeutic window when used as an adjunct to thrombolysis in clinical trials, producing unacceptable major bleeding when administered at 0.6 mg/kg, i.v. bolus, plus 0.2 mg/kg/hr (Antman et al. 1996; GUSTO Investigators 1996). When the dose of hirudin was adjusted to avoid major bleeding (0.1 mg/kg and 0.1 mg/kg/hr), no significant therapeutic advantage over heparin was observed. If the relative improvement in the ratio between efficacy and bleeding observed preclinically with Xa inhibitors compared to thrombin inhibitors such as hirudin is supported in future clinical trials, this will establish an important safety advantage for FXa inhibitors and provide valuable information for evaluating the safety of new antithrombotic agents in preclinical experiments.

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Antman EM, TIMI 9B Investigators (1996) Hirudin in acute myocardial infarction: Thrombolysis and thrombin inhibition in myocardial infarction (TIMI) 9B trial. Circulation 4:911–921

Bernardi MM, Califf RM, Kleiman N et al. (1993) Lack of usefulness of prolonged bleeding times in predicting hemorrhagic events in patients receiving the 7E3 glycoprotein IIb/IIIa platelet antibody. Am J Cardiol 72:1121–1125

Bick RL (1995) Laboratory evaluation of platelet dysfunction. Clin Lab Med 15:1–38

Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) IIb/IIIa Investigators (1996) A comparison of recombinant hirudin with heparin for the treatment of acute coronary syndromes. N Engl J Med 335:775–782

Rodgers RPC, Levin J (1990) A critical reappraisal of the bleeding time. Sem Thromb Hemost 16:1–20

Sloan MA, Gore JM (1992) Ischemic stroke and intracranial hemorrhage following thrombolytic therapy for acute myocardial infarction: a risk-benefit analysis. Am J Cardiol 69:21A–38A

Stieg PE, Kase CS (1998) Intracranial hemorrhage: diagnosis and emergency management. Neurol Clin 16:373–390

I.M.6.3 Selection of Models Based on Species-Dependent Pharmacology/Physiology

As alluded to earlier, species selection for animal models of disease is often limited by the unique physiology of a particular disease target in different species or by the species specificity of the pharmacological agent for the target. For example, it was discovered relatively early in the development of platelet GPIIb/IIIa antagonists that these compounds were of limited use in rats (Cox et al. 1992) and that there was a dramatic species-dependent variation in the response of platelets to GPIIb/IIIa antagonists (Bostwick et al. 1996; Cook et al. 1993; Panzer-Knodle et al. 1993). This discovery lead to the widespread use of larger animals (particularly in dogs, whose platelet response to GPIIb/IIIa antagonists resembles humans) in the evaluation of GPIIb/IIIa antagonists. Of course the larger animals required more compound for evaluation, which created a resource problem for medicinal chemists. This was especially problematic for companies that generated compounds by combinatorial parallel synthetic chemistry in which many compounds can be made, but usually in very small quantities. However, some pharmacologists devised clever experiments that partially overcame this problem. Cook et al. (1996) administered a GPIIb/IIIa antagonist orally and intravenously to rats, and then mixed the platelet-rich plasma from the treated rats with platelet-rich plasma from untreated dogs. The mixture was then evaluated in an agonist-induced platelet aggregation assay and the resulting inhibition of canine platelet aggregation (rat platelets were relatively unresponsive to this GPIIb/IIIa antagonist) was due to the drug present in the plasma obtained from the rat. Using this method, only a small amount of drug is required to determine the relative bioavailability in rats. However, the animal models chosen for efficacy in that report (guinea pigs and dogs) were selected based on their favorable platelet response to the GPIIb/IIIa antagonist.

Similarly for inhibitors of FXa, there are significant variations in the activity of certain compounds against FXa purified from plasma of different species and in plasma-based clotting assays using plasma from different species. DX-9065 is much more potent against human FXa (Ki = 78 nM) than against rabbit (Ki = 102 nM) and rat (Ki = 1980 nM) FXa. Likewise, in the PT assay, DX-9065a was very potent in human plasma (concentration required to double PT, PT × 2, was 0.52 μM) and in squirrel monkey plasma (PT × 2 = 0.46 μM), but was much less potent in rabbit, dog, and rat plasma (PT × 2 = 1.5, 6.5, and 22.2 μM, respectively). Other FXa inhibitors have also demonstrated these species-dependent differences in activity (Tidwell et al. 1980; Nutt et al. 1991; Taniuchi et al. 1998). Regardless, the investigator must be aware of these differences so that appropriate human doses can be extrapolated from the laboratory animal studies.

Although in many cases the exact mechanism for the species-dependent differences in response to certain therapeutic agents remains unclear, these differences must be examined to determine the appropriate species to be used for preclinical pharmacological evaluation of each agent. This evaluation can routinely be performed by in vitro coagulation or platelet aggregation tests prior to evaluation in animal models.

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Bostwick JS, Kasiewski CJ, Chu V et al. (1996) Anti-thrombotic activity of RG13965, a novel platelet fibrinogen receptor antagonist. Thrombosis Research 82:495–507

Cook JJ, Holahan MA, Lyle EA et al. (1996) Nonpeptide glycoprotein IIb/IIIa inhibitors. 8. Antiplatelet activity and oral antithrombotic efficacy of L-734,217. J Pharmacol Exp Ther 278:62–73

Cook NS, Zerwes H-G, Tapparelli C et al. (1993) Platelet aggregation and fibrinogen binding in human, rhesus monkey, guinea-pig, hamster and rat blood: activation by ADP and thrombin receptor peptide and inhibition by glycoprotein IIb/IIIa antagonists. Thromb Haemostasis 70:531–539

Cox D, Motoyama Y, Seki J et al. (1992) Pentamadine: a non-peptide GPIIb/IIIa antagonist - in vitro studies on platelets from humans and other species. Thromb Haemostasis 68:731–736

Nutt EM, Jain D, Lenny AB et al. (1991) Purification and characterization of recombinant antistasin: a leech-derived inhibitor of coagulation factor X_a. Arch Biochem Biophys 285:37–44

Panzer-Knodle S, Taite BB, Mehrotra DV et al. (1993) Species variation in the effect of glycoprotein IIb/IIIa antagonists on inhibition of platelet aggregation. J Pharm Tox Meth 30:47–53

Taniuchi Y, Sakai Y, Hisamichi N et al. (1998) Biochemical and pharmacological characterization of YM-60828, a newly synthesized and orally active inhibitor of human Factor Xa. Thromb Haemost 79:543–548

Tidwell RR, Webster WP, Shaver SR, Geratz JD (1980) Strategies for anticoagulation with synthetic protease inhibitors. X_a inhibitors versus thrombin inhibitors. Thromb Res 19:339–349

I.M.6.4 Selection of Models Based on Pharmacokinetics

Much debate surrounds the issue as to which species most resembles humans in terms of gastrointestinal absorption, clearance, and metabolism of therapeutic agents. Differences in gastrointestinal anatomy, physiology, and biochemistry between humans and commonly used laboratory animals suggest that no single animal can precisely mimic the gastrointestinal characteristics of humans (Kararli 1995). Due to resource issues (mainly compound availability) and animal care and use considerations, small rodents, such as rats, are usually considered for primary in vivo evaluation of pharmacokinetics for novel agents. However, there is great reservation about moving a compound into clinical trials based on oral bioavailability data derived from rat experiments alone. Usually, larger animals such as dogs or non-human primates, which have similar gastrointestinal morphology compared to humans, are the next step in the evaluation of pharmacokinetics of new agents. The pharmacokinetic characteristics of FXa inhibitor, YM-60828, have been studied extensively in a variety of laboratory animals. YM-60828 demonstrated species-dependent pharmacokinetics, with oral bioavailability estimates of approximately 4, 33, 7, and 20 % in rats, guinea pigs, beagle dogs, and squirrel monkeys, respectively. Although these results suggest that YM-60828 has somewhat limited bioavailability, evaluating the pharmacokinetic profile of novel agents in a number of species (Sanderson et al. 1998) is a well-established approach used to aid in identifying compounds for advancement to human testing. That is, acceptable bioavailability in a number of species suggests that a compound will be bioavailable in humans. Which of the laboratory species adequately represents the bioavailability of a specific compound in humans can only be determined after appropriate pharmacokinetic evaluation in humans. Nevertheless, preclinical pharmacokinetic data are important in selecting the appropriate animal model for testing the antithrombotic efficacy of compounds because the ultimate proof-of-concept experiment is to demonstrate efficacy by the intended route of administration.

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I.M.6.5 Clinical Relevance of Data Derived from Experimental Models

Animal models of thrombosis have played a crucial role in the discovery and development of a number of compounds that are now successfully being used for the treatment and prevention of thrombotic diseases. The influential preclinical results using novel antithrombotics in a variety of laboratory animal experiments are listed in the table below, along with the early clinical trials and results for each compound. This table intentionally omits many compounds that were tested in animal models of thrombosis, but failed to be successful in clinical trials or, for other reasons, did not become approved drugs. However, these negative outcomes would not have been predicted by animal models of thrombosis because the failures were generally due to other shortcomings of the drugs (e.g, toxicity, narrow therapeutic window, or undesirable pharmacokinetics or pharmacodynamics) which are not always clearly presented in the scientific literature due to proprietary restrictions in this highly competitive field.

Nonetheless, it is clear that animal models have supplied valuable information for investigators responsible for evaluating these drugs in humans, providing pharmacodynamic, pharmacokinetic, and safety data that can be used to design safe and efficient clinical trials. For detailed applications see the following references: Bugge et al. (1995, 1996); Carmeliet et al. (1993, 1994, 1996); Christie et al. (1999); Connolly et al. (1996); Cui et al. (1996); Evans et al. (1989); Healy et al. (1995); Herzog et al. (1999); Hodivala-Dilke et al. (1999); Huang et al. (1997); Kahn et al. (1998); Kay et al. (2000); Kung et al. (1998); Law et al. (1999); Leon et al. (1999); Lin et al. (1997); Offermans et al. (1997); Ploplis et al. (1995); Subramaniam et al. (1996); Thomas et al. (1998); Toomey et al. (1996); Vassalli and Dichek (1997); Waugh et al. (1999); and Zoldhelyi et al. (2000).

Table 10 Animal models of thrombosis and their clinical correlates.

Compound	Preclinical Animal Model	Preclinical Results	Reference	Clinical Indication	Clinical Result	Reference
Recombinant tissue plasminogen activator (Activase)	Rabbit pulmonary artery thrombosis	Lysis of preformed pulmonary thrombus	Matsuo et al. 1981	Acute myocardial infarction – thrombolysis	Improved recanalization	Collen et al. 1984
Abciximab (ReoPro)	Canine coronary cyclic flow reduction (Folts et al. 1991)	Significant inhibition of platelet-dependent thrombosis	Coller et al. 1986	High-risk coronary angioplasty	Reduction in death, myocardial infarction, refractory ischemia, or unplanned revascularization	EPIC Investigators, 1994
Tirofiban (Aggrestat)	Canine coronary cyclic flow reduction (Folts et al. 1991)	Significant inhibition of platelet-dependent thrombosis	Lynch et al. 1995	Unstable angina	Reduction in death, myocardial infarction, refractory ischemia	PRISM Investigators, 1998
Eptifibitide (Integrilin)	TPA-induced coronary thrombolysis	Significant improvement in lysis of occlusive thrombus	Nicolini et al. 1994	Acute myocardial infarction – thrombolysis with tPA	Improvement in incidence and speed of reperfusion	Ohman et al. 1997
Enoxaparin (Lovenox)	Canine coronary cyclic flow reduction (Folts et al. 1991)	Significant inhibition of platelet-dependent thrombosis	Leadley et al. 1998	Unstable angina	Significant decrease in death, myocardial infarction, and need for revascularization at 30 days	Cohen et al. 1998
Hirudin (Refludan)	Rabbit jugular vein thrombus growth	Inhibition of thrombus growth compared to standard heparin	Agnelli et al. 1990	Deep vein thrombosis after total hip replacement	Significantly decreased rate of DVT	Hansson et al. 1997
Argatroban	Canine coronary artery electrolytic-injury (TPA-induced thrombolysis)	Accelerated reperfusion and prevented reocclusion	Fitzgerald et al. 1989	Unstable angina	No episodes of MI during drug infusion	Garabedian et al. 1994

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Chapter I.N
Ocular Toxicity Tests

Beth L. Schultz

I.N.1
General Considerations

Ocular drugs, cosmetics or all chemicals, which can come in contact with the eye carry the potential of harming various ocular structures. Thus, their possible toxic effects on ocular structures must be evaluated in tests before use in humans.

At present, it has been suggested that this be done in 3 steps. First, a thorough review of the literature of similar compounds should be conducted to obtain theoretical knowledge about ocular toxicity. Second, tests of this substance are performed in various in vitro procedures to obtain possible serious toxic effects, which might stop further testing. Third, if no serious toxic effects have been observed tests are conducted in the eyes of one animal followed by subsequent tests in more animals. Fourth, the substance if an ocular drug will then be subjected to clinical trials. An important feature of these pre-clinical tests is that they correctly predict or are in agreement with findings in humans. A recent study concluded that there is indeed a relatively good agreement between certain pre-clinical and clinical observations (Dart 2003).

A comprehensive review of various in vitro and in vivo procedures has been published recently by Wilhelmus in 2001.

Introduced by Wolfgang H. Vogel, Jefferson Medical College, Thomas Jefferson University, Department of Pharmacology, Philadelphia, USA

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I.N.2
Dendritic Cell Culture

PURPOSE AND RATIONALE

This test uses an in vitro human dendritic cell culture to obtain information of the potential for various chemicals to induce allergic contact dermatitis. This test is used as an alternative to the Local Lymph node assay (LLNA) to minimize or replace the use of live animal testing for predicting skin sensitization (Kimber et al. 2002, see below). The test allows for evaluation of skin sensitization by examining the presence of cell surface markers on Peripheral Blood Mononuclear Cell (PBMC)-derived dendritic cells (DC) that are known to be involved in the development of allergic contact dermatitis.

PROCEDURE

The method contains three stages including first establishing a cell line followed by test chemical exposure and finally evaluated for expression of cell surface markers. To establish a cell line, human leukocyte preparations are attained from a plasma distributor. The leukocyte preparations as described by Ryan et al. (2004), are diluted with an equal part of complete medium (RPMI 1640 containing 1 × L-glutamine, 1 × penicillin-streptomycin-neomycin antibiotic mixture), 30 μ2-mercaptoethanol and 10 % heat inactivated fetal bovine serum. The diluted preparation is layered onto a Ficoll-Paque gradient to

attain the PBMC. According to protocol, the PBMC concentration is adjusted with complete medium and a proportion of the cell suspension is plated in T75 flasks and incubated for 2 h at 37 °C/CO_2. Non-adherent cells are removed and discarded. A mixture containing 10mL complete medium with 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) is added to the adherent cells remaining in the flask. The flasks are incubated for 48hrs at 37 °C/CO_2. Following incubation the cells are collected, centrifuged and resuspended in fresh complete medium containing GM-CSF and IL-4 for another incubation for 72 h. Residual T cells and B cells are removed from the cultures on day 5 by two passages over CD2 (pan T cell) and CD19 (pan Bcell) immunomagnetic beads. The T and B-cell depleted DC are resuspended in fresh cytokine containing complete medium, replated and incubated for 48 h.

The prepared cell line is collected on the 7th day is washed, resuspended in complete medium containing GM-CSF and IL-4 and 2 mL and is plated in the wells of a six-well culture plate. 1mL of test chemical also prepared in complete medium with GM-CSF and IL-4 is added to each well and the plate is incubated for 24 h. Viability of the DC after test chemical exposure is then assessed by propidium iodide dye exclusion using a Coulter Epics XL flow cytometer (Ryan et al. 2004).

EVALUATION

Measurement of cell surface marker expression by flow cytometry after exposure to chemical allergens has been previously established by Hulette et al. (2004). Expression is assessed by flow cytometry using saturating concentrations of fluorochrome-conjugated monoclonal antibodies such as CD86-fluorescein isothiocyanate (FITC), CD83-FITC, CD40-phycoerythrin (PE), CD54-PE, CD80-FITC, and CD1a-FITC. The measurement of the flow cytometer represents the fluorescence intensities of the surface markers. Single parameter histograms are produced and analyzed for changes in mean fluorescence intensity (MFI) of allergen-treated DC as a percentage of the MFI of an untreated control DC (Ryan et al. 2004).

CRITICAL ASSESSMENT OF THE METHOD

Cytotoxicity may decrease cell surface expression and transcription of markers. Thus, if concentration of test chemical induces low cell viability then measurement of cell surface expression and transcription is limited. In addition, donor variability may introduce differences in fluorochrome-conjugated monoclonal antibody expression (Aiba et al. 1997).

PBMC-derived DC serve as a surrogate marker for Langerhans cells (LC) and the chemical allergen-induced changes in cell surface markers of DC produce a similar pattern to those that occur in LC which are the antigen-presenting cell in the skin that plays a key role in the development of allergic contact dermatitis (Ozawa et al. 1996).

MODIFICATION OF THE METHOD

The fluorochrome-conjugated monoclonal antibodies used may be varied. Investigation on one, a combination or all may be performed. In addition RNA Isolation to confirm the quality of RNA and Genochip Microarray for transcript profiling may be performed after flow cytometry evaluation.

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I.N.3 Corneal Epithelial Organ Culture

PURPOSE AND RATIONALE

This test uses an ex vivo model of corneal organ culture, preferentially porcine, to obtain information of the possible ocular toxicity of various chemicals. This test is used as an alternative to the Draize Test to minimize or replace the use of live animal testing of ocular irritancy (Symposium, Proceeding 1996). The test allows for determination of reversibility of corneal injury following exposure to chemicals, drugs or cosmetics (Xu et al. 2004).

PROCEDURE

The eyes are collected, prepared and maintained through specific organ culture technique prior to toxicity study. The porcine or bovine eyes may be acquired

from an abattoir. Following enucleation the eyes must be kept on ice in a moisture chamber and processed for cell culture within 6 hours (Xu et al. 2000) to 24 hours (Xu et al. 2004). The eyes are prepared for excision by rinsing in sterilized phosphate-buffered saline (PBS) then dipping in Betadine and rinsing again with PBS. Next the eyes are exposed to a 0.1 % gentamicin-PBS solution. Then a corneal-scleral rim with 4 mm of surrounding limbal conjunctiva is excised according to organ culture technique (as developed by Foreman et al. 1996). The excised tissue is rinsed in sterilized PBS and put epithelial–side down into a sterile container with Minimum Essential Medium (MEM). MEM plus 1 % agarose and 0.5 mg/mL rat tail tendon collagen is placed in the upward facing endothelial concavity and allowed to gel at 42 °C. The combined corneal-gel mixture is inverted and placed in a 35-mm dish. 2 mL of MEM culture medium is added dropwise to the central cornea allowing for coverage of the limbal conjunctiva and leaving the cornea exposed to the air (Xu et al. 2000).

For acute toxicity studies, a small round 7 mm diameter filter paper is saturated with a test chemical in MEM. The filter paper is then placed onto the center of the corneal organ culture surface. After 2 minutes it is removed and the cornea is washed with PBS. Readings are then taken 0, 24, 48, 72, or 96 h after exposure and are evaluated.

EVALUATION

The evaluation of corneal injury recovery to ocular irritants may be measured subsequently by corneal epithelial barrier disruption and transactivation of stress-related genes. Corneal epithelial barrier disruption readings are recorded specifically through Surface Biotinylation-Tight Junction Permeability Assay (Xu et al. 2000) and Fluorescein Isothiocyanate-Dextran Retention. Transactivation of stressed-related genes is assessed through Electrophoretic Mobility Shift Assay (EMSA) through NF-κB and AP-1 DNA binding activities (Abdulkadir et al. 1995). These subsequent assessments of corneal injury recovery will be covered individually in following assays.

CRITICAL ASSESSMENT OF THE METHOD

Porcine cornea are similar to human cornea in that both have 5–7 layers of epithelial cells and a Bowman's membrane. Bovine cornea differs in that it has 10 layers of epithelial cells and no Bowman's membrane. Porcine and Bovine corneal cultures both differ in certain aspects from in vivo animal cornea. The latter has the presence of an overlying mucin layer and its underlying apical epithelial layer (Bowman's membrane). This forms a highly impermeable line of defense through tight junction barriers, against biological and chemical insults (Argueso et al. 2001). Although corneal epithelial cell culture lacks the protective mucin and apical epithelial layer, results of its recovery to ocular irritancy have been correlated to the in vivo animal model of The Draize Test (Sina et al. 1995).

Ethical concerns over the use of animals may be addressed by the use of ex vivo or in vitro cell culture models such as this. In addition corneal cell culture combined with objectively quantifiable assays for corneal epithelial barrier disruption and transactivation of stress-related genes may reduce the high variability associated to the subjectively scored Draize Test.

MODIFICATION OF THE METHOD

The dose of chemicals applied to filter paper may be modified to view concentration-dependent parameters of test substances. By establishing the dose response to ocular irritancy, comparisons can be made to the mild, moderate and severe grading systems previously validated (Stokes 2003). An alternative exposure of test chemicals may be performed by direct application of test solution dropwise onto the center surface of the corneal (Xu et al. 2000). The test solution then dissolves into the culture media.

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I.N.4 Surface Biotinylation-Tight Junction Permeability Assay

PURPOSE AND RATIONALE

This test uses the ex vivo model of corneal organ culture previously discussed combined with surface biotinylation technique. The corneal epithelium functions as a barrier that separates the internal ocular tissues from the external environment and is therefore vulnerable to chemical insult (Gipson and Sugrue 1994). The joint assessment of corneal organ culture and surface biotinylation is a measure of ocular toxicity that is intended to reveal disrupted tight junction barriers following chemical exposure.

PROCEDURE

A filter paper saturated with a test chemical in MEM or direct application of test chemical solution is placed onto the center of the corneal organ and washed with PBS as noted prior. After test chemical exposure, PBS rinse and culturing, the corneas are processed for biotinylation study on day 0, 1, 2, 3 and 4. The process (as developed by Chen et al. 1997) involves wetting the corneas with 1 mg/mL sulfo-NHS-LC-biotin in Hank's balanced salt solution (bss). To complete the method of labeling, the corneas are incubated for 30 minutes. Subsequently the corneas are rinsed with PBS and embedded in optimum cutting temperature (OCT) compound and frozen in liquid nitrogen. 6 μm sections are cut by cryostat are then fixed in ice cold acetone and conjugated with rhodamine-avidin D in PBS containing 1 % bovine serum albumin (BSA) for 1 hour. Slides are then visualized under a Fluorescent microscope. Preferred instrumentation is a Nikon Eclipse E-800 fluorescence microscope equipped with a SPOT digital camera.

EVALUATION

The method allows visualization of the epithelial barrier and penetration of small molecules into cell layers indicating disruption of tight junctions (Saitou et al. 1998). Slides are examined under the fluorescent microscope for bound biotin and rhodamine staining in deeper epithelial and stromal layers. Staining indicates penetration of biotin molecules into the tissue through disrupted tight junction barriers (Xu et al. 2004). Recovery from disruption is tracked on day 0, 1, 2, 3, and 4.

CRITICAL ASSESSMENT OF THE METHOD

Corneal organ culture combined with objectively quantifiable assays for corneal epithelial barrier disruption reduces the high variability associated to the subjectively scored Draize Test. Although the surface biotinylation allows for an objective outcome measure, the scoring system is not yet quantitatively comparable for assessment of ocular irritancy to multiple test products. As it is utilized more extensively in varied laboratories with numerous test chemicals a standardized scoring system can be elicited similar to the familiar Draize Test.

MODIFICATION OF THE METHOD

The concentration of test chemicals applied directly and dissolved in culture media may be modified to generate extended biotinylation of the corneal surface caused by test chemical exposure in a concentration-dependent manner (Xu et al. 2000). Xu et al. also suggests modifying exposure time of test chemicals from 2 minutes to longer test durations to examine further disturbed biotin surface labeling and stromal labeling which would indicate breakdown of tight junctions and penetration of biotin into the epithelial layer.

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I.N.5 Fluorescein Isothiocyanate-Dextran Retention

PURPOSE AND RATIONALE

This test uses the ex vivo model of corneal organ culture previously discussed combined with Fluorescein Isothiocyanate-Dextran Retention technique. Chemical insult may interfere with the corneal epithelial barrier function by disturbing the integrity of tight junctions. Disruption of tight junctions would

allow chemicals to diffuse into deeper, innervated epithelial layers, causing eye irritation (Rein 2003). The joint assessment of corneal organ culture and Fluorescein Isothiocyanate-Dextran Retention is a measure of ocular toxicity that is intended to reveal disrupted tight junction barriers following chemical exposure.

PROCEDURE

A filter paper saturated with a test chemical in MEM or direct application of test chemical solution is placed onto the center of the corneal organ and washed with PBS as noted prior. After test chemical exposure, PBS rinse and culturing, the corneas are processed for Fluorescein Isothiocyanate-Dextran Retention study on day 0, 1, 2, 3 and 4. The methods that follow have been described by Xu et al. (2004). The corneas are incubated with 4 kD, 10 mg/mL of Fluorescein Isothiocyanate-Dextran (FITC-Dextran) at room temperature for 10 minutes and then washed. The center of the cornea is marked with an 11 mm trephine and epithelial cells within are scraped off with a scalpel blade under a dissecting microscope. The cells are then lysed in 100 μL extracting buffer (20 mM Hepes-KOH at pH 7.9, 1 mM DTT, 1 mM EDTA, 200 mM KCl, 20 % glycerol, 0.1 mM PMSF, 0.1 % NP 40 and proteinase inhibitors. The mixture is shaken with periodic agitation at 4 °C for 1 hour. It is placed in a Centrifuge at 14 000 rpm and 4 °C for 20 minutes. Cell debris is pelleted and supernatants are collected, aliquoted, quick-frozen in liquid nitrogen and stored at –80 °C. This is used to determine protein concentration and measure presence of FITC-Dextran within the epithelium. Protein concentration is determined by using a micro bicinhoninic acid (BCA) protein assay reagent kit. To measure FITC-Dextran presence within the epithelium 2 μL of aliquoted cell lysate is pipetted to a 96-well plate, mixed with 198 μL of 1 % Triton X-100 and subjected to fluorescence microplate reading (Xu et al. 2004).

EVALUATION

Fluorescein retention measurements are converted to relative amount of fluorescence normalized against protein concentration. Test chemical exposure causing disruption of tight junctions will allow fluorescein molecules to penetrate the corneal tissue and become trapped within its epithelial layer. Thus, fluorescein molecules are used as a permeability tracer and applied in defining corneal epithelial and endothelial permeability (Watsky et al. 1989). FITC-retention provides a quantitative assessment for paracellular leakage secondary to tight junction disruption by chemical injury.

CRITICAL ASSESSMENT OF THE METHOD

Corneal organ culture combined with objectively quantifiable assays for corneal epithelial barrier disruption reduces the high variability associated to the subjectively scored Draize Test. The FITC-Dextran retention has been studied as a quantitative evaluation of the corneal epithelial barrier (Lopez et al. 1991) following chemical exposure of benzalkonium chloride (BAC), Polyquad, and Thimerosal. Sodium dodecyl sulfate (SDS) has also been tested for disruption of the tight junctions via FITC-Dextran retention assay. However, as an objective outcome measure for ocular toxicity, the scoring system is not yet quantitatively comparable for assessment of ocular irritancy to multiple test products. This limitation is similar to surface biotinylation assays. As fluorometry is utilized more extensively in varied laboratories with numerous test chemicals a standardized scoring system can be elicited similar to the familiar Draize Test.

MODIFICATION OF THE METHOD

The concentration of test chemicals applied directly or dissolved in culture media may be modified to test concentration-dependent alterations in epithelial barrier properties. Also different molecular weights of fluorescein may be substituted to alter permeability and retention relationships as a function of the test chemical used.

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I.N.6 Electrophoretic Mobility Shift Assay

PURPOSE AND RATIONALE

This test uses the ex vivo model of corneal organ culture previously discussed combined with Electrophoretic

mobility shift technique (EMSA). The corneal epithelium may demonstrate a stress response following chemical insult. The stress response is mediated by transcription factor proteins. These proteins alter gene expression and initiate the healing response (Camhi et al. 1995). The joint assessment of corneal organ culture and EMSA is a measure of ocular toxicity that is intended to reveal DNA binding of transcription factors responsible for triggering cellular stress and subsequent healing following chemical exposure.

PROCEDURE

A filter paper saturated with a test chemical in MEM or direct application of test chemical solution is placed onto the center of the corneal organ and washed with PBS as noted prior. The test chemical exposed organ culture is prepared for EMSA measurements on day 0, 1, 2, 3, and 4. Following the test chemical exposure and wash a scalpel blade is used to remove epithelial cells from the corneal organ culture placed beneath a dissection microscope. A method to prepare the epithelial extract has been recommended by Abdulkadir et al. 1995 and repeated by Xu et al. 2000. The extracted epithelial cells are resuspended in a mixture containing 100 μ of 20 mM HEPES-KOH, 1 mM DTT, 1 mM EDTA, 200 mM KCl, 20 % glycerol, 0.1 mM PMSF, 0.1 % NP40 and incubated with periodic agitation at 4 °C for 1 h. After incubation the cells are pelleted via centrifuge by 10-min 14,0000 rpm in a microfuge at 4 °C. Supernatents are then removed, aliquoted and quick-frozen in liquid nitrogen then stored at –80 °C. Protein concentrations are determined with a protein assay reagent kit. Next the nuclear and cytosol proteins contained within the epithelial extract are assessed for binding activity of oligonucleotides AP-1 and NFκB by EMSA.

The protocol for EMSA as recently noted by Xu et al. 2004 is as follows. Oligonucleotides are labeled using T4 polynucleotide kinase and [γ -^{32}P] ATP. The labeled probes are purified from free nucleotides by ethanol precipitation. 5 μL of extracted proteins is mixed with 2 μL of 5 × gel shift binding buffer and incubating at room temp for 15 min. Then 1 μL of ^{32}P-labeled oligonucleotide is added and 20 min is given for a binding reaction to occur at room temp.

EVALUATION

Samples from the binding reaction are loaded on a 4 % nondenaturing polyacrylamide gel and run in 1 × TGE. The gel is fixed in 7 % acetic acid and 10 % methanol for 15 minutes, dried, and autoradiographed to detect DNA binding of AP-1 and NFκB. Radiolabeling is quantified as the sum of all shifted bands in each lane as determined by PhosphorImager Analysis.

CRITICAL ASSESSMENT OF THE METHOD

DNA binding of AP-1 and NFκB is concentration-dependent and corresponds to Draize scores for ocular irritancy. Test chemical exposure time and probe-labeling efficiency may introduce variance to results. Despite the variance, detection of altered expression and/or activation of stress-response transcription factors like AP-1 and NFκB can serve as an early marker for subsequent deteriorative outcomes (Ramesh et al. 1999) of ocular toxicity.

MODIFICATION OF THE METHOD

DNA binding of transcription factors is present for concentrations of test chemicals that elicit mild and moderate scores of ocular irritancy via the Draize. The binding is greatly reduced or absent for concentrations producing a severe ocular irritancy score by the Draize. The lack of binding indicates loss of viability and poor recovery. Concentration of test chemical used may be varied to reach a non-binding outcome to reveal stages of stress and recovery or lack there of.

DNA binding detection may be performed for distinct transcription factors or for sets of transcription factors.

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I.N.7 Murine Local Lymph Node Assay

PURPOSE AND RATIONALE

This test using animals seems to be the most frequently used predictive test for the identification of skin-sensitizing chemicals or for chemicals prone to cause allergic contact dermatitis. While it is most frequently used for general dermatological investigations, results

can be applied to the eye lids as well (Kimber and Basketter 1992; Kimber et al. 1994).

PROCEDURE

Mice (often female mice of the CBA/CA or CBA/J strains) are exposed on the dorsum of both ears with 25 μl of 2–5 concentrations of the test chemical or to the same volume of the vehicle. Treatment is performed daily for 3 consecutive days. Five days following the start of the test all mice are injected via the tail vein with 250 μl of phosphate buffered saline, which contains 20 μc Ci of tritiated methyl thymidine. Five hours later, the animals are sacrificed. The draining auricular lymph nodes are excised and pooled. A cell suspension is prepared by gentle disaggregation through a 200-mwesh stainless steel gauze. The cells are washed twice with phosphate buffered saline and precipitated with 5 % trichloroacetic acid at 4 degree Celsius. After 12 h the pellets are re-suspended in 1 ml of trichloroacetic acid and this volume is transferred to scintillation vials. Incorporation of tritiated methyl thymidine is determined.

EVALUATION

Activity is measured according to the increase in lymph node proliferation measured in methyl thymidine incorporation. Chemicals, which cause a 3 fold or greater response than the vehicle are considered to have skin-sensitizing potential.

CRITICAL ASSESSMENT OF THE METHOD

Allergens can cause vastly different responses in individuals or the allergic response is very individualized. Nevertheless, this method will identify the majority of skin-sensitizing chemicals and has shown good agreement with human patch tests (Schneider and Akkan 2004).

MODIFICATION OF THE METHOD

The test is performed by using single lymph nodes from individual animals, which allows statistical evaluation of the results (Kimber et al. 1995).

A modification of the procedure uses 125-iododeoxy-uridine but seems to give the same results (Kimber et al. 1995)

A non-radioactive assay has been developed based on the release of IL-2 from lymph nodes of chemically exposed lymph nodes (Hariya T et al. 1999). However, this assay is only valid for strong but not weak sensitizing chemicals.

A more recently developed non-radioactive test uses the incorporation of 5-bromo-2'-deoxyuridine as a measure of cell proliferation (Takeyoshi et al. 2004).

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I.N.8 The Draize Test

PURPOSE AND RATIONALE

This test uses the eyes of a live animal, preferentially rabbits, to obtain information of the possible ocular toxicity of various chemicals. This test is most closely related to the human situation where these chemicals will later be used as ocular drugs or cosmetics or where the human eye might be exposed accidentally to these substances. This is a classical test developed about 60 years ago (Draize et al. 1944).

For further description of methods see chapters I.H (Peripheral Nervous System) and I.P (Safety Assays in Skin Pharmacology).

PROCEDURE

For acute toxicity studies, 0.1 ml of a solution or ointment of the test substance is instilled into the conjunctival sacs of 3–6 rabbits. Readings are then taken 1, 24, and 48 h after instillation and are evaluated. If residual injuries still exist, another reading is performed after 96 h.

EVALUATION

The evaluation occurs according to a slight variation of the scoring system developed by Friedenwald et al. (1944) and presented in abbreviated form in Table 1.

Table 1 The total score is the sum of all the individual scores.

Part	Tissue Score
Cornea	A) Opacities 1 or 2 B) Area of cornea involved 3 or 4 **Score equals A × B × 5; maximum = 80**
Iris	A) Folds, swelling, injections 1 No reaction to light, hemorrhage 2 **Score A × 5; maximum = 10**
Conjunctivae	A) Redness, beefy red 1, 2 or 3 B) Chemosis, swelling, lid closing 1, 2, 3 or 4 C) Discharge, amounts 1, 2 or 3 **Score (A+B+C) × 2; maximum = 20**

CRITICAL ASSESSMENT OF THE METHOD

The eyes of the rabbit differ in certain aspects from the eyes of humans. They are more sensitive, have a lower tear production and blink frequency and posses a nictitating membrane. Nevertheless, the Draize test predicts human ocular toxicity correctly in 85 % cases but overestimates in 10 % and underestimates in 5 % (Gad and Chengelis 1991). In addition, ethical concerns have been raised in the use of animals and benefit vs risk of these tests for the protection of the human eye must be carefully evaluated.

MODIFICATION OF THE METHOD

Although not mentioned directly for the eye in the original paper, modifications suggested for skin testing are also applicable to the eye. These include varying the dose, using multiple applications and washing the eyes after an application to study reversibility of observed toxicity (Draize et al. 1944).

Instead of applying the test substance topically, the substance can be injected into the eye and they can then be observed for pathological changes (Veckeneer et al. 2001).

This test has been used since then many times each with slight variations. For instance, exposure of only one eye to the test substance and no exposure of the other eye or exposure of the other eye to the vehicle. To minimize discomfort to the animal, a local anesthetic is sometimes instilled before use of the test substance. The qualitative scoring system hasbeen extended using also measures of eye blinks or wipes. Examinations can also include magnifying glasses, slit lamp examination, fluorescein staining and photodocumentation. After observed toxicity, animals are sacrificed, the eyes removed and subjected to microscopic and histological examinations.

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Chapter I.0
Assays in Endocrine Safety Pharmacology

Jürgen Sandow

I.O.1 General Considerations

Studies on Endocrine Safety Pharmacology are part of the "Supplemental Safety Pharmacology Studies" described in the ICH guideline S7A. These supplemental studies are meant to evaluate potential adverse effects on organ system functions which are not addressed by the general methods of the core battery, or by repeated-dose toxicity studies. The definition clearly requires that the specific pharmacology of the test substance be known before any decisions on the test articles of endocrine safety pharmacology are taken, for this specific case. The general pharmacology profiling of new drugs nowadays is predominantly performed by in vitro testing, which may be quite an effective procedure for compounds with hormonal activity. The established primary interaction of hormones and substances with hormonal activity with an ever increasing set of membrane receptors and nuclear receptors, as well as binding proteins and enzymes can be approached by a number of high troughput procedures and also by spe-

cific in vitro testing. This evidence will be available at the time of designing the final strategy for toxicological evaluation, including Endocrine Safety Pharmacology. For all activities predicted from the in vitro testing, some evidence for hormone-related effects should be available from the early animal studies with repeated dose administration for several days. This approach very much facilitates the safety pharmacology strategy.

Endocrine Safety Pharmacology studies are performed for compounds designed to enter Clinical Pharmacology Phase I. They are needed to detect undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above (ICH-guideline S7A, 2001). The main objective is to decide whether the test compound can be administered to humans, which effects need to be monitored specifically, and which clinical investigation is required for a risk evaluation based on the safety pharmacology findings. In Endocrine Pharmacology, the design of specific studies will be based on the individual properties and intended clinical uses of the test substance. Safety pharmacology endpoints (biomarkers and other specific tests) can be incorporated into ongoing or planned toxicology, toxicokinetics, and clinical studies. Where this is not feasible, endpoints related to suspected hormone activity should be evaluated in separate safety pharmacology studies, and in mechanistic toxicology studies (Harvey et al. 1999).

Endocrine Safety Pharmacology studies investigate the pharmacodynamic effects of a substance on physiological functions in relation to exposure in the biologically effective range ("therapeutic range") and above (ICH S7A). They are usually initiated (1) to evaluate adverse pharmacodynamic and/or pathophysiological effects of a substance observed in toxicology and/or clinical studies, and (2) to investigate the mechanism of the adverse pharmacodynamic effects observed and/or suspected. This indicates that evidence from specific pharmacology studies is already available at that time of designing the safety related tests.

In this chapter, pharmacological methods are described which can be applied to the characterization of candidate compounds, their effects on endocrine functions (hormone secretion and synthesis) and their effect on target organ functions. Most frequently, these effects are directed to the reproductive system, the thyroid gland, and the adrenal glands, or to pituitary hormones, gastro-intestinal hormones or hormones which regulate growth, and metabolism. There is a close relation to a pharmacological methods designed for carbohydrate und lipid metabolism, insulin resistance, atherosclerosis and obesity (described in the Metabolism section of this book).

Due to the complexity of endocrine investigations and the time and expense of classical bioassays for hormone effects, such studies are no longer recommended for a standard protocol. Evidence for endocrine risks is usually obtained from in vitro studies on hormone receptors and enzymes, which should lead to the inclusion of specific study parameters into the Regulatory Toxicology studies. In some instances, a targeted investigation is a performed for the release or inhibition of release of specific hormones, by single-dose tests measuring the response of hormones, by acute changes in the serum concentration or associated tissue contents of hormones. Some of the classical bioassays, described in detail by Vogel (2002) can be used for the safety pharmacological characterization, listed in Chapter N "Endocrine Pharmacology" of the book on Drug Discovery And Evaluation (Vogel 2002). Here, reference is made to the selection of procedures and assays which may be required to meet the ICH guideline S7A (2001) which are appropriate in the context of Regulatory Toxicology studies (added to the standard protocol) and for mechanistic studies on the observed interference with hormone systems.

I.0.2 Regulatory Toxicology Studies

The unexpected evidence is generally provided by organ weight changes (frequently testes or ovaries, thyroid gland or adrenal glands), and by significant changes in clinical chemistry (for instance enzymes or electrolytes). In regulatory studies, the animals should not be overburdened with accessory examinations, satellite groups are needed frequently to obtain sufficient serum and tissue samples, or to obtain material for ex-vivo studies in addition to the necessary histology specimens. With paired organs (testes, ovaries, adrenals) this should represent no problem, and anterior pituitaries may be bisected by a median-sagittal cut to obtain tissue for hormone analysis.

There are some changes in hormone contents which may be detected in regulatory toxicology studies by inclusion of the recommendations in the study protocol, mostly related to the anterior pituitary, thyroid gland, adrenals, and the reproductive system, less frequently to changes in growth hormone and prolactin secretion. Interference with glucose regulation is not easily detected unless frequent samples are taken, because animals compensate for hyperglycaemia and show no related symptoms. Major changes in electrolyte

balance are readily detected with interim collection of urine specimens under controlled conditions, or in serum samples at autopsy, with adequate sampling and immediate processing. There may be organ weight changes and specific findings in the histopathology, which require a follow-up endocrine analysis in a mechanistic study using immunostaining or markers of cell proliferation. Frequent findings which require explanation but may not necessarily be related to similar effects in humans are thyroid enlargement, changes in ovarian or testis morphology (for instance Leydig cell hyperplasia), adrenal hyperplasia or pancreatic tumours (for instance beta-cell hyperplasia). The observations are mostly from one month treatment studies, less frequently from 90 days or six months treatment. Estrogenic effects are indicated by uterine weight, androgenic effects by epididymal weight or more frequently by histology of the testis. In each case it is useful to obtain and store a sufficient blood sample volume from treated animals and controls to be able to look for distinct hormonal changes, this is important for long-term studies where blood sampling can be performed at some time during the study without interfering with the condition of the animals at the final autopsy. It is then advisable to perform an evaluation of a similar type as outlined in "Drug Discovery And Evaluation " (Vogel 2002, section on Endocrinology).

I.O.3 Mechanistic Studies

A different set of questions arises from drug candidates which belong to a class that has already shown some endocrine effect, or is suspected of having endocrine-mediated effects (Harvey et al. 1999). No general recommendation can be given for the situation when specific endocrine investigations need to be performed in separate non-GLP studies. Such studies are summarised as mechanistic studies.

A detailed ex vivo biochemical analysis of drug induced changes requires hormone determinations in brain, hypothalamic and pituitary tissue, peripheral target organs, and measurement of circulating plasma concentrations of hormones and hormone-dependent substances. Hypothalamic hormones in tissue samples are identified by direct measurement (for instance TRH, GnRH, somatostatin, GHRH, CRF). There are however strict conditions for autopsy, tissue samples need to be frozen immediately, and enzyme inhibitors are added during processing. Results are interpreted in the context of the associated peripheral changes in pituitary hormones, organ weight changes and histological findings.

The suprahypothalamic neurotransmitter level can be assessed by a determination of catecholamines in circumscribed brain areas, the technique requires preparation of frozen tissue and isolation of specific nuclei by the micropunch technique. The catecholamines and indolamines can be measured by a radio-enzymatic methods and by a high-pressure liquid chromatography (HPLC) with electrochemical detection. These mechanistic investigations are mostly initiated due to questions arising from the receptor interaction profile of the drug candidate, they may be required to prove that such receptor interactions truly change the functional state of neurotransmitters (functional expression). Mostly, however, the peripheral effects of such neurotransmitter mechanisms (for instance prolactin secretion) are sufficiently distinct.

Mechanistic Studies (Single-Dose)

These will be in general the endocrine experiments directed at specific hormone measurement (time action profile). For such acute experiments, standard operating procedures are required based on the secretory kinetics of the hormones to be measured (Heinrich-Hirsch et al. 2001). Frequently, the anaesthetic has a marked effect on hormone secretion, in some cases animals with indwelling cannulas will be required for meaningful assessment. Retroorbital punction in un-anaesthetised rats was found to be a suitable procedure. The rat has some limitations and dogs provide better experimental conditions for frequent sampling. Unless the investigator knows about the physiology of hormone secretion in the species to be examined, consultation with an endocrine pharmacologist is helpful, reference may also be made to similar procedures which are established for the human.

For the pituitary hormones, and pancreatic hormones, analytical methods are in general available with species specific reagents for the mouse, rat, dog, pig and to a limited extent for rabbit. Steroid hormones can be measured readily by an array of analytical methods. Application of gene arrays and studies on gene expression are not recommended for single-dose experiments, repeated dose exposure is required (3–7 days).

Mechanistic Studies (Repeated-Dose)

These studies are designed to investigate the context of changes, adapting experimental conditions to the questions raised by the regulatory studies (Harvey et al. 1999). The dose range should include the anticipated therapeutic range (physiological range), and does not

need to know include very high doses. The purpose is to compare base-line conditions with changes after stimulation (or inhibition, as the case may be). In relation to drug administration, several time points need to be included. At the end of the study, an important decision concerns when to sacrifice the animals in relation to the preceding last treatment. This item addresses changes in the tissue content of hormones. Such changes are valuable indicators of the extent of the endocrine effect, however changes in tissue content may occur within hours and not be detected 24 hours after the last treatment (the usual interval for autopsy in regulatory studies).

The biochemical parameters for evaluation of changes in hypothalamic – pituitary – target organ function are derived from hormone concentrations in tissue or in serum and plasma. Both factors (tissue and/or plasma concentration) are influenced by the stress of handling and the technique of sacrificing the animals. Plasma levels are more susceptible to modification by stress, but tissue content may also be changed during the procedure preceding autopsy. Decapitation is recommended as the established method of sacrificing animals with a minimum of endocrine interference.

In regulatory studies and in mechanistic studies, one may rely on changes in tissue content for hormones like corticotropin (ACTH), prolactin and growth hormone (GH), because the tissue content of these hormones changes slowly and in a characteristic direction, whereas their plasma levels may fluctuate widely both with handling stress and due to circadian rhythm. This also indicates that one condition for mechanistic studies is to select time points of investigation for which comparative laboratory data are available, or at least publications that provide evidence that circadian rhythm does not interfere during the selected time period.

For the more stable secretion of thyroid stimulating hormone (TSH, thyrotropin) and the gonadotropin (LH and FSH), plasma determinations are adequate provided that autopsy is completed within a short time, and the time of autopsy selected based on data which minimise artifacts due to circadian hormonal changes. In our laboratory, rats are sacrificed by decapitation (one experimental group at a time), and autopsies are performed during the morning period of 08:00–10:00. In the mechanistic studies however, it may be necessary to use groups of animals spaced at increasing intervals after treatment, to obtain sequential tissue samples. These recommendations are also valid for basal concentrations of gonadal steroids and adrenal steroids.

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I.0.4 Endocrine Survey

This test is applied for the detection of biological effects related to previous indications from in vitro pharmacology, and from animal studies performed with the tests substance.

The Endocrine Profile of a new test, days determined in a repeated-dose study of least 7 days duration. The study plan is similar to a subchronic toxicology study (14–28 days duration in two animal species, usually the rat and dog). The guideline states “effects of the test compound on organ systems not investigated elsewhere should be assessed when there is a reason for concern”. Reasons for concern are in most instances related to the reproductive system (gonadal function), the adrenal system (adrenocortical function), or to the thyroid gland. Concern may be caused by histopathological findings, organ weight changes or findings in clinical chemistry.

When a distinct endocrine profile of a compound is present before starting the toxicology studies, a targeted investigation can be added to the standard toxicology protocol, satellite groups may be added, or specific mechanistic studies may be initiated in the context of toxicological evaluation of the test substance.

In this method, the repeated dose administration with a design similar to toxicology studies is preferred. Studies of up to four weeks duration need to include function tests for hormone secretion (basal concentrations, stimulation or inhibition), and for hormone contents of endocrine organs and tissues (e.g. hypothalamus, pituitary gland, reproductive organs). In the Endocrine Survey, 5 to 7 days of treatment are mandatory because adaptation and response of hormone-dependent organs to the treatment requires that a steady state be reached. Repeated-dose treatment with at least two doses is also needed for a sufficient

effect size to achieve biologically relevant and statistically significant differences between controls and treatment groups.

The inclusion of untreated or a vehicle control groups is always mandatory. The additional inclusion of comparator groups (comparators of known endocrine activity) may be considered. In the majority of cases this is however not necessary, because there is an extensive data base in the literature about the effect size of established drugs that affect the endocrine systems. This agrees with the guideline statement "in well-characterised in vivo test systems, positive controls may not be necessary".

I.O.4.1 Endocrine System Evaluation in Rats

PURPOSE AND RATIONALE

This study is designed for a test substance which has shown some effects on endocrine systems during the preceding toxicology evaluation (28 days treatment in rats, or in dogs), however without a distinct profile related to a specific endocrine system.

This study is generally performed in rats, less frequently in mice, because extensive reference data are available for hormones and substances acting on hormone systems, including serum concentrations and tissue contents. The rationale of the Endocrine Survey is to investigate several endocrine systems simultaneously, preferably in rats of a strain that is also used in the regulatory toxicology studies. Parameters to be investigated (see Table 1) are organ weight changes, histology or histopathology of hormone-producing organs and hormone-responsive tissues, and specific clinical chemistry (biomarkers). During this study and before autopsy, hormone concentrations in blood and tissue are determined (related to function of the reproductive system, adrenal and and thyroid glands). The survey may include effects on the endocrine pancreas (see section on Metabolism).

PROCEDURE

Adult male and female rats, body weight preferably 200 grams are randomly assigned to control and treatment groups, at least six animals per group, and at least two doses. Dose levels for treatment are selected based on pharmacodynamic information (these may be in vitro findings using receptors or enzymes, or single-dose studies in animals). The low dose should elicit the primary pharmacodynamic effect in the test species (if possible related to the proposed therapeutic effect in humans), the high dose should elicit the adverse effect(s) anticipated based on previous study results in vitro and in vivo. In case of only two test doses, the dose increment should be at least fivefold. In accordance with guideline ICH S7A, the highest test dose should produce moderate adverse effects in this study or in other studies of similar route and duration, and the adverse effects can include dose-limiting pharmacodynamic effects or other toxicity.

The duration of the treatment period should be at least seven days. It may be extended to the duration of a preceding toxicology study (28 days) where some observations for concern-were made, but no satellite groups were included for specific investigation.

Before treatment and during the treatment period, the hormone concentrations in controls and treatment groups may be a determined at baseline (always at the same time of the day, to avoid confounding effects of diurnal hormone fluctuations). Blood sampling should be done sparingly (every week) and discontinued at least three days before the end of the study period. Dynamic function tests (stimulation or inhibition) may be performed preferably at seven days intervals, by using established diagnostic reagents that act on hormone secretion (eg. LHRH, TRH, CRF/ACTH, somatostatin). In a similar manner as for human diagnostic procedures, reagents may be combined when the hormonal effects do not interfere (e.g. combined TRH/LHRH test).

Measurement of the effects by a non-invasive procedure is often possible by collecting urine samples and using urinary excretion data. During the treatment period, collection of urine samples may be performed in metabolism cages during the night period, in rats which have been adapted to this procedure, at least three days before the first diagnostic sampling. Biomarkers in the urine samples are analyzed e.g. for endogenous compounds (non-invasive diagnostic procedure), electrolytes, or for the test substance excreted in the urine (pharmacokinetics).

At the end of the treatment period, groups of animals are killed under minimum stress conditions, preferably by rapid decapitation. The time point "24 hours after last dosing" is not always suitable, e.g. when changes in pituitary hormone contents need to be assessed, the time point "2 hours after last dosing" is preferable. Satellite groups need to be included if necessary for the exact time course of changes after dosing.

Endocrine organs are dissected out, and their weight is recorded. In the case of paired organs, one organ is assigned to histology (with suitable fixation), and the other organ is immediately frozen for further hormone analysis, receptor determination, or molecular biology

Table 1 Endocrine Survey study, hormone systems to be investigated after repeated-dose treatment, optional biomarkers to be included in the investigation during the study and at autopsy

Endocrine system	Optional Biomarker
Pituitary-adrenal axis	
Pituitary weight	Corticotropin-releasing hormone in hypothalamus
Adrenal weight	Pituitary content of adrenocorticotropin and vasopressin
Thymus weight	Adrenal content of corticosterone and aldosterone
	Corticosterone and aldosterone blood level, electrolyte (Na^+, K^+, Ca^{2+}, Cl^-) concentrations in serum
	Urinary excretion of corticosterone and aldosterone
Pituitary-gonadal axis	
Male reproduction	
Pituitary weight	LH-RH in hypothalamus
Weight of testes	Pituitary content of FSH, LH and prolactin
Weight of seminal vesicles	Blood levels of FSH, LH and prolactin
Weight of ventral prostate	Testosterone content in testes and serum
Weight of musculus levator ani	Growth hormone in pituitary and serum
Female reproduction	
Pituitary weight	LH-RH in hypothalamus
Weight of ovaries	Pituitary content of FSH, LH and prolactin
Weight of ovaries	Pituitary content of FSH, LH and prolactin
Weight of uterus	Blood levels of FSH, LH and prolactin
	Content of estradiol and progesterone in ovaries and serum
Pituitary-thyroid axis	
Pituitary weight	TRH in hypothalamus
Thyroid weight	Pituitary content of thyrotropin
	Serum content of thyrotropin, T3 and T4

(gene expression profiling). Organs of interest are the adrenal glands, thyroid gland, male reproductive organs (testes, seminal vesicles, ventral prostate, levator ani muscle), female reproductive organs (uterus, ovaries).

Specific dissection is performed on the brain and pituitary gland. Hypothalamic fragments may be dissected and shock frozen immediately in liquid nitrogen, for later extraction and determination of hypothalamic peptides. The pituitary gland is dissected, the posterior pituitary is stored separately, and the anterior pituitary is halved by a median-sagittal cut to obtain separate tissue samples for histology (fixation), and for subsequent analysis of hormone contents (stored frozen at –20 °C until hormone assay).

At the time of autopsy, other tissues of interest may be obtained and processed either for histology or for biochemical analysis. The decision on these specific supplementary selections depends on information from previous studies about possible endocrine-mediated adverse effects, or toxic pharmacodynamic effects.

The selection of the blood sampling procedure depends on the hormone to be measured. Most hormones can be reliably measured e.g. during brief carbon monoxide anaesthesia, or preferably during brief ether anaesthesia by retroorbital venipuncture. Repeated blood sampling is not advisable in this method, when necessary satellite groups need to be added e.g. to be sampled for several hours during anaesthesia with pentobarbital or ketamine.

EVALUATION

For all quantitative parameters such as organ weights, group means are calculated and the significance of differences is assessed by the appropriate statistical methods.

Histology may be reported in descriptive terms, evaluated by a semi-quantitative methods,. or by computer-assisted histomorphometric methods. This information then can be used for quantitative statistical analysis. For the very specific add-on methods of in situ hybridization and gene expression, a description of the observed changes is generally appropriate together with quantitative data generated by computer-assisted analysis of gene expression profiles.

MODIFICATIONS OF THE METHOD

In each case, this method needs to be adapted to the new test substance (dose level and duration), and to the biological/biochemical information which gave "reasons for concern" in the planning of the study. The age of the test animals (or initial body weight) may be selected according to the objective of investigation, mainly adult animals (100–200 gram initial body weight) are recommended. However, the method is also applicable

to studies on pubertal development (30–40 gram initial body weight). For compounds which affect e.g. body composition (antiobesic drugs) or bone density (e.g. bisphosphonates), the selection of rats 300–400 gram body weight is preferable, and markedly longer treatment periods may be required (mechanistic studies beyond the Endocrine Survey Design).

CRITICAL ASSESSMENT OF THE METHOD

The Endocrine Survey is a flexible approach to the investigation of several hormonal systems in one study, it is closely related to the design of subchronic toxicology studies, however with the specific focus on endocrine systems.

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I.O.4.2 Determination of Hypothalamic Hormones

PURPOSE AND RATIONALE

Many tests substances change the secretion of hypothalamic hormones, either by direct action or more generally by a feedback effects at the hypothalamic level. Under these conditions, the tissue concentration in hypothalamic specimens of treated rats is of interest, especially because several hypothalamic peptide assays are available and can be measured in the same specimen. In contrast to the tissue concentration, for many hypothalamic hormones it is difficult to measure the circulating concentrations, due to analytical problems of low concentration and rapid inactivation by enzymes. Circulating concentrations would also change immediately due to the interference of anesthesia and of the autopsy procedure. It is therefore recommended to take samples of hypothalamic tissue, and control samples of cerebral cortical tissue.

PROCEDURE

Specific dissection is performed on the brain and pituitary gland. Hypothalamic fragments may be dissected and shock frozen immediately in liquid nitrogen, for later extraction and determination of hypothalamic peptides. The procedure is suitable for the rat and for the rabbit. Immediately after sacrifice, the skull is opened, the brain is removed and the hypothalamic fragment at the base of the brain is clipped with scissors, and stored deep-frozen. Alternatively, specimens may be shock frozen and homogenized immediately in an ice-cold buffer containing peptidase inhibitors (e.g. bacitracin or aprotinin) Specimens of cerebral cortical tissue are subsequently taken and analysed as controls.

The pituitary gland is then dissected out and stored separately. There are numerous publications which refer to the measurement of luteinising hormone releasing hormone (LHRH – Adams and Spies 1981a–c, Aubert et al. 1980, Berault et al. 1983, Catt et al. 1980, Chan et al. 1981, Clayton 1982a,b, Clayton and Catt 1979, Clayton et al. 1979a,b, 1980a, Conn and Hazum 1980, Conn et al. 1980, Dalkin et al. 1981, Dufau et al. 1979, Hazum 1981, Heber and Odell 1978, Kuhl et al. 1980, Loughlin et al. 1981, Loumaye and Catt 1982, Naor et al. 1981, Perrin et al. 1980, Sandow 1982, Sandow et al. 1979, 1981), corticotropin releasing hormone (CRH – Aguilera et al. 1986, Brown et al. 1988, DeSouza and Battaglia 1986, DeSouza 1987, Flores et al. 1990, Grino et al. 1986, Hauger et al. 1987, 1988, Millan et al. 1986, Perrin et al. 1986), and growth hormone releasing hormone (GHRH – Abribat et al. 1990, Audhya et al. 1985, Bohlen et al. 1984, Gelato et al. 1985).

EVALUATION

The peptide concentration in the individual specimens is determined by radioimmunoassay, using a synthetic reference peptide for the standard curve. Several commercial assays are now available for hypothalamic peptides. An alternative method is the determination of peptide content in tissue extracts by an HPLC method or by mass spectrometry.

Group means are calculated and appropriate tests for significance are applied.

MODIFICATIONS OF THE METHOD

In the context of safety pharmacology, measurement of hypothalamic hormone concentrations is performed in the context of repeated dose treatment, to assess changes after a several days period of biological adaptation. These measurements can be added to any of the conventional subacute toxicology protocols.

CRITICAL ASSESSMENT OF THE METHOD

The main difficulty is experience with rapid processing of the tissue samples, and selection of an enzyme inhibitor to prevent spontaneous degradation by tissue peptidases (Elkabes et al. 1981, Griffiths and Kelly 1979, Hazum et al. 1981, Horsthemke and Bauer 1981, 1982, Horsthemke et al. 1981, McDermott et

al. 1982, Posner et al. 1982). The result needs to be assessed in context with changes in pituitary hormone contents, frequently the adaptation to treatment at the pituitary level is more dose-related than at the level of the hypothalamic tissue contents.

In many publications, changes in receptors for hypothalamic peptides have been described at the target organ level e.g. the gonads (Harwood et al. 1981a,b, Pieper et al. 1980, Sharpe 1982, Sharpe and Fraser 1981), and in non-endocrine tissues e.g. the gastrointestinal tract (LHRH and GHRH receptors, Bruhn et al. 1985). These studies however have identified no major regulatory mechanisms which could be activated by test compounds to be developed for phase I clinical pharmacology. Application of the measurement in safety pharmacology should therefore be restricted to hypothalamic tissue, as an additional level of information when pituitary hormone changes are identified.

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I.O.4.3 Determination of Pituitary Hormone Contents

- Secretion of LH and FSH
- Secretion of TSH
- Secretion of GH and stimulation of IGF
- Secretion of prolactin
- Secretion of ACTH and MSH

PURPOSE AND RATIONALE

This section describes the measurement of pituitary hormones in the context of repeated dose studies of 1–4 weeks duration. When performing an endocrine safety pharmacology study in rats it is advisable to determine pituitary hormone concentrations at autopsy, because in this species specific pituitary hormone assays are readily available. The time point two hours after last dosing is in general suitable, when changes in pituitary hormone contents need to be assessed.

For toxicology studies in dogs, preparation of the pituitary glands at autopsy may be performed, but this is time-consuming and less advisable because of the small numbers of animals in such studies. In general, homologous assays need to be a applied for the rat luteinizing hormone (LH) (Daane and Parlow 1971, Niswender et al. 1968, Seki et al. 1971), follicle-stimulating hormone (FSH) (Beastall et al. 1987, Migdley 1967) and thyroid stimulating hormone (TSH) (Kieffer et al. 1975, Garcia et al. 1976, 1977, DeVito et al. 1987), which are glycoprotein hormones exhibiting species specificity. For pharmacological experiments in rats, such as evaluation of gonadotropin releasing hormone activity, a homologous assay is necessary. The reagents are provided by the National Pituitary Agency, Bethesda, Md. The assay procedures can be adapted from the standard operating procedure proposed by the National Pituitary. Agency, USA. For growth hormone (GH) (Schalch and Reichlin 1966) and prolactin (PRL) (Niswender et al. 1969), species specific assays are needed. Determination of corticotropin (ACTH) is less frequently required. For specific research investigation, there are several other methods available, such as assays for melanotropins and other specific pituitary peptides.

PROCEDURE

The skull is opened by scissors, the pituitary gland is dissected out, the posterior pituitary is stored separately, and the anterior pituitary is halved by

a median-sagittal cut to obtain separate tissue samples for histology (fixation), and for subsequent analysis of hormone contents (stored frozen at –20 centigrade until hormone assay). Pituitary glands are homogenized in phosphate buffered saline pH 7.4 and diluted to the concentration required for the radio-immunoassay. The initial homogenate may be stored deep-frozen for sequential assay of several hormones.

EVALUATION

Hormone concentrations in dilutions of pituitary homogenates are calculated from standard curves using the appropriate reference standard for rat pituitary hormones, sample data are calculated as hormone per pituitary gland or per pituitary weight (mg). Group means are calculated and the significance of differences is assessed by the appropriate statistical methods.

MODIFICATIONS OF THE METHOD

This approach provides a fast and flexible assessment of several pituitary hormones, results may be compared with morphometric evaluation and immuno-histochemistry for one or several hormones of interest.

CRITICAL ASSESSMENT OF THE METHOD

Changes in pituitary hormone contents are frequently characteristic and may be followed up by measurement of hormone secretion profiles if required. Analytical methods for the rat are often also applicable to mice. For toxicology studies in dogs, preparation of the pituitary glands at autopsy may be performed, but is time-consuming and less advisable because of the small numbers of animals in such studies.

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I.O.4.4 Dynamic Function Tests

The changes in base-line hormone secretion are useful for interpretation of drug effects, it is often advisable to include dynamic function tests based on stimulation of pituitary hormone secretion, or of gonadal and adrenal hormone secretion. Examples for pituitary testing have been mentioned above and secretion. Typical pituitary function tests are the TRH test for TSH secretion, the LHRH test for secretion of FSH and LH, and the mono iodo-tyrosine (MIT) test for prolactin secretion. Synthetic CRF may be injected to stimulate secretion of corticotropin (ACTH), more frequently the injection of corticotropin is used to stimulate the adrenal directly. At the target organ level, similar

function tests can be performed e.g. using thyrotropin (TSH) to stimulate thyroid hormone secretion (T4 and T3), human chorionic gonadotropin (hCG) to stimulate testosterone or ovarian steroid secretion, and corticotropin (ACTH) to stimulate adrenal steroids secretion. All reagents for these tests are readily available for tests in rats and dogs, the tests can be built into a standard toxicology protocol, several days before the end of the study. Conversely the inhibition of increased hormone secretion due to treatment may be tested in treated animals at the end of the study, e.g. with inhibitors of prolactin secretion (bromocriptine), this is usually reserved for mechanistic studies.

I.O.4.5 Gonadotropin Release from Anterior Pituitary Cells

PURPOSE AND RATIONALE

Anterior pituitaries may be incubated directly, kept in culture or used for cell lines (Mittler and Meites 1964, 1966; Mittler et al. 1970; Sandow et al. 1972) in order to study the synthesis and release of gonadotropins in response to LH-RH.

PROCEDURE

Female Sprague-Dawley rats weighing 100–150 g are used as donors. Each anterior pituitary is removed and cut into 4–6 pieces of approximately equal size. The cultures are performed in 3.5–1-cm sterile disposable plastic Petri dishes each containing 3 ml medium consisting of 9 parts Difco medium 199 and 1 part of newborn calf serum. Twenty-five U/ml penicillin and 25 μg/ml streptomycin are added. In each dish, the explants are supported at the gas interface. An atmosphere of 95 % oxygen and 5 % carbon dioxide and a temperature of 36 °C are maintained. Opposite sides of the same pituitaries provide matched control and experimental preparations. The pituitaries are incubated for a total time of 5 days. After the first two days, the medium is removed and discarded. Fresh medium is then added with the LH-RH solutions. Approximately 12 h after the first change of medium and addition of LH-RH, media are removed and frozen. Fresh medium with LH-RH is again added; this procedure is repeated until 6 samples of medium representing the last 3 days of culture are obtained. Media are assayed for LH content by radioimmunoassay according to Niswender et al. (1968) and for FSH content according to Parlow et al. (1963).

For preparation of pituitary cells, there are several modifications which have been successfully applied, enrichment of specific cell types has been reported, and cell lines in culture are more stable and practical than the freshly dispersed pituitary tissue.

EVALUATION

Several increasing concentrations of test preparation and LH-RH standard preparation are tested, dose-response curves are obtained allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Pituitary halves or quarters were initially used for the assay of LH-RH as well as for the assay of TRH, cultures of enzymatically dispersed anterior pituitary cells from rats were also used (Vale et al. 1972; Martin and Sattler 1979). Loughlin et al. (1981) used perifused pituitary cultures as model for LH-RH regulation of LH secretion. O'Connor and Lapp (1984) studied the effect of pulse frequency and duration of luteinizing hormone releasing hormone in anterior pituitary cells attached to Cytodex I beads. Functional integrity of anterior pituitary cells separated by a density gradient has been studied (Scheikl-Lenz et al. 1985). These studies have shown that the time course of the pituitary response in vitro is quite different from the response observed in experimental animals. Receptor binding ability to rat pituitary and human breast cancer membranes of different agonists and antagonists of luteinizing hormone-releasing hormone was studied by Fekete et al. (1989). Vigh and Schally (1984), Czernus and Schally (1991) described in detail a cell superfusion system consisting of a Sephadex column with dispersed pituitary cells. LH response of anterior pituitary cells to 3 min exposure to various concentrations of LHRH at 30 min intervals as well as growth hormone response to human growth hormone releasing hormone (GHRH) resulted in excellent dose-response curves. The effect of growth hormone releasing hormone was inhibited by somatostatin. Likewise, the effect of LHRH was inhibited by pretreatment with LHRH antagonists.

The method is suitable for studies of interaction with synthetic steroid hormones and endogenous compounds which are modulating the response to releasing hormones (pituitary feedback studies).

CRITICAL ASSESSMENT OF THE METHOD

The stimulation of LH release and FSH release from pituitary cells in vitro does not reflect the time course of the pituitary hormone response found in vivo. The pituitary incubation methods are however useful early safety evaluation of compounds that have shown

endocrine activity in the initial toxicology evaluation in rats, and the incubation methods are also useful for comparison of compounds and potency estimates.

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I.0.4.6 TSH Release from Anterior Pituitary Cells

PURPOSE AND RATIONALE

The in vitro bioassay method of Saffran and Schally (1955a,b), developed for detecting CRF activity in hypothalamic extracts was modified to measure TRH activity in vitro (Guillemin et al. 1963; Bowers et al. 1965; Schally and Redding 1967). The assay is also applicable to other hypothalamic hormones, their analogues, and to the testing off compounds which modulate the pituitary response to hypothalamic hormones, e.g. gonadal steroids and adrenal steroids as well as synthetic compounds with endocrine modulating activity.

PROCEDURE

Male Sprague-Dawley rats weighing 150–200 g serve as donors. After removal each pituitary is cut in half, transferred to a 15 ml beaker containing 1.5 ml Krebs-Ringer bicarbonate medium with 200 mg% glucose and incubated for three 60-min periods. The media used in the first 2 incubations are discarded. At the beginning of the third incubation period, various amounts of test preparation or TRH standard are added to individual beakers. At the end of the third incubation period the media from both control and experimental beakers are carefully aspirate, and the remaining pituitary tissue may be stored frozen for later determination of hormone contents. The incubation media are assayed by RIA for content of TSH, and other pituitary hormones of interest.

EVALUATION

Dose-response curves are established for test preparation and standard allowing calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

For the assay of TRH analogues, cultures of enzymatically dispersed anterior pituitary cells from rats can be used instead of pituitary halves (Vale et al. 1972).

Barros et al. (1986) studied the effect of TRH on cultured GH3 rat anterior pituitary cells using the whole-cell voltage clamp technique.

CRITICAL ASSESSMENT OF THE METHOD

The method of testing in vitro on dispersed pituitary cells is essentially a static method for the comparison of hormones and hormone analogs. It has also been applied to the testing and pituitary tissue ex vivo, from animals that have undergone a period of treatment with compounds that are known to modify the pituitary response, e.g. synthetic corticoids.

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I.0.4.7 GH Release from Anterior Pituitary Cells

PURPOSE AND RATIONALE

For testing of pituitary cell stimulation, isolated pituitary glands or cell cultures may be used. In the investigation of growth hormone regulation, antagonism of GH release is tested by adding somatostatin analogs at increasing concentrations in the presence of a standard concentration of GHRH. Human GHRH and the related peptides with growth hormone releasing activity may be tested. The effect of GHRH analogues can be tested avoiding the interference of counterregulatory somatostatin secretion which limits the duration of GH release in vivo.

PROCEDURE

The pituitaries of male Sprague-Dawley rats weighing about 100 g are quickly removed after decapitation. The posterior lobe is discarded and the anterior lobe is divided into two halves by a midsagittal cut. Five bisected hemipituitaries are incubated in plastic vials containing 4 ml TCM 199 with 0.1 % BSA, 15 μg/ml penicillin and 25 μg/ml streptomycin. The vials are gassed with 95 % O_2 and 5 % CO_2. After 30 min of control incubation, the medium is changed and various doses of standard and test substances are added for an incubation of 90 min. GH content in the medium and in the pituitary tissue after incubation is determined by a specific radioimmunoassay (Schalch and Reichlin 1966). Other of hormones may be tested in the same procedure, for example prolactin.

EVALUATION

Dose-response curves are established for standard and test compounds measuring GH release into the medium and GH depletion from the pituitaries. Potencies ratios with confidence limits are calculated from dose-concentration curves.

MODIFICATIONS OF THE METHOD

Superfused pituitary cells may be used measuring activity and duration of effect, as well as interaction of stimulatory and inhibitory factors. Growth hormone releasing factor from tumors in human pancreas and from rat hypothalami as well as analogs of growth hormone-releasing hormone were evaluated in a superfused pituitary cell system (Vigh and Schally 1984; Czernus and Schally 1991; Halmos et al. 1993). Anterior pituitaries of two young adult male Sprague Dawley rats were digested with 0.5 % collagenase CLS2 (Worthington) for 50 min. After incubation, the fragments were digested into cell clusters (5–40 cells) by mechanical dispersion, transferred onto two columns and allowed to sediment simultaneously with 0.8 ml Sephadex G-10. The dead volume of the system was set to 1 ml. Medium 199 containing BSA (2.5 g/l), $NaHCO_3$ (2.2 g/l) and gentamycin sulfate (85 μg/ml) was equilibrated with a mixture of 95 % air and 5 % CO_2 and used as the culture medium. The medium was pumped at a flow rate of 0.33 ml/min. During an overnight recovery period, the baseline stabilized and the cells regained their full responsiveness. The samples were then infused through a four-way valve at 5×10^{-10} M concentration for 3 min (1 fraction) at 45-min intervals. Rat GH was determined by radioimmunoassay. The same system was used by Rekasi and Schally (1993) and Kovács et al. (1996a) to evaluate the activity of growth hormone-releasing hormone antagonists. For determination of the antagonistic activity, the cells were exposed to GHRH antagonist simultaneously with GHRH or to GHRH antagonist combined with 100 mM KCl (controls for potassium stimulated GH secretion) for 3 min. After 30 min, the duration of the inhibitory effect of GHRH antagonist was also tested by repeated 3-min infusions of 10^{-9} M GHRH. Using this system with pituitaries of transgenic mice overexpressing the human GHRH gene, Kovacs et al. (1997) evaluated the effects of growth hormone-releasing hormone antagonists. Horváth et al. (1995) also determined cAMP release from superfused rat pituitary cells stimulated by growth hormone-releasing hormone.

GH release was determined using cultured rat pituitary cells (Brazeau et al. 1982; Perkins et al. 1983; Scheikl-Lenz et al. 1985). Pituitary cells were prepared by enzyme dispersion with collagenase, DNAase and pancreatin. The cells were cultured for 3 days in microbiological Petri dishes in Dulbecco's modified essential medium with 20 mM HEPES, 15 % fetal calf serum, 100 mU/ml penicillin-G and 100 μg/ml streptomycin at 37 °C and 10 % CO_2.

Cheng et al. (1993) tested time- and dose-dependent growth hormone release by a non-peptide growth hormone secretagogue in rat pituitary cells. Sanchez-Hormigo et al. (1998) tested growth hormone-releasing hexapeptide on growth hormone secretion from cultured porcine somatotropes.

As a follow up from the in vitro pituitary cells studies Kovács et al. (1996b) measured the effects of chronic administration of a growth hormone-releasing hormone agonist on body weight, tibia length and tail length in growth hormone-deficient (monosodium glutamate-lesioned) rats. Jacks et al. (1996) evaluated an orally active growth hormone secretagogue in dogs.

Serum growth hormone levels were dose-dependently increased after oral and intravenous administration. Moreover, an increase of insulin-like growth factor and serum cortisol was found. Clearly, the pituitary incubation method can only identify compounds which are suitable for further in vivo characterization, because the responses found in test animals at the target organ level are vast different from those found at the pituitary cell level.

CRITICAL ASSESSMENT OF THE METHOD

The advantage of pituitary cell incubation with test compounds of suspected endocrine activity is that useful initial information can be provided rapidly, especially for compounds that show neuroendocrine modulating activity, e.g. neuroleptics and dopamine receptor agonists. The response at a clearly defined cellular level needs than to be compared with the response in intact animals, where multiple feedback loops are operating, and may markedly changed their response profile when the test result is compared in vitro and in vivo. The possibility of using pituitary cells ex-vivo from treated animals should always be kept in mind.

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I.0.5 Determination of Gonadal and Adrenal Steroid Hormones

PURPOSE AND RATIONALE

The gonadal steroid hormones are not species specific, and can be readily determined by analytical method in mice, rats, rabbits, dogs and other animal species used in safety evaluation. For investigation of receptor concentrations in tissue samples (Lieberman 2001), and for circulating concentrations, many methods are available (Wheeler and Hutchinson 2005). In the Endocrine Survey protocol, testosterone, estradiol and progesterone are usually required for the interpretation of effects in the reproductive system. Reproductive organ weights (testes, seminal vesicles, uterus and ovaries) are recorded at the end of the study. In general, effects of the test compound will be related to changes in steroid biosynthesis. In rat and dog repeated dose studies, it is advisable to correlate the changes in organ weights with circulating concentrations of target organ hormones. Serum testosterone is determined in male rats at time of autopsy, 2 hours or 24 hours after last treatment. In case of relevant changes, serum LH may also be determined. Serum estradiol and progesterone are determined in female rats. There is however the problem that the ovarian hormone concentrations change with the estrous cycle, their determination in a general procedure is relevant when major changes are found in weight of the uterus and the ovaries.

PROCEDURE

Blood samples are taken during the study or at autopsy. Gonadal steroid hormones are determined by specific immunoassays requiring very small sample volumes, extraction procedures are no longer needed. Steroid receptors can be determined in tissue samples storage at autopsy, for later decision on necessary investigations.

EVALUATION

Hormone concentrations are calculated from standard curves using the appropriate steroids as reference standards. Sample data are calculated as hormone concentrations in serum or per unit tissue weight (mg), when gonadal tissue is extracted. Group means are calculated and the significance of differences is assessed by the appropriate statistical methods.

MODIFICATIONS OF THE METHOD

For the steroid hormone assays, they are are many suppliers nowadays, an internet search for suitable reagents and methods is indicated (e.g. Linco Research Reagents 2005, DSL Diagnostic Systems 2005).

CRITICAL ASSESSMENT OF THE METHOD

In the general procedure in female rats, determination of serum estradiol is useful when changes in the reproductive system are detected. In adult male rats, changes in reproductive organ weights are distinctive and generally do not require an additional measurement of testosterone for confirmation. For repeated dose studies in dogs, testosterone measurement was found useful whereas measurement auf estradiol- and progesterone concentrations did not contribute, due to the specific problems of the dog estrous cycle, and due to low circulating concentrations. An alternative approach is the processing of gonadal tissue by ex-vivo procedures or in vitro methods (Powlin et al. 1988).

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I.0.6 Hypothalamic-Pituitary-Gonadal Function

I.0.6.1 Repeated Dose Study in Male and Female Rat

PURPOSE AND RATIONALE

This investigation is a general outline for repeated dose studies which have is similar design as the regulatory toxicology studies in rats. In the book on Drug Discovery (Vogel 2002), there is a simplified description designated "Endocrine Survey". The duration of treatment can vary from seven days to 28 days, depending on the study objective. Issues of safety pharmacology to be addressed are:

Group 1: direct effects on female and male reproductive tract due to gonadal steroid activity (direct hormone action).

Group 2: indirect effects caused by the secretion of gonadotropins and their effect on female and male reproductive tract of (indirect hormone action).

Group 3: indirect effects caused by the secretion of hypothalamic hormones, which in turn stimulate synthesis and secretion of gonadotropins.

In effects of group 3, the gonadotropins in turn induce the secretion of gonadal steroids. when more complex interactions are present, they are detected for instance by the effect of changes in the secretion and synthesis of prolactin, either on ovarian function or on the mammary gland. There is considerable overlap with the findings of reproduction toxicology, and the investigation of pituitary-gonadal function should always be in the context of female reproduction as assessed by the reproduction toxicology methods, and to a lesser extent concerning male reproduction.

PROCEDURE

Male and female rats of suitable age (depending on study objectives) are assigned to groups of six to eight animals, including control groups. For safety pharmacology, initial body weight 100 grams is preferred, and duration of treatment should be 14–28 days (in some cases a seven-day treatment period is acceptable when a very clear indications about the anticipated effects by information available from previous in vitro pharmacology investigation, or derived from structure activity considerations). The strain of rats needs to be the same as used in the regulatory toxicology of the same institution, to avoid problems caused by strain-specific reactions.

For specific questions, selection of rats of 60 grams initial body weight or 300 gram initial body weight may be advisable. This depends on the study objective concerning pubertal development, or effects on the reproductive system of animals having reached full maturation, (plateau of weight development for female animals, or phase of slow body weight gain for male animals).

The dose range is selected based on the biological dose known to induce a effect related to the proposed therapeutic indication (low dose or "therapeutic dose"), the high dose should at least be a tenfold increment of the biological dose. The route of application is selected by the intended route of treatment in humans, groups with additional routes of application may be included to increase exposure by achieving high initial concentrations. Pharmacokinetic monitoring of the achieved drug concentrations is essential, at least one time point near the end of the study, with selection of adequate sampling time based on available pharmacokinetic information. It may be necessary to treat the animals more than once daily, if sufficient exposure is to be achieved. At the end of this study (24 hours after the last drug dosing), the animals are weighed (final body weight), the organs of the contralateral reproductive tract (see below) are dissected out, weighed and processed for histology. In case of paired organs, the contralateral organ may be used for biochemical analysis (hormone contents), or other specific investigation such as in situ hybridization or gene expression pattern.

For hormone determinations, tissues are stored frozen at –20 °C. For analysis of gene expression, specific conditions apply of rapid refrigeration and storage at –80 °C, tissue handling should be done by specialised investigators who need to be present during the procedure of sacrificing the animals. Due to the complexity of such investigations, inclusion of satellite groups is advisable.

I.O.6.1.1 Male Reproductive System

Reproductive organ weight and histology: dissect out and determine weight of the testes, seminal vesicles, ventral prostate and levator ani muscle (optional). Preserve for histology one testis, seminal vesicles and ventral prostate. Optional: store one testis at –20 °C for determination of gonadal steroid content, perform ex-vivo stimulation test with one testis by incubation for three hours with human chorionic gonadotropin (hCG), isolate Leydig cells by collagenase and perform stimulation tests on the Leydig cell preparation.

Hypothalamus

Take hypothalamic tissue fragment at autopsy (clearly visible at base of brain, excise with curved scissors, shock freeze immediately and store at –20 °C), record weight of fragment. For control tissue, take sample of brain cortex (about same size and weight). An alternative way of handling sample is to homogenise immediately in cold saline containing bacitracin as an inhibitor of peptidase activity, and store homogenate at –20 °C. Tissue content of luteinising hormone releasing hormone (LHRH gonadorelin) is determined by specific radioimmunoassay (RIA).

Anterior Pituitary

Anterior and posterior pituitary are found together at the base of the skull, once the brain has been removed. The neurohypophyseal part (posterior pituitary) is discarded and the anterior pituitary is bisected to

provide one half for histology and the other half for hormone tissue content (median-sagittal cut through the anterior pituitary). Store pituitary tissue at –20 °C for measurement of hormones.

In principle, all hormones can be determined from pituitary homogenates. Specific RIA methods are available from several suppliers, and hormone assays may also be performed using reagents of the National Pituitary Program, USA. For the reproductive system of rats, species specific reagents and assays are available, as well as some cross-reacting RIA methods based on hormones of others species. Make sure that in the analytical department, there is some prior experience with rat hormone determination. Contents of follicle stimulating hormone (FSH) and luteinising hormone (LH) are mandatory to be determined, prolactin (PRL) and growth hormone (GH) are optional, these hormones may change due to alterations of the gonadal steroid secretion, in particular due to estrogenic effects or to stimulation of estrogen secretion.

Testes

In addition to weight determination and histology, there are several available methods for obtained additional information about the site of action and mechanism of a change in androgen biosynthesis.

The tissue content of the testis can be analysed by a several methods for androgens, either by a high-performance liquid chromatography (HPLC), or by specific radioimmunoassays for testosterone, progesterone, and the testosterone precursors in the biosynthetic pathway.

Receptors in the testis for FSH and LH may be determined for specific investigations, but the information for risk assessment provided by this determination is limited. Incubation of the testis ex-vivo with human chorionic gonadotropin (hCG) is of considerable importance, and that biosynthesis of androgens may be assessed by measurement of the incubation media.

I.0.6.1.2
Testis Incubation and Androgen Biosynthesis

PURPOSE AND RATIONALE

Androgen biosynthesis (secretory capacity for androgen precursors and testosterone) is assessed by incubation of the testis in vitro with hCG 250 mU for 3 hours.

PROCEDURE

The testes of each rat were decapsulated and gently washed in tissue culture medium (TCM) 199 (Sigma biochemicals #M 2154) previously gassed with carbogen (95 % O_2, 5 % CO_2). Each decapsulated testis was transferred into a 20 ml glass vial containing TCM 199 at +35 °C and pre-incubated for a minimum of 10 minutes in a temperature controlled water bath (+35 °C) with shaking at 80 cycles per minute. After 10 minutes the incubation medium was removed and replaced with 6 ml of pre-gassed TCM 199 at +35 °C containing 250 mU hCG (Sigma biochemicals CG5, 5000 IU/vial). Throughout the incubations each vial was continuously gassed via a plastic tube, with carbogen (3 L per min supplied to 8 vials). After 3 hours, the incubation was terminated by removing the vials from the incubator, decanting the incubation medium into refrigerated test tubes and transferring the testis tissue to separate vials. The incubation medium and testis tissue was stored frozen at – 20 °C for subsequent steroid hormone assays.

EVALUATION

The steroids in the incubation medium and in the tissue are determined (testosterone, progesterone, 17-OH-progesterone, delta-4-androstenedione. Steroids measured in medium and tissue may be testosterone (testo), progesterone (prog), 17alpha-OH-progesterone (17a-OH-prog), delta-4-androstenedione (4-A-dione) and estradiol-17beta (E2). Testosterone and progesterone in unextracted media and ether extracts of testicular homogenates can be determined by radioimmunoassays (RIA). The steroids 17a-OH-progesterone, 4-alpha-androstene-dione and E2 can be measured by specific 125-I-RIA methods using commercially available reagents and procedures suitable for steroid hormones without prior extraction (for example Biermann Co., Bad Nauheim, Germany).

Group means are calculated, and the significance of differences between groups is assessed by analysis of variance and appropriate tests of significance.

CRITICAL ASSESSMENT OF THE METHOD

This method can be applied ex vivo, in repeated-dose studies for clarification of mechanisms of action, and as part of subacute toxicology studies. The immediate processing of the decapsulated testis is an advantage when compared with the assays based on a enzymatic dispersion of Leydig cells.

Testosterone secretion in vivo

The stimulation test can be performed as a single-dose test in rats during the treatment period, preferably three to four days before the end of the study, or as a single dose test in satellite groups of animals. Stimulation of testosterone in rats can be indirect via the release of luteinizing hormone by injection of LHRH (gonadore-

lin) or an LHRH agonist (e.g. buserelin at an equipotent dose). This stimulation test can be combined for the measurement of FSH, LH and testosterone after a single dose of LHRH injected subcutaneously in conscious rats, or by infusion of LHRH for in rats during anesthesia. another possibility is to stimulate testosterone secretion directly in conscious rats by the injection of hCG. These stimulation tests are similar to the procedures used in clinical pharmacology.

I.0.6.1.3 Female Reproductive System

Reproductive organ weight and histology : dissect out and determine weight of the ovaries, and uterus, and take tissue samples of the mammary glands (optional). Preserve for histology. for any specific investigation of cell proliferation, satellite groups need to be added. This is especially important for investigation of the mammary gland.

Preparation of hypothalamic and pituitary tissue and storage for assay is identical with the procedures for male animals.

Ovaries

There all many possibilities for a detailed investigation of ovarian function. For details, the section on ovarian hormones in "Drug Discovery And Evaluation. Safety Assays" may be consulted. Determination of receptor binding can be added to the morphological investigation of follicular function and corpus luteum formation. In the context of endocrine safety studies, the information from the segments I–III on reproduction and fertility in the regulatory toxicology protocol should be considered before deciding on specific investigation of the ovaries in rats. Interpretation of these findings (relevance for the clinical situation) is difficult.

Mammary Glands

For the evolution of histological findings, measurement of pituitary prolactin concentrations should be considered as an additional source of information.

EVALUATION

For all quantitative parameters, group means are calculated and the significance of differences is assessed by at the appropriate statistical methods.

Histology may be reported it in descriptive terms or evaluated by a semi-quantitative methods. Frequently, the use of histomorphometric methods provides information that can be quantitated induced for statistical analysis. For the add-on methods of in situ hybridization and gene expression, a description of the observed changes is generally appropriate.

MODIFICATIONS OF THE METHOD

The investigation may be restricted to female or male animals of reproductive age (initial body weight 100 grams), or may be targeted to prepubertal rats, to detect interference with pubertal development. The most important consideration is sufficient duration of treatment of at least two weeks, preferably four weeks. Inclusion of histology of the reproductive tract is essential, any additional application of ex-vivo investigation (cell proliferation, in situ for hybridization, spectrum of gene expression) adds to the complexity and should be included only if the primary investigation of histology warrants such detailed follow up.

CRITICAL ASSESSMENT OF THE METHOD

The treatment period should be at least 14 days. It may the shorter than in the preceding toxicology study (28 days) where the observations were made, provided that there is the inclusion and application of biomarkers of endocrine function. Many endocrine effects require exposure for 7, 14 or 28 days to become visible and significant. it is helpful to have the confirmation of a distinct weight change or a histological change in addition to the observed change of biomarkers.

Suitable modifications of this method are used when unexpected findings concerning the reproductive function are found in the four weeks toxicology study with a new compound, or in reproduction toxicology. Testing of a new compound with classical bioassays is a very unusual, due to the availability of many short term in vitro tests for receptor affinities (gonadotropin receptors and steroid receptors), and would only be performed if the hormone activity is known from structure activity information. More frequently, unexpected effects of new compounds are observed in the toxicology studies in rats and dogs, requiring an explanation for the relation of the therapeutic dose range to overdosing, in order to include targeted investigation in the early clinical pharmacology program.

For this purpose, it is advisable to investigate the reason and mechanism for the observed changes, enabling prediction of the relevance for the proposed clinical indication. There are certain areas where the prediction is very limited, for instance in relation to human reproductive function when changes in the estrous cycle of rats are observed. On the other hand, any observation of dose-related endocrine interference is valuable for the risk assessment, concerning clinical pharmacology.

The exploration of ovarian- testicular function is sometimes done in prepubertal rats (Davies et al. 2000, Marty et al. 2001) or in young rats. In female rats, monitoring of the oestrus cycle by vaginal cytology is important but can rarely be included in the standard protocol. For quantitative measurement of steroid hormone contents, all animals should be sacrificed at the same stage of the oestrous cycle in mechanistic studies, whereas this is impractical in routine toxicology studies. Weight of the uterus and ovaries frequently indicates endocrine disruption at the pituitary or gonadal level, additional parameters are listed in Table 1 for inclusion in the standard protocol.

Testing in animals shortly before puberty (immature rats) often reveals pituitary-gonadal activities more readily than testing in adult animals (Kim et al. 2002). The use of in vitro methods (receptor binding, cell lines) raises many problems of interpretation of the results, the biological relevance of receptor data needs to be confirmed in each case by bioassay (Rogers et al. 2000, Beresford et al. 2000, Fang et al. 2000). These methods are an advantage only when compounds are selected at an early-stage, not for safety pharmacology and risk assessment of advanced compounds. Histology of the uterus is very important, it reflects the classical bioassay findings for estrogenic and progestational activity. Ex-vivo studies with short term incubation of ovaries are conceivable but have not been frequently investigated. Their purpose would be information on steroid biosynthesis, at specific times of the oestrus cycle.

In female rats, measurement of steroid hormones is less useful due to the precise reflection by histology, and the rapid fluctuations during their oestrous cycle. Pituitary hormone contents is more reliable and informative. At autopsy, the neurohypophyseal part (posterior pituitary) is discarded and the anterior pituitary is bisected to provide one half for histology and the other half for measurement of hormone tissue content, which changes often in a dose related manner, whereas it is difficult to quantitate the histological changes in the pituitary gland by histomorphology. LHRH (GnRH) stimulation tests are recommended near the end of each study, by a subcutaneous injection of 0.1 μ/kg measuring the LH and FSH concentrations 60 minutes later. These tests can also be performed once per week during the study without compromising the end result.

Basal LH and FSH concentrations may be measured two hours after medication with the test drug or 24 hours after medication. Changes are often more readily detected shortly after medication, and pharmacokinetics of the test drug should be the basis of decision for selecting the time point. In general, measurement of LH is the more sensitive parameter, hypothalamic LHRH content is rarely useful for diagnosis. Pituitary prolactin content 24 hours after last medication and mammary gland histology need to be included for specific classes of drugs, the prolactin stimulation test can be performed by oral administration of mono iodo-tyrosine (MIT) 1 mg/kg with serum prolactin determination 20 to 30 minutes later.

In male rats, weight changes of the testes and androgen dependent organs (seminal vesicles, ventral prostate, levator ani muscle) readily detect interference with androgen biosynthesis and secretion (Leydig cell function). The effect on spermatogenesis and Sertoli cell function it is readily detected by histology but difficult to quantitate. Before going to detailed histomorphology, effects on pituitary hormones should be evaluated by measuring the tissue content of LH, FSH and prolactin (as in females), the serum concentration of testosterone, and the testosterone content in testis tissue. It is advisable to use one testis for steroid hormone analysis, and the contralateral testis for histology. When there are indications for an effect on androgen biosynthesis, the testis can be studied ex-vivo by incubation with human chorionic gonadotropin (pattern of androgen biosynthesis), including tissue contents at the end of the incubation period. Leydig cells isolated by collagen dispersion may also be to perform similar stimulation tests.

In order to assess pituitary function, inclusion of the LHRH stimulation test for LH and FSH is recommended in male rats and female rats, and the mono-iodo-tyrosin (MIT) stimulation test for prolactin secretion is recommend at least for the female animals. Pituitary content of LH, FSH and prolactin should always be determined at autopsy (half of the pituitary for hormone assays and the other half for histology).

In our experience, many examinations described here for the rat can also be performed in dog toxicology studies, the major limitation for later interpretation being the small number of animals per group in the regulatory dog studies.

Any detailed examination on intrauterine development, sex differentiation, and pubertal development is beyond the scope of this discussion. Highly specialised methods need to be applied, and there is usually no indication for such effects from the early regulatory toxicology studies (28 days in rats and dogs). The special aspects of toxicological findings with xenobiotics have been reviewed for the female reproductive tract

by Creasy (1999) and for the male reproductive tract by Newbold (1999).

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I.O.6.1.4
FSH Receptor Binding and Effect on FSH Receptors

PURPOSE AND RATIONALE

This evaluation can be performed a s a single-dose in vitro test using e.g. cell membranes containing FSH receptor, or as an ex vivo test measuring the concentration of FSH receptors in organ tissue of treated animals, after a suitable treatment period of at least seven days. The application of ex-vivo testing for gonadal tissue is an interesting extension of repeated dose studies where changes in the target tissue need to be quantitated. The method is applicable to studies of ovarian function.

PROCEDURE

Membrane preparations from bovine testes are used according to the methods of Cheng (1975) and Andersen (1983). Fresh bovine testes or testes from rats weighing 220–280 g are decapsulated and rinsed with cold 0.025 M Tris-HCl buffer at pH 7.2, containing 0.3 M sucrose, and then minced and homogenized with a Polytron homogenizer at maximum speed for 30 sec at a concentration of 5 ml buffer per g of tissue. The homogenate is first filtered through 4 layers, and the filtrate is again filtered through 8 layers of cheesecloth. The filtrate is then centrifuged at 12 000 g for 30 min at 4 °C. The pellet is discarded and the supernatant is further centrifuged at 100 000 g for 1 h at 4 °C. The supernatant is discarded and the pellet resuspended in cold 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM $MgCl_2$, at a concentration of 1 ml buffer per g of the original weight of the testis. The isolated membranes are stored at –70 °C in aliquots of 10 ml per vial until use.

For assays, 12/75 mm glass disposable tubes are used. To each tube, 0.2 ml of 0.025 M Tris-HCl buffer at pH 7.2, containing 10 mM $MgCl_2$ and 0.1 % BSA, 0.1 ml of standard FSH or unknown samples in the same buffer, 0.1 ml of ^{125}I-hFSH tracer labeled by the lactoperoxidase method (50 000 cpm, approximately 2 ng), and finally 0.1 ml of plasma membrane receptors of appropriate dilution (approximately 1–2 mg/ml) are added to reach a final volume of 500 µl per tube. The tubes are then shaken vigorously and incubated at room temperature for 20 h. Following incubation, the reaction is stopped by adding 3.0 ml of cold 0.025 M Tris-HCl buffer containing 0.1 % BSA. After centrifugation at 4 000 rpm for 30 min, the supernatant is drained. The pellet remaining at the bottom of the tube is counted in an automatic gamma counter.

EVALUATION

Specific binding to the testicular receptor (pellet), and nonspecific binding (nondisplacable by 1000-fold excess of unlabeled hFSH) arec determined. The specific binding of 125I-hFSH in the presence of a range of standard hFSH concentrations is used for calculating a standard curve, and the sample concentrations are calculated from the FSH receptor standard curve, using suitable computer programs.

MODIFICATIONS OF THE METHOD

For the use in safety pharmacology, selection of a specific method for determination of the FSH receptor concentration is not important, however biological validation of the method is very important. In reference experiments, the effect of a preceding treatment on the concentration of FSH receptors should be detected, e.g. reduction in receptor concentration after inhibition of gonadotropin secretion.

CRITICAL ASSESSMENT OF THE METHOD

Significant differences between biological activity and receptor binding activity of FSH preparations have been found by Marana et al. (1979), Zaidi et al. (1981),

Foulds and Robertson (1983), Burgon et al. (1993). This is attributed to the assay principle of measuring binding activity, but not subsequent intracellular signaling. Several receptor binding assay procedures have been described, e.g. Cheng (1975), Andersen (1983) using bovine testes; Reichert (1976) using rat testes tubule tissue.

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I.O.7 Hypothalamic-Pituitary-Adrenal System

GENERAL CONSIDERATIONS

The methods described here are applied for Supplemental Safety Pharmacology Studies (ICH S7A), if the test substance has shown indications of effects on the hypothalamic-pituitary-adrenal system in the preceding pharmacology studies. The in vitro studies are generally performed with several increasing doses to investigate concentration-effect relationships. The preliminary information from these in vitro studies is then compared with the biological information available from in vivo for studies, to assess the need for additional animal studies based on repeated dose administration of the test compound.

In the safety pharmacology protocol, the interaction of multiple endocrine systems in test animals is addressed. As discussed by Harvey (1996) and by Harvey and Everett (2003), effects on adrenocortical function are frequently found in toxicology studies, sometimes related to enzyme induction and effects on steroid biosynthesis (Loose et al. 1983, Nebert and Russell 2002, Weber et al. 1993). Test procedures in animals are required when there is a reason for concern. Frequently however the effects observed are due to stress rather than specific interaction with the target organ, and may involve effects on catecholamine release from the adrenal medulla (Tucker 1996). Recently, much new evidence has been accumulated from the testing of industrial chemicals with effects on adrenal steroid biosynthesis (Harvey and Johnson 2002).

I.O.7.1 Adrenal Steroid Excretion in Rats in a Repeated Dose Study

PURPOSE AND RATIONALE

This investigation is performed in adult male and female rats. It may be applied in the context of a toxicology study as an added investigation, or performed as a separate study to detect changes in adrenal func-

tion during treatment. Assessment of the change in adrenal function is performed in an minimal invasive procedure, by measuring the excretion of adrenal steroid hormones in the urine during the night period. Successful application of the method critically depends on suitable housing conditions for the animals, the animal house should have a controlled light dark cycle and well controlled air-conditioning. Animals need to be adapted to metabolism cages for collection of urine. This can be achieved by transferring the animals for each night period to metabolism cages, with the final urine collection at the end of the treatment period under conditions of established adaptation.

PROCEDURE

Male and female rats (preferably of 200 gram initial body weight) are kept in their light and temperature controlled animal house, under standard conditions, on a pelleted rat food, with free access to drinking water. Starting two days after the beginning of treatment, the animals are transferred to metabolism cages for the night period (2 rats per cage to avoid isolation stress). Urine is collected for the night period during the final three days of treatment, and stored deep-frozen for the analysis of adrenal steroid hormones.

In a toxicology study, groups of animals are randomly assigned to one control group and three treatment groups (three increasing dose levels). Treatment is administered in the morning, and animals are transferred to their metabolism cages in the evening.

For a more specific evaluation, treatment should be administered in the evening, before the onset of the dark period. This ensures that effects on adrenal steroid secretion of limited duration and at lower dose levels are also detected in the urine samples. For each cage and collection night, the volume of the urine from to rats collected during the night is recorded.

At the end of the treatment period, the animals are killed by decapitation, preferably 24 hours after the last treatment injection. Body weight and organ weights are recorded, and organs of interest are dissected out at autopsy for histological examination and/or measurement of hormone contents.

In male animals, weight of the adrenals, testes, seminal vesicles and ventral prostate is recorded (because the effects on steroid biosynthesis frequently involve both the adrenal and gonadal system). In female animals, weight of the adrenals, ovaries and uterus is recorded. As an added investigation, the hypothalamus of the animals may be dissected out and shock frozen immediately for assay of corticotropin releasing hormone (CRH), and the adenohypophysis (pituitary) is dissected out for assay of corticotropin content (ACTH) by radioimmunoassay. The pituitary tissue is stored frozen at –20 centigrade until the assay of hormone content can be performed.

During a treatment period of seven days or up 28 days (toxicology study), the excretion of corticosterone and of aldosterone during the night period is monitored on the last three days of treatment, or on two consecutive days of each week (toxicology study). This approach ensures a check on consistency of changes in adrenal steroid excretion.

EVALUATION

The following parameters are evaluated at the end of the study period by calculation of the group means and standard deviation, applying suitable methods for analysis of variance, and tests for significance of differences between groups. In most instances, the significance of differences for treatment groups versus control groups is calculated at the 95 per cent level by analysis of variance and a distribution-free rank test. Here is a list of the important parameters which are to be calculated in the evaluation of studies on adrenocortical function:

- Excretion of corticosterone, in the urine of at least 6 rats but control and treatment groups (pooled urine of 2 rats per metabolism cage).
- Excretion of aldosterone in the same samples.
- Weight of adrenal glands at autopsy.
- Tissue content of corticosterone and aldosterone, expressed as total content per gland (ng/ adrenal), and as relative concentration (ng/g adrenal tissue).

Supplementary information about the reproductive system is obtained in the same study to investigate the possibility of interference by the test Substance with the secretion and synthesis of steroid hormones of the male and female gonads.

- Male rats: weight of testes, seminal vesicles and ventral prostate.
- Female rats: weight of the ovaries and uterus.

Optional parameters of hypothalamic-pituitary function to be determined are the hypothalamic content of CRH, and the anterior pituitary content of ACTH (group means and standard deviation). Final data may be calculated as hormone content per mg or gram of organ tissue (pituitary hormone contents), or as hormone content per tissue equivalent (hypothalamic fragments).

MODIFICATIONS OF THE METHOD

It is important to realise that this test described for rats is also performed in dogs under special conditions, since a similar methodology can be applied to the dog. The principal adrenal steroid in rats is corticosterone whereas in the dog cortisol is secreted. Collection of urine samples during the night under minimum invasive conditions has been successfully performed in dogs.

The core of this method is the assessment of adrenal steroid excretion during minimum invasive conditions, during repeated-dose administration of the test substance.

Modifications of the method are the duration of treatment (not less than seven days), timing of treatment injections (conventional regimen in the morning, or injections late in the evening shortly before starting the urine collection period after transfers to metabolism cages).

Adrenal Steroid Content

Determination of adrenal gland steroid content may be included but is of limited value. Determination of serum concentrations of corticosterone and aldosterone may be included during up week 3 for studies of 4 week-duration, but needs to be performed under well controlled conditions which reduce stress to a minimum.

Serum Corticosterone Measurement

For serum corticosterone, preferably a stimulation test with ACTH after low dose dexamethasone blockage of spontaneous secretion may be included. Rats are injected with 0.5 mg/kg oft dexamethasone 18 hours before the stimulation test. The test is then performed by a injection of synthetic ACTH (1-24), which is readily available as a clinical diagnostic tool. The clinical test procedures have been reviewed (Weiss and Patel 2002). Extended investigations are the assessment of effects on other steroid secreting organs (gonadal system), and the assessment of hypothalamic-pituitary changes at the end of the treatment period. Determination of pituitary ACTH content may be particularly useful in case of inhibitory effects on adrenal steroid biosynthesis.

CRITICAL ASSESSMENT OF THE METHOD

Changes in the secretion of adrenal steroids are often induced by stress effects, which needs to be differentiated from drug effects on the adrenals steroid secretion (Harvey and Everett 2003). This is the reason for selecting the minimum invasive procedure of monitoring adrenal steroid excretion in urine, which avoids blood sampling and reduces the stress on animals by adequate adaptation periods for the night time sampling procedure.

It is always important to compare the effect of well established drugs which affect adrenal cortical steroid secretion and synthesis (comparator drugs). The best reference compound for adrenal steroid excretion is synthetic corticotropin, which readily induces adrenal hyperplasia at high doses or when given by sustained subcutaneous infusion (osmotic minipumps). There are many synthetics steroids with some adrenocortical activity. The problem as with other hormonal evaluations is the pronounced circadian rhythm of corticosterone secretion and excretion in the urine (Miki and Sudo 1996, Hilfenhaus et al. 1980). To avoid these difficulties of sampling under stress conditions, in a system constantly changing and responding immediately to any stress, the night period is the most adequate selection of minimum stress and interference especially in well-controlled animal houses.

The equivalent of cortisol secreted by monkeys can also be detected by a the non-invasive procedure of urine sampling (Pal 1979), many studies of this kind are available for humans. Blood sampling needs to be done during specific times of the day, for advance stimulation tests endocrine essays have been developing based on a temporary suppression of the pituitary adrenal response by pre-treatment with dexamethasone. Such tests may be included during a study of 28 days duration, but should be done at least seven days before the end of the study in order not to interfere with the final hormonal evaluation. Stimulation tests with synthetic corticotropin may be built into the study. For the evaluation of stimulatory drug effects, there is a non-invasive procedure for corticosterone secretion which could the same time can be applied to electrolyte balance studies (Haack et al. 1978). During the night period (14 or 16 hours), the animals are placed in metabolism cages for urine collection, the amount of corticosterone and aldosterone excreted during this period is determined in the urine samples, together with the electrolyte concentration. The principle is derived from studies on diuretics (Hilfenhaus 1977).

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I.O.7.2 Corticosterone Secretion in Dexamethasone Blocked Rats

PURPOSE AND RATIONALE

Corticotropin activity can be measured by the increase of corticosterone in venous blood of hypophysectomized or dexamethasone-blocked rats, as a single-dose test when receptor affinity for ACTH receptor is found with a test compound. The test can be used to measure time-response curves of corticotropin preparations, and compounds with a adrenal stimulating activity (Vogel 1969a,b). The use of hypophysectomized rats for evaluation of the pituitary response to compounds with corticotropin activity has been entirely replaced by the dexamethasone-blocked model.

In the context of repeated dose studies for endocrine safety evaluation, a modification of this test is built into the procedure, as described above (A4-1).

PROCEDURE

For the ACTH assay Male Sprague-Dawley rats weighing 150–200 are injected subcutaneously 24 h and 1 h prior to subcutaneous injection of the ACTH-preparation or the standard with 5 mg/kg dexamethasone in oily solution. Eight rats are used for each dose of test preparation or standard. Various time intervals after ACTH injection, the rats are anesthetized with 60 mg/kg pentobarbital i.p. and blood is withdrawn by cardiac puncture. One ml plasma is diluted with 2 ml distilled water and extracted (washed) with 5 ml petrolether to remove the lipids. The petrol ether is discarded. Two ml of the water layer are extracted twice with 5 ml methylene chloride by vigorous shaking for 15 min. The methylene chloride phase is separated by centrifugation. Both methylene chloride extracts are unified and shaken with 1 ml ice-cold 0.1 N NaOH. The water phase is immediately removed and the methylene chloride extracts dried by addition of dry sodium sulfate. An aliquot of 5 ml of the methylene chloride extract is mixed with 5 ml of the fluorescence reagent (7 parts concentrated sulfuric acid, 3 parts 96 % ethanol, v/v). After vigorous shaking, the methylene chloride phase is removed and fluorescence is measured with primary filters of 436 mm and secondary filters of 530–545 mm. For calibration, concentrations of 0, 20, 50, 100, and 250 mg/ml corticosterone are treated identically and measured in each assay. The synthetic ACTH(1-24) is used as the reference standard, this compound has full biological activity (Schuler et al. 1963) when compared with natural ACTH(1-39).

EVALUATION

Using 3 doses of test compound and standard, activity ratios with confidence limits can be determined after each time interval with the 3 + 3 point assay giving evidence for the duration of action (Vogel 1969a,b).

MODIFICATIONS OF THE METHOD

In contemporary methods, the assay of corticosterone is performed by immunoassay in unextracted serum samples or in serum and tissue extracts by HPLC. In the classical methods, fluorometry was applied. Pekkarinen (1965) used fluorometric corticosteroid

determinations in guinea pigs resulting in highly deviating activity ratios of synthetic and commercial corticotropins as compared with the international working standard of ACTH.

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I.0.7.3 Corticosteroid Release from Adrenal Cell Suspensions in vitro

PURPOSE AND RATIONALE

An in vitro assay of corticotropin was described by Saffran and Schally (1955). This test has been modified by Van der Vies (1957) and used by several authors (Staehelin et al. 1965; Vogel 1969).

PROCEDURE

There are many modifications of this method, based on incubation of small fragments of adrenal glands, or on the use of dispersed adrenal cells. Cells are incubated in a physiological buffer. The final solution is gassed with a mixture of 95 % O_2 and 5 % CO_2 for 10 min. As with similar methods – for example for pituitary cells or dispersed Leydig cells – there is a pre-incubation period, followed by the test incubation with corticotropin or test compound. The vessels are incubated and gassed with a mixture of 95 % O_2 and 5 % CO_2 at 38 °C under continuous shaking for 2 h. The initial analytical procedure for corticosterone has been replaced by measuring aliquots in extracts of incubation medium by HPLC or directly in the media by specific immunoassays.

EVALUATION

The concentrations in the incubation flasks are compared (duplicates or triplicates), potency ratios with confidence limits may be calculated from a 2 + 2 design or from dose-response curves.

MODIFICATIONS OF THE METHOD

Corticosterone is now conveniently determined by RIA or HPLC, and dispersed adrenal cells are incubated instead of adrenal tissue fragments.

CRITICAL ASSESSMENT OF THE METHOD

The method is suitable to detect changes in steroid in biosynthesis induced by test compounds. In endocrine safety evaluation, it is however recommended to apply measurement of corticosterone and adrenal steroid in metabolites in the tissue as an ex-vivo investigation to test material from animals previously treated in a repeated dose protocol.

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I.O.7.4 ACTH Receptor Affinity

PURPOSE AND RATIONALE

For all new compounds which have some structural similarity or relation to the corticoid scaffold. There are now many such test systems which measure the initial membrane binding to the ACTH receptor, and to define the initial steps of hormone-related activation. However the binding affinity does not necessarily imply biological activation e.g. of adrenal cortical cells. Therefore, such tests always need to be followed up by a in-vitro testing on adrenal glands or dispersed adrenal cells.

Corticotropin (ACTH) receptors have been used as the test preparations for comparison of the binding affinities of adrenocorticotropin peptides using a cloned mouse adrenocorticotropin receptor expressed in a stably transfected HeLa cell line (Kapas et al. 1996). Such assays measure the initial membrane binding but not strictly the biological activation e.g. of adrenal cells or melanocytes.

PROCEDURE

Hela cells are seeded into 12-well culture plates at a density of 10^6 cells/well. On the second day of culture, the cells are washed as follows: 2 × in 1 ml of ice-cold 0.9 % NaCl, 1 × in 1 ml of ice-cold glycine (50 mM glycine, 100 mM NaCl, pH 3.0) for 5 min, 2 × in 0.5 ml of ice-cold 0.9 % NaCl. Cells are then incubated for 60 min at 20 °C with increasing concentrations of nonradioactive ACTH or various ACTH analogues and the reactions initiated on the addition of [^{125}I-iodotyrosyl^{23}]ACTH[1-39] (2000 Ci/mmol; final concentration 0.1 pmol/liter) in DMEM. At the end of the incubation, the medium is removed and the cells washed three times with 0.9 % NaCl and then dissolved in 0.5 M NaOH/0.4 % sodium deoxycholate. Each point is determined in triplicate. Specific binding is determined as the difference of total radioactivity bound minus non-specific binding radioactivity determined in the presence of an excess of 10^{-5} M nonradioactive ACTH.

EVALUATION

Binding parameters are calculated from computer programs, one example being the ligand program of Munson and Rodbard. Estimates of binding affinity are then compared with the binding affinity of natural corticotropin, corticotropin analogues, and more importantly with the drug concentrations of the compound to be evaluated reached in the toxicology studies, or similar pharmacokinetic studies (level of exposure).

MODIFICATIONS OF THE METHOD

There are many different modifications of the method, early technology used membrane preparations, modern methods use solubilized receptors and receptor of constructs which enable fast signal detection.

Penhoat et al. (1993) reported the identification and characterization of corticotropin receptors in bovine and human adrenals, using cultured bovine adrenal fasciculata reticular cells and crude plasma membrane fractions. Lebrethon et al. (1994), Penhoat et al. (1995) studied the regulation of ACTH receptor mRNA and binding sites by ACTH and angiotensin II in cultured human and bovine adrenal fasciculata cells. Picard-Hagen et al. (1997) found, that glucocorticoids enhance corticotropin receptor mRNA levels in ovine adrenocortical cells, as an equivalent of receptor regulation by circulating glucocorticoid concentrations.

Naville et al. (1996, 1997) developed a stable expression model in order to characterize the human ACTH receptor by binding studies and functional coupling to adenylate cyclase.

Schioth et al. (1996) described the pharmacological distinction of the ACTH receptor from other melanocortin receptors in the mouse adrenocortical cell line Y1. Melanocortin receptors do not have a binding epitope for ACTH beyond the sequence of alpha-MSH (Schioth et al. 1997).

CRITICAL ASSESSMENT OF THE METHOD

This test is to be performed if the specific pharmacology of the compound indicates the potential for interaction with the ACTH receptor. It is used to prove that the compound achieves highly specific receptor targeting (Behrens and Ramachandran 1981, Oelofsen and Ramachandran 1983) or modulates the receptor density by changing peripheral glucocorticoid it concentrations. For specific compounds, the extent of safety pharmacology studies to be performed with the product is reduced (Stocco and Clark 1996, Abdel-Malek 2001, Vaisse et al. 2000).

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I.0.7.5 ACTH Secretion and Tissue Content

PURPOSE AND RATIONALE

Numerous assay procedures have been developed for the measurement of ACTH concentrations in clinical diagnostics (eg in tumour related secretion, for ectopic hormone production, Krieger 1983, Genazzani 1975, Ganong 1974). This principle can also be applied to safety pharmacology studies, when a reduction in glucocorticoids secretion leads to up regulation of ACTH secretion, by removing the inhibitory feedback. This is the primary diagnostic application in repeated dose studies with test compounds which may interfere with adrenal steroid biosynthesis.

PROCEDURE

Several methods have been described, starting with radio-immunoassay procedures (Rees 1971, Kao 1979, Krieger 1975), and proceeding to immunoradiometric methods (Hodgkinson 1984, White 1987, Zahradnik 1989, Raff 1989, Gibson 1989, Fukata 1989).

EVALUATION

Hormone concentrations in serum extracts or in unextracted serum and ACTH concentrations in dilutions of pituitary homogenate are calculated from standard curves using an appropriate reference standard for pituitary corticotropin (for the biologically active sequence the ACTH analogue ACTH 1-24 may be used). Sample data are calculated as ng ACTH/ml serum, as ACTH per pituitary gland, or per pituitary weight (mg). Group means are calculated and the significance of differences is assessed by the appropriate statistical methods.

MODIFICATIONS OF THE METHOD

For practical application, a method should be selected that can be applied to unextracted serum, provided that ACTH degradation is prevented by adding an enzyme inhibitor at the time of blood collection.

CRITICAL ASSESSMENT OF THE METHOD

A very critical item has been a tendency of ACTH to fluctuate widely and rapidly under conditions of stress (Meeran 1989, Lambert 1985, Goverde 1989). This is especially a problem in studies with dogs, where the experiments need to be performed under stress-free conditions and even then there will be the difficulty of rapidly fluctuating concentrations.

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I.0.7.6
Adrenal Steroid Activity

GENERAL CONSIDERATIONS

In the endocrine safety pharmacology for the hypothalamic pituitary adrenal system, the levels to be examined are:

1. CRH activity, by determination of the hypothalamic peptide content – the information obtained by this measurement is usually limited and and will be controlled by the feedback action of pituitary hormone release or inhibition and adrenal steroid secretion
2. ACTH secretion and synthesis, which may be modified by compounds affecting pituitary gland – this information is valuable and reflects the level of secretion of glucocorticoids over several days of treatment, and
3. Adrenal corticoid activity which is in general known from the of pharmacological profiling of the test compounds for their glucocorticoid and mineralocorticoid activity in single dose in vitro tests.

Certainly, such compounds might directly affect the target organ functions subserved by the adrenal steroids, and their activities would be detected in the classical in-vitro and in-vivo bioassays for adrenocortical activity. However, it is assumed that indications for such activity have already been found in the pharmacological profiling, and have given a reason for concern which led to the decision of performing an endocrine safety evaluation (Sullivan and Kinter 1995). Therefore, the direct assessment of steroid activity will not be discussed here. At the biochemical level of investigation, signalling mechanisms have often also been investigated (Wittliff and Raffelsberger 1995), the purpose of the endocrine safety pharmacology is then to assess the relevance of these in-vitro findings by and their manifestation of biological effects in the intact animal test, by repeated-dose administration.

PURPOSE AND RATIONALE

There are numerous classical bioassay as for the glucocorticoid activity and mineralocorticoid activity of steroidal and nonsteroidal compounds. For the scope of endocrine safety pharmacology however, it may be assumed that in the pharmacology procedures used for this selection of these compounds, modern methods were applied such as receptors screening and screening for enzyme activities (see reviews by Barnes and Adcock 1993, Berger et al. 1992, Jensen 1996, Lieberman 2001).

CRITICAL ASSESSMENT OF THE METHODS

Direct tests for adrenal steroid activity are at the entry level of endocrine safety pharmacology, such activities are usually expressed by a feedback regulation at the pituitary level, changing the ACTH response, or by one of the multiple biological effects of the adrenal steroid hormones ranging from anti-inflammatory to immunosuppressive activity (Schimmer and Parker 1995).

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I.0.8
Hypothalamic-Pituitary-Thyroid Function

I.0.8.1
Pituitary Thyroid Evaluation in Rats in a Repeated-Dose Study

PURPOSE AND RATIONALE

Inhibitory effects on pituitary-thyroid function are readily detected in toxicology studies by changes in thyroid weight and histology of the thyroid and

pituitary gland. Their interpretation regarding relevance for human however is a difficult, because in many cases there are underlying mechanisms such as enzyme induction which were subsequently not found in clinical study (Capen 1998, 1999). When effects on the thyroid are suspected, the inclusion of serum concentrations of TSH, T3 and T4 is recommended, duration of treatment should be 28 days for reliable detection of changes. A treatment period of 15 days in intact male rats has been recommended based on testing of endocrine active compounds (EAC), comparing daily treatment by a intraperitoneal injection or by gavage (O'Connor et al. 2002). Hormones measured were TSH, T3 and T4. The endocrine safety evaluation may use the 28 days toxicology study for orientation, using findings in pituitary histology as the relevant and reliable source of information. The relevant parameters can be readily built into the study design, based on basal blood sampling at the end of the treatment period, and determination of the pituitary TSH and prolactin content (using one half pituitary for the hormone assay). An increase in the serum concentration of TSH is the most frequent relevant finding, a stimulation test can be included for the secretion of TSH and prolactin, by injection of thyroid stimulating hormone (TRH). Changes in biosynthesis of thyroid hormones due to enzyme inhibition are usually detected by the rise in TSH. Measurements of free T3 and free T4 do not contribute to diagnostic relevance in rats. The uptake of radioiodine (131-I) into the thyroid of treated animals is a very useful additional test.

PROCEDURE

Separate groups of 5–10 male and female Sprague-Dawley rats weighing 100 g are used (juvenile rats). For some compounds, rats of 200 g body weight may be required (adult rats). They are treated daily over a period of 6–12 days with the test compound by the intended route (usually orally by gavage or by subcutaneous injections, sometimes by exposure to test compounds in the feed). For toxicological studies, a treatment period of 4 weeks is preferable. A similar protocol is applied in chronic toxicity studies in rats and dogs. On the day after the last application (alternatively 2 hours after the last dosing), the animals are sacrificed, weighed, and the following parameters determined: Pituitary weight and thyroid weight, pituitary content of thyrotropin (TSH and prolactin (PRL), TRH content in hypothalamic tissue (optional), serum concentrations of TSH, T3 and T4. Determination of free thyroid hormones does not contribute to diagnostic evidence, and is not recommended. Samples should always be taken at the same time of the day, preferably in the morning, to avoid artefacts by diurnal fluctuation of thyroid hormones (Weeke 1973, Brabant et al. 1990).

There are nowadays numerous possibilities for additional exploration, using ex-vivo tissue samples. The classical approach is histology of the anterior pituitary gland (Tucker 1999), this can be extended to histomorphometry, in situ hybridization for enzymes, and gene expression profiling. In general however, pituitary hormone contents are sufficiently informative, when pituitary TSH content and serum TSH concentration are determined.

EVALUATION

For each parameter, means and standard error calculated, an analysis of variance is performed and the appropriate tests of significance are applied. The mean values of each parameter of the treated groups are compared with the values of the vehicle control group. It is important to compare the results with reference to their use for the rat strain commonly-used in the Laboratory. For many studies it may be advisable to include groups treated with reference compounds of established endocrine activity.

They are many published toxicology studies with xenobiotics, which may serve as examples, and facilitate the selection of reference compounds for defined mechanisms of action (Capen and Martin 1989).

MODIFICATIONS OF THE METHOD

The main modifications are selection of the age of the animals, duration of treatment, and time of autopsy related to the last drug application.

CRITICAL ASSESSMENT OF THE METHOD

Several reference compounds have been explored in the OECD endocrine disrupters programme, phenobarital and propylthiouracil being the most frequently studied (O'Connor et al. 2002, Mellert et al. 2003, Cooke et al. 2004, Cho et al. 2003). The interpretation of positive findings in the rat concerning inhibition of thyroid function (which has been frequently found) presents considerable difficulty and there are many cases where such findings in rats have been shown to be of no relevance for the human (Akhtar et al. 1996, Waritz et al. 1996, Capen 1998, Poirier et al. 1999). Many drugs that act on thyroid function in rats share a mechanism of action based the induction of microsomal enzymes, which in turn may enhance the biliary excretion of thyroid hormones (Vansell and Klaassen 2001).

The response of rats of different strains with regard to the TSH response when treated with thyroid

inhibitors appears to be in a similar range (Fail et al. 1999), the commonly used Sprague-Dawley and Wistar rat also share a similar response of thyroid hormone inhibition as found with propylthiouracil. The mechanism of thyroid inhibition by interference with iodine uptake is frequently found, the reference component being perchlorate (Kyung et al. 2002). A separate mechanistic study is required to clarify this question.

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I.O.8.2 TRH Radio-Immunoassay

PURPOSE AND RATIONALE

In repeated dose studies, hypothalamic tissue is readily available at autopsy of rats and can be used for the determination of hypothalamic hormone contents. it is important to take samples are rapidly and stop enzyme

inactivation in the tissue immediately (Griffiths et al. 1975, Jeffcoate and Whiter 1976, Eskay et al. 1976) by the addition of suitable enzyme inhibitors. The hypothalamic TRH content has been measured by numerous groups (Mitsuma et al. 1976, Montoya et al. 1975, Pease et al. 1980, Utsumi et al. 1975).

PROCEDURE

The TRH radio-immunoassay was developed as a double antibody assay, with modifications mainly depending on the antiserum used, and on the conditions of incubation. Addition of an enzyme inhibitor to the assay tubes is essential to avoid degradation of TRH in samples and of radioiodinated TRH.

EVALUATION

As with other radioimmunoassays, sample concentrations are calculated from a or standard curve of synthetic TRH. Using various doses of standard and test preparation, dose-response curve scan be established allowing calculation of sample concentrations, or potency ratios for tissue extracts tested at several concentrations.

MODIFICATIONS OF THE METHOD

Modifications of this assay have been applied to the measurement in serum, in hypothalamic tissue homogenates and extracts, and in human urine.

CRITICAL ASSESSMENT OF THE METHOD

The method is of interest because the results can be correlated with measurements of pituitary TSH and prolactin secretion, and tissue content of these hormones. In a number of studies it has been shown, that changes in the secretion of thyroid hormone in the treated animals as well as administration of hormonally active compounds can change the hypothalamic TRH content in characteristic manner (Emerson and Utiger 1975, Schwinn et al. 1976, Simpkins et al. 1976).

Synthetic TRH is used as a diagnostic tool in in animals and in the human. There is a response of the secretion of TSH as well as of prolactin (van Landehem and van de Wiel 1979, Andreassen et al. 1980). Pituitary cells in culture respond readily to adding of TRH with the secretion both of TSH and prolactin (Queen et al. 1975, Haugh and Gautvik 1976, Tal et al. 1978, Snyder et al. 1978)

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I.O.8.3 TSH Receptor Assay and TSH RIA

GENERAL CONSIDERATIONS

Traditionally, TSH activity was determined by bioassays. There is now sufficient evidence for the correlation of biological activity and results of immunoassays. Reference standards are available for rat TSH, dog TSH and human TSH (DSL Diagnostic Systems 2005). Immunometric measurements of TSH are available (Utiger 1979, Spencer 1994, Meinhold et al. 1994), for rat radio-immunoassay methods see section I.O.4.3 of this Chapter. Large scale synthesis of recombinant human thyrotropin has been reported (Cole et al. 1993, Hussain et al. 1996). The role of

the thyrotropin receptor has been reviewed by Vassart and Dumont (1992). Castagiola et al. (1992) described a binding assay for thyrotropin receptor autoantibodies using the recombinant receptor protein. A brain-derived TSH receptor has been cloned and expressed (Bockmann et al. 1997). Binding characteristics of antibodies to the TSH receptor were described by Oda et al. (1998). the diagnostic procedures used in clinical assessment of thyroid function have been reviewed (Spencer 1994, Spencer 2004), this internet site provides an extensive overview of the analytical methods for assessment of thyroid function. An internet resource for hypothalamic-pituitary-thyroid physiology is available (Mariotti 2002).

Application of the TSH receptor assay is recommended for assessment of the functional state of the pituitary gland after repeated dose treatment, and serum TSH radio-immunoassay is important for the detection of inhibition of thyroid hormone synthesis by test compounds.

PURPOSE AND RATIONALE

The TSH radioimmunoassays procedures have replaced other methods for the quantitative assessment of changes in pituitary responsiveness after repeated dose treatment. test compounds which enhance the secretion of thyroid hormones lead to a suppression of the serum TSH concentrations, a clinical example being hyperthyroidism (Connors et al. 1981, Pekary et al. 1980). On the other hand, a reduction in circulating concentrations of thyroid hormones (T3 and T4) will release the pituitary gland from feedback inhibition, and the serum concentration of TSH may arise in an exponential manner.

They are now many modifications of different sensitivity and limit of detection (Utiger 1979, Spencer 2004). In the endocrine safety pharmacology in rats and dogs, the TRH test injection with measurement of the serum TSH response is an established tool for assessment of changes at the level of thyroid hormone secretion, with consecutive modification of the pituitary TSH and prolactin response.

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I.O.8.4 Iodine Uptake and Release in Rats

PURPOSE AND RATIONALE

This method may be used in the context of repeated dose treatment, on satellite groups of rats which show changes in thyroid uptake of labelled iodine. It may be sufficient to determine radio-iodine uptake in controls and in the high-dose group.

A modification of this method was previously used for evaluation of thyrostatic drugs, and responds to inhibition of the iodine transport mechanism in the thyroid gland.

For conventional drug evaluation, all rats were pre-treated with the same dose of radio-iodine (pre-loading). The release of 131-I from the pre-loaded thyroid in rats is inhibited by treatment with thyroxin

(Wolff 1951), and the degree of inhibition is related to the dose administered (Perry 1951). This phenomenon was used to compare thyroid hormone derivatives with the standard thyroxine. It is superseded by direct assay of thyroid hormones by radioimmunoassay and HPLC, and by measuring feedback inhibition directly via the decrease in serum TSH following administration of high doses of triiodothyronine and thyroxine.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g are fed a commercial laboratory with low concentrations of thyroid hormones, containing constant amount of unlabelled iodine. The animals are treated for at least seven days with the test compound to be evaluated, at a pharmacologically active dose determined by a previous biological studies. On the morning of the test day, rats are injected with a test dose of 131-I (intravenously or intraperitoneally), and the concentration of radioactivity in the thyroid glands is measured after 1–4 hours. The blood concentration of 131-I at these time points is measured, and the tissue to blood ratios are calculated for individual animals.

EVALUATION

Statistical evaluation depends on the experimental design for such studies with pre-labelling of the thyroid gland and release by thyroid stimulating drugs, or single dose uptake of radio-iodine into the pituitary of treated rats, for example after several days of exposure to a thyrostatic drug.

From the dose-response curves, potency ratios and confidence limits are calculated. This approach may be modified for short term uptake of ^{131}I or ^{125}I as a parameter of thyroid peroxidase inhibition by antithyroid drugs.

MODIFICATIONS OF THE METHOD

The main modification for practical use in endocrine safety pharmacology is the application of this method to satellite groups of animals in a repeated dose study. Most pelleted animal feeds nowadays contain a standard amount of iodine (it may be useful to check the specification). Therefore, the addition of potassium iodine to the drinking water is not required. In this satellite groups, the animals are injected with a standard dose of radio-iodine after the last treatment, and the dose of radioactivity accumulated in the thyroid glands after a standard time interval (e.g. two hours) is determined and evaluated.

CRITICAL ASSESSMENT OF THE METHOD

Determination of 131-I in the thyroid of rats at the end of a treatment period is a useful additional test, which can be applied to satellite groups, preferably control and high dose. This is a sensitive test which readily detects inhibition of thyroid hormone synthesis e.g. by propylthiouracil.

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I.O.8.5 Inhibition of Iodine Uptake into the Thyroid of Rats

PURPOSE AND RATIONALE

Propyl-thiouracil (PTU) and a wide spectrum of drugs may inhibit thyroid hormone synthesis. Some of these drugs can be used for treatment of thyrotoxicosis. As consequence of thyroid peroxidase inhibition, the iodine uptake and content of the thyroid is decreased. This phenomenon is dose-dependent and may occur at lower doses than those increasing thyroid weight in rats (McGinty and Bywater 1945). The historical parameter of iodine content was replaced by measuring uptake and release of radio-iodine (131-I).

PROCEDURE

Groups of male Wistar rats age 26–28 days, weighing 40–45 g, are placed into metabolism cages. They are fed normal diet, and potassium iodide is added to the drinking water, in order to minimise differences between thyroid status of the animals during treatment. Test compounds may be injected daily, or added to the feed (mainly for testing of industrial chemicals, in this procedure, the test compounds and the reference standard are added in various concentrations to the diet over a period of ten days). The amount of compound which each rat receives is calculated from the total food consumption over 10 days and expressed as milligram ingested daily per kilogram of body weight. After 10 days of treatment, the rats are sacrificed and the thy-

roids dissected free from adjacent tissue and capsule. The thyroid is weighed and iodine content determined (conventional procedure). In daily doses between 0.1 and 10.0 mg/kg, thiouracil decreases the iodine content of the thyroid dose-dependent. Definitely higher doses are necessary to to increase thyroid weight.

When the uptake of a standard dose of radio-iodine is tested at the end of the treatment period, the radioactivity found in the thyroid gland is used as the parameter instead of the iodine content.

EVALUATION

Dose-response curves of test compounds are compared with those of the reference standard for calculation of potency ratios with confidence limits.

MODIFICATIONS OF THE METHOD

Walker and Levy (1989) used implantable pellets of propylthiouracil to induce thyroid dysfunction in rats. In the modification based on uptake of labelled iodine, as standard dose of radio-iodine 131-I is injected in each animal, and the amount of radioactivity in the thyroid gland is determined in a gamma counter.

Release of labelled iodine may be stimulated by injection of TRH (TSH releasing hormone, protirelin).

CRITICAL ASSESSMENT OF THE METHOD

This test is valuable as single-dose uptake test, and maybe performed when changes in the serum concentrations of T3 and T4 if questionable relevance have been found, without that corresponding increase or decrease in the serum TSH concentrations. It may help to differentiate changes which occur or predominantly at the level of thyroid hormone synthesis.

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I.O.8.6 Thyroid Hormone Assays (T3 and T4)

PURPOSE AND RATIONALE

Measurement of thyroid hormone concentrations in the serum has become an important final step in the endocrine safety pharmacology procedures (Capen 1992, O'Connor et al. 2002). Basal concentrations are measured in serum samples in the morning before treatment (to avoid artefacts by the in diurnal fluctuation of thyroid hormone concentrations). In contrast to the rise in TSH after a test injection of TRH (dynamic function test), there is no pronounced rise after TRH stimulation. However, in specific situations, the secretion of thyroid hormones may be stimulated by the injection of a biosynthetic TSH preparation.

PROCEDURE

Standard radio-immunoassay procedures are applied, nowadays mainly solid phase assays which can be rapidly performed and evaluated. they are also several enzyme-linked immunoassay (ELISA) procedures from commercial suppliers. It is recommended to perform an internet check for the most appropriate method at the time of the study (for example DSL 2005). Methods have been described for triiodothyronine (Nejad et al. 1975, Chopra et al. 1972a, Larsen 1972a, 1972,) and for thyroxin (Chopra et al. 1971, Chopra 1972). The use of assays based on thyroxin binding globulin (Chopra et al. 1972b) is no longer recommended and cannot be applied to the rat, because the rat does not have this binding protein. However, for the human, measurement

of the thyroxine binding globulin by radioimmunoassay has been successfully applied (Levy et al. 1971).

EVALUATION

The usual procedure for radio-immunoassay data is calculation by a computer programmme, from standard curves of the hormone to be measured. it is important to have a sufficient number of animals per group, to be able to perform analysis of variance and statistical evaluation by tests of significance

CRITICAL ASSESSMENT OF THE METHOD

There are now many methods of RIA and ELISA design available for the rat and dog thyroid hormones. It is recommended that each laboratory performs an on-site validation for the methods to be selected for routine evaluation of endocrine safety pharmacology.

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I.0.8.7 Thyroid Function in Chronic Toxicology Studies

PURPOSE AND RATIONALE

The problems of evaluating thyroid function during high dose drug exposure are well known for several classes of compounds. In endocrine safety pharmacology, this represents a recurrent difficulty which can be resolved by early inclusion of functional parameters into the long term toxicology studies. To take premptive action on possible effects disclosed by long term toxicity studies, particularly carcinogenicity, it is advised to perform hormone measurements early in the development programme, viz. during 6–12 month toxicity studies and carcinogenicity studies. To start mechanistic studies at a later date maybe required of if special aspects of enzyme induction and changes in pharmacokinetics need to be investigated for drugs affecting thyroid function.

Mechanistic Studies

Test should include measurement of basal concentrations for TSH, total T3, and total T4 – at several time points during the study. Measurement of the free fractions of T3 and T4 is in our experience not required and does not contribute to interpretation of the organ related findings e.g. in thyroid histology. A TRH test may be included for the stimulation of TSH secretion by subcutaneous injection of TRH.

TRH Test

The TRH test is performed in a minimal invasive manner, 24 h after dosing on the next morning. The test dose is s.c. injection of 1 μg/ kg body weight synthetic TRH followed by blood sampling in unanaesthetized rats 30 min later. For mechanistic studies, it is also possible to use another specific TRH test blocking basal TSH by injection of levothyronine (L-T3) 18 hours 1 μg /rat s.c. before the test. On the next morning, the TRH dose is injected i.v. followed by blood sampling at 15 min or (in anaesthetized rats) after 15 and 30 min.

MODIFICATIONS OF THE METHOD

In carcinogenicity studies, such minimal invasive procedures (basal hormones, TRH test for stimulated TSH) can be included in small groups of animals.In other studies (chronic toxicity), pituitary hormone contents (autopsies 24 hours after last dosing) of THS, PRL and GH are a useful parameter (median-sagittal sectioning of pituitary gland with half going to histology and half to hormone assay). Iodine uptake cannot be assessed in the toxicology studies and must remain a specific item satellite groups or for a mechanistic study. The thyroid content of iodoproteins can be assessed if thyroid glands (one half as for pituitaries) are made available at autopsies, or from added groups included in the study.

CRITICAL ASSESSMENT OF THE METHOD

Inclusion of these investigations of endocrine safety pharmacology is a useful addition to chronic toxicology studies.

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Chapter I.P
Safety Assays in Skin Pharmacology

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I.P.1
In Vivo Percutaneous Absorption Assays

PURPOSE AND RATIONALE

In vivo studies have been conducted in man and in several species to compare absorption fates of numerous compounds. Percutaneous absorption rates in the rat and rabbit were generally higher than in human while the skin permeability of monkeys and swine more closely resembles humans. Although these differences are not predicted by any single factor, such as epidermal thickness, they are not unexpected in light of differences in skin characteristics. There are interspecies differences in routes of excretion of some chemicals as well. This may be due in part to metabolism of the chemical, and the metabolic capabilities of the species should be considered when selecting an animal model and designing the experiment.

PROCEDURE

Percutaneous absorption can be determined by applying a known amount of chemical to a specified surface area and then measuring the level of the chemical in the urine and/or feces. To correct for incomplete excretion of the material in urine/feces levels are measured following parenteral administration of the chemical. Radioactive-labeled chemicals, usually carbon 14 or tritium, are widely used for analytic convenience. Ingestion of the test material by the animal must be prevented, and this may require restraint of the animal or design of specialized protective apparatus for the site of application. Because urine and feces are collected for analysis, specialized cages are also required.

EVALUATION

Although studies with radiolabeled compounds accurately reflect absorption, they may not provide accurate estimates of bioavailability. For example, comparison of bioavailability from nitroglycerin (unmetabolized drug) and level of radioactive tracer indicates that use of the tracer overestimates available drug by as much as 20 %. This corresponds to the metabolism of the drug to an inactive form.

The greater the mass balance ($> 90\,\%$), the higher the reliability.

CRITICAL ASSESSMENT OF THE METHOD

There is clear relationship between the mass of a chemical residing in the stratum corneum that has been washed 30 min after application and the eventual penetration that can be measured in urine and/or blood. This principle has led to a facile method for estimating percutaneous absorption in animals and man. One samples and measures stratum corneum content with cellophane tape sampling. This method

is used to estimate bioequivalence, but does not have a regulatory imprimatur (Rougier et al. 1999).

MODIFICATIONS OF THE METHOD

The difficulties in conducting these pharmacokinetic assays – collecting excrement for relatively long period of time (24 h +), requirements for specialized cages and specialized protective apparatus, and the increased space requirements for housing animals individually – has led to the use of other in vivo assays and to the development of in vitro models.

Loss of radioactive material from the skin surface has been used to estimate in vivo percutaneous absorption. The difference in applied dose and residue on the skin is assumed to be absorbed. The characteristics of the radioisotope, penetrant, and vehicle may limit the usefulness of this procedure. Volatile materials may leave the surface without penetrating, and it is difficult to recover all material from the skin surface. In addition, skin may retain a reservoir of the penetrant that has not entered the circulation.

Robert et al. (1999) provides extensive details.

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I.P.2 In Vitro Percutaneous Absorption Assays

PURPOSE AND RATIONALE

The excised skin of humans or animals can be used to measure penetration of chemicals. In vitro assays using excised skin use specially designed diffusion cells.

PROCEDURE

The skin is stretched over the opening of a collecting receptacle, epidermal side up. The chemical to be studied is applied to the epidermis, and fluid from the receptacle is assayed to measure the penetration of the chemical, which usually is radio-labeled. The preferred method for toxicological relevance is a one-chambered cell in which the stratum corneum is exposed to the air. The underside of the skin is bathed with saline or other receptacle fluid, which should be constantly stirred and maintained at physiological temperature. Either full-thickness skin or epidermis alone may be used in vitro assays.

EVALUATION

This type of in vitro assay offers advantages over in vivo assays. Highly toxic compounds can be studied in human skin and large cell numbers can be run simultaneously. Diffusion through the membrane can be studied. In addition, these assays may be less expensive and easier to conduct. In vitro penetration rates through skin of various species have been compared. Skin of the weanling pig and miniature swine appear to be good in vitro models for most compounds. The skin of monkeys appears to be a good model as well. For most compounds, mouse and hairless mouse skin appears more permeable than skin of other species. Rat skin is a reasonable model for some compounds while large differences have been noted for others.

CRITICAL ASSESSMENT OF THE METHOD

The presumed simplicity of in vitro penetration assays has led to their universal acceptance for preclinical and other screening purposes. However, they do not mimic human exposure in some important areas. Excised skin must often be stored prior to us and thus retains only some enzymatic activity. In intact skin, chemical penetrating the epidermis would enter the circulation through vessels and lymphatics located just below the epidermis. In excised full-thickness skin, the dermis is also involved in absorptive process. Storage conditions and procedures for preparing the tissue may affect skin absorption and metabolism. This is no opportunity to qualify key aspects of penetration: cell turnover and desquamation and rub off.

MODIFICATIONS OF THE METHOD

The influence of the dermis can be minimized by using heat-separated epidermis, or by removal of the skin with a dermatome at the level of the upper dermis.

Bronaugh (1999) collated the experimental variables leading to discrepancies. His text provides

a catalogue of variables that can lead to experimental designs that may have in vivo relevance, especially for hydrophilic materials.

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I.P.3
Guinea Pig Sensitization Tests

PURPOSE AND RATIONALE

Predictive animal tests to determine the potential of substances to induce delayed hypersensitivity in man are conducted most often in guinea pigs. Several tests will be described here. Each offers its own advantages and disadvantages; most have features in common.

PROCEDURE

All tests use young (1–3 months old or 250–550 g), randomly bred albino guinea pigs. Animals are maintained in the facilities with temperature of approximately 20 ± 1 °C, 40 %–50 % relative humidity, 12-h automatic light cycle, a standard vitamin C-supplemented chow, and water available at all times. Test sites are clipped free of hair with electric clippers; some assays specify chemical depilation as well. Almost all evaluate the response as production of visible dermatitis, using descriptive scales for erythema and edema. Some assays specify use of one sex or one half of each sex. The tests differ significantly in route of exposure, use of adjuvants, induction interval, and number of exposures. The principal features of the most commonly used assays and the assays acceptable to regulatory agencies to predict sensitization are summarized in Table 1.

EVALUATION

The Draize Test (DT)

The Draize sensitization test was the first predictive sensitization test accepted by the regulatory agencies, and is still widely used. One flank of 20 guinea pigs is shaved and 0.05 ml of a 0.1 % solution of test material in saline, paraffin oil, or polyethelene glycol is injected into the anterior flank on day 0. The next day, and every other day through day 20, 0.1 ml of test solution is injected into a new site on the same flank. Challenge follows a 2-week rest period. The opposite untreated flank is shaved and 0.05 ml of test solution is injected into each animal. Twenty previously untreated controls are injected at the same time. The test site is visually evaluated 24 and 46 h after injection. A larger or more erythematous response in test animals than that of controls is considered a positive response. Results are expressed as the percentage of animals positive or as the ratio of positive animals to the number tested.

Open Epicutaneous Test (OET)

This test simulates the conditions of human use by using topical application. The procedure determines the dose required to induce sensitization and to elicit a response in sensitized animals. The irritancy profile is evaluated by testing various concentrations (undiluted, 30 %, 10 %, 3 %, and 1 %) in ethanol, acetone, water, polyethylene glycol, or petrolatum. In 6 to 8 guinea pigs, 0.025 ml of the dosing solutions are applied to 2 cm^2 areas of the shaved flanks. Vehicle solubility and use conditions (e.g., direct application to skin or dilution during normal use) are considered in selecting the concentrations to be tested. Test sites are evaluated visually 24 h after applying test solutions for the presence of erythema. The dose not causing a reaction in any animal and the dose causing a reaction in 25 % of the animals are determined. During induction, 0.10 ml of test solution is applied to an 8 cm^2 area of flank skin of 6 to 8 guinea pigs for 3 weeks, or 5 times a week for 4 weeks. Groups of animals are treated with different doses; a control group is treated with vehicle only. The highest dose tested usually is the minimal irritant concentration; lower doses are based on usage concentration or a stepwise reduction (e.g., 30–10–3–1). Solutions are applied to the same site each day unless a moderate inflammatory response develops. Each animal is challenged on the previously untreated flank 24–72 h after the last induction treatment. The minimal irritant concentration, the maximum nonirritant concentration, and five solutions of lower concentrations are applied, 0.025 ml to a 2 cm^2 area. Skin reactions are read on an all or none

Table 1 Principle features of guinea pig sensitization assays.

Feature of test	Draize	Open Epicutaneous Tests (OET)	Beuher Assay	Freund's Complete Adjuvant Test (FCA)	Optimization Test	Split Adjuvant	Guinea Pig Maximization (GPMT)
Number in test group	20	6–8	10–20	8–20	20	10–20	20–25
Number in control group	20	6–8	10–20	8–20	20	10–20	20–25
INDUCTION							
Exposure route	id	Open epicutaneous	Patch	id	id	Patch	id and patch
Number of exposures	10	20–21	3	3	9	4	1 id; 1 topical
Duration of each patch	No patch	Continuous (no patch)	6 hours	–	–	48 h each	48 h patch
Concentration	0.2	Non-irritatitng	Sligthly-irritatitng	5–50 %	0.1 %	0.1–0.2 ml	Maximum tolerated
Test group(s)	TS	TS	TS	TS in FCA	TS in FCA		TS, TS + FCA, FCA
Control group	None	Vehicle only	Vehicle only	FCA only	–		FCA, FCA + V, V
Site for dosing	Left flank	Right flank	Left flank	Shoulder	Back (flank 1^{st} injection)	Mid-back	Shoulder
Frequency of exposure	Every 2^{nd} day	Daily	Every 5–7 days	Every 4 days	Every other days	Day 0, 2, 4, 7	0 (id); day 7 patch
Duration (days)	1–18	0–20	0–14	0–9	0–2	0–9	0–9
Misc.			9 exposure version			Day 0 dry ice treatment FCA (id) day 4	Irritant dose of SLS pretreatment
Rest Period (days)	19–34	21–34	15–27	9–21; 22–34	22–34	10–21	9–30
CHALLENGE							
Exposure Route	id	Open	Patch	id; patch	id	Patch	Patch
Number of exposures	1	2	1	2	2	1	1
Duration of exposure	–	–	24 h			24 h	24 h
Exposure day(s)	35	21 and 35	28	22; 35	14–28	22	21

FCA – Freund's complete adjuvant; SLS – sodium lauryl sulfate; TS – test substance; V – vehicle; id – interdermal. Reproduced from Maibach and Patrick (2001) with permission.

basis at 24, 48, and 72 h after solutions application. The maximum nonirritating concentration in the vehicle-treated group is calculated. Animals in test groups that develop inflammatory responses to lower concentrations are considered sensitized. The dose required to sensitize is determined by comparing the number of positive animals in the test groups. The minimal concentration necessary to elicit a positive response in a sensitized animal is apparent from the challenge responses.

Buehler Test

This test also employs topical application of the test material. An absorbent patch, Webril 20 × 20 mm, backed by Blenderm tape and saturated with 0.4 ml of the test material, is placed on the shaved flanks of

10–20 guinea pigs. Test concentration varies from undiluted to usage levels. An optimum concentration that produces slight erythema is selected based on an irritancy screen conducted in other animals. The patch is held in place by wrapping the animal with an occlusive wrapping, then placing the animal in a special restrainer fitted with a rubber dam to maintain even pressure over the patch for a 6-h exposure period. This procedure is repeated 7 and 14 days after the initial exposure. A control group of 10–20 animals is patched with vehicle only. Two weeks after the last induction patch, animals are challenged with patches saturated with a nonirritating concentration of test material applied to both flanks and with the vehicle (if other than water and acetone). Wrapping and restraint are as during induction. After 6 h, the patch is removed and the area depilated. Test sites are visually evaluated 24 and 48 h after patch removal. Animals developing erythematous responses are considered sensitized. The incidence of positive reactions and the intensity of the response are calculated.

Freund's Complete Adjuvant Test (FCAT)

FCAT is an intradermal technique incorporating test material in a 50/50 mixture of FCA and distilled water. A 6 × 2 cm area across the shoulders of two groups of 10 to 20 guinea pigs is shaved and injected with a 5 % solution of the test material in FCA/water; injection volume is 0.1 ml. Control animals are injected with FCA/water. Injections are repeated every 4 days until 3 injections are given. The minimal irritating and maximum non irritating concentration following topical application of 0.025 ml solutions to a 2 cm^2 area of skin is determined on a minimum of 4 naive guinea pigs. Twenty-one days after the first induction injection, 0.025 ml of the minimal irritant concentration, and two lower concentrations are applied to 2 cm^2 areas of the shaved flank. Test sites are not covered and are evaluated for the presence of erythema at 24, 46, and 72 h after application. The minimum nonirritating concentration in FCA/water-treated controls is determined. Animals injected with the test material during induction that respond to lower doses are considered sensitized. The incidence of sensitization and the threshold concentration for elicitation of the response in these animals are calculated.

Optimization Test

This test incorporates the use of adjuvant for some induction injections and both intradermal and topical challenges. Injections during induction are 0.1 ml of 0.1 % concentration of test material in 0.9 % saline or in 50/50 FCA/saline. A total of 10 injections are given. On day #1, 1 injection is given into the shaved flank and one into a shaved area of dorsal skin. The test material is administered in saline during the first week. During the second and third weeks, test material is administered in FCA/saline every other day to a shaved area over the shoulders. Twenty test animals are treated; twenty controls are injected with saline during week 1 and FCA/saline during weeks 2 and 3. The intensity of the 24-h responses during week 1 is calculated as reaction volume. Thickness of a skin fold over the injection site is measured with a caliper (mm), and the two largest diameters of the erythematous reaction are recorded (mm).

The Split Adjuvant Test

This test uses skin damage and FCA as adjuvants by topical application of the material. The skin on the back, just behind the scapula of 10–20 guinea pigs is clipped, shaved to glistening, and then treated with dry ice for 5–10 sec. A dressing of a layer of loose mesh gauze and stretch adhesive with a 2 × 2 cm opening over the shaved area is placed around the animal and secured with adhesive tape. This dressing remains in place throughout induction. Around 0.2 ml of creams or solid test material, 0.1 ml if liquid, is spread over the test site and covered with two layers of #2 filter paper backed by occlusive tape and attached to the dressing by adhesive tape. The concentration tested varies by irritancy potential, use conditions, etc. Two days later, the filter paper is lifted from the test site, the test material is reapplied, and the filter paper covering is replaced. On day 34, the filter paper cover is removed; two injections of 0.075 cc FCA are given into the edges of the test site, the test material is reapplied, and on day 9 the dressing is removed. Twenty-two days following the start of treatment, animals get challenged by topically applying 0.5 ml of test material to a 2 × 2 cm area of the shaved mid-back. The test site is covered by filter paper backed with adhesive tape, held in place by wrapping the animal with an elastic adhesive bandage secured with adhesive tape. A group of naive controls, 10–20 animals, is treated by the same procedure at challenge. The dressing is removed 24 h after application, and the test site id evaluated at 24, 48 and 72 h, visually using a seven-point descriptive visual scale. Individual animals having significantly stronger reactions than controls are considered sensitized.

Guinea Pig Maximization Test (GPMT)

The Guinea Pig Maximization Test combines FCA, irritancy, intradermal injection and occlusive topical

application during the induction period. The shoulder region of two groups of 20–25 guinea pigs is shaved. Two identical sets of intradermal injections of 0.1 ml 50/50 FCA/water, test material in water, paraffin oil, or propylene glycol, and the same dose of the test material in FCA/vehicle, are placed in a 2 × 4 cm area. Seven days later the test article is placed on filter paper over the injection site. The filter paper is covered with approximately 4 × 8 cm occlusive surgical tape and secured in place with an elastic bandage wrapped around the animal. If the test material is nonirritating, the test site is pretreated with 10 % sodium lauryl sulfate in petrolatum on day 6 to provoke an irritant reaction. Control animals are patched with the vehicle alone. The dressing is removed from the animals 48 h after application. Test and control animals are challenged on the shaved flank with the highest nonirritating concentration, with approximately one half of the highest nonirritating concentration, and with the vehicle. Solutions are applied to 1 × 1 cm pieces of filter paper secured in place; patches are removed 24 h later. If needed, the challenge area is shaved 21 h after patch removal. Twenty-four and 48 h after removal of the patch the reactions are visually evaluated and compared to controls. Reactions are considered positive when they are more intense than the response to vehicle and the responses to the test material in controls. Based on the incidence of positives in the test group, test materials are rated as a weak to extreme sensitizer.

CRITICAL ASSESSMENT OF THE METHOD

Almost all of the tests described above evaluate the response as production of visible dermatitis, using descriptive scales for erythema and edema. Some assays specify use of one sex or one half of each sex. The tests differ significantly in route of exposure, use of adjuvants, induction interval, and number of exposures. The principal features of the most commonly used assays and the assays acceptable to regulatory agencies to predict sensitization are summarized in Table 1.

MODIFICATIONS OF THE METHOD

These methods can be adopted to define dose relationships – as was the intent of the open epicutaneous test (OET). The availability of the threshold for induction (TIC) and elicitation (TEC) provides a valuable tool in clinical risk assessment.

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I.P.4
Sensitization Tests In Mice

I.P.4.1
Local Lymph Node Assay (LLNA)

PURPOSE AND RATIONALE

LLNA consists of a topical induction followed by measurement of the mitotic activity of the draining lymph node. It involves application of multiple topical in vivo doses of the material of interest to mouse ear, followed by in vitro evaluation of mitotic activity of cells from the draining lymph nodes.

PROCEDURE

Experimental animals of either sex, 8–12 weeks of age are selected. Each individual assay should use a single sex only. At least 3 dose levels are evaluated in separate groups of diluents include 4:1 acetone/olive, methyl ethyl ketone, dimethylformamide, propylene glycol, dimethyl sulphoxide, and 2.5 % hydroxypropyl cellulose in methanol. Three consecutive concentrations from the series 100 %, 50 %, 25 %, 10 %, 5 %, 2.5 %, 1.0 %, 0.5 %, 0.25 %, 0.1 %, 0.05 % and 0.001 % are tested.

Twenty-five μl of test solution or the vehicle alone is applied to dorsal surface of the pinnea every day of the 3 consecutive days. Five days after the 1st exposure, 250 ml phosphate buffered saline containing 20 μci methyl thymidine is injected via the tail vein of each animal. Five hours after injection, animals are euthanized by carbon dioxide asphyxiation. Draining auricular lymph nodes are excised and pooled with nodes from other animals in the same group. A single-cell suspension is prepared by gently passing the nodes through stainless steel, 200-mesh gauze with the plunger of a syringe. The cell suspension is centrifuged at 190Xg for 10 min, and then the pellet is washed twice with 10 ml PBS. Cells are resuspended in 3 ml of 5 % trichloroacetic acid (TCA) and incubated overnight at 4 °C. Centrifuge, remove supernatant, resuspend the precipitate in 1 ml 5 % TCA. Transfer the suspension to 10 ml scintillation fluid, count disintegrations/min using β scientillation counter. Disintegration per lymph node is calculated for each experimental group and the control group. The ratio of 3H TdR incorporation into the test group and the control is calculated for each dose. Some investigators prefer to pool the lymph nodes from all animals in the dosage group. If the ratio is 2–3 for any dose, the material is considered a sensitizer.

EVALUATION

Standardized validating predictive assays in mice have been developed in the last 10 years. LLNA as one of such assays offers advantages in the low number of animals used, lower cost, and less time required for conducting the assay in comparison to various guinea pig assays.

CRITICAL ASSESSMENT OF THE METHOD

LLNA is unique of all predictive assays in only evaluating the response of the efferent phase of the response.

MODIFICATIONS OF THE METHOD

Several investigators have compared LLNA with various guinea pig assays and have suggested variations of the method. LLNA is not as stringent as some guinea pig assays; however, it is expected to retain utility as a rapid screening assay for materials with strong sensitization potential. A validation meeting to review the strengths and weaknesses of the assay has been reported. Certain materials, such as metals, have not been reliably identified as allergens. The validation attempt matched LLNA results with guinea pig Buehler and maximization assays. Both have false-positives and false-negatives. No attempt was made to determine the clinical relevance of either the LLNA data nor the benchmarks used. Further experience will be needed to place this assay in toxicologic.

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I.P.4.2
Mouse Ear Swelling Test (MEST)

PURPOSE AND RATIONALE

The MEST uses both topical and injection exposures for induction and a topical challenge of the pinnea in which visual evaluation is replaced by measuring ear thickness with an engineer's micrometer. Its purpose is to evaluate the response of mice following the challenge with potential sensitizers.

PROCEDURE

Six to eight week old female CF-1, Balb/c, or SW female mice are gang housed in direct bedding cages. Following a 5–7 day quarantine period, the fur of the abdomen is shaved by electric clippers from 10–15 test animals and 5 controls. Next, the area is tape stripped with Dermaclear, a surgical adhesive tape, until the skin appears glossy. Then a divided dose of 0.05 ml Freund's complete adjuvant is injected intradermally with a tuberculin syringe fitted with a 30-gauge needle. The Freund's complete adjuvant is injected into two sites within the shaved area but along the borders. Following the injection of adjuvant, 100 μl of vehicle containing the test material or vehicle alone (controls) is applied to the center of the shaved area. The abdomen is allowed to dry, and then the mouse is returned to its cage. The tape stripping and application of appropriate material to the abdomen is repeated each day for the next 3 days.

Acetone, methyl ethyl ketone or 70 %, 80 %, and 95 % ethanol in water have been shown to be acceptable vehicles based on solubility and chemical compatibility with the test substance. The dose selection is based on dermal irritation toxicity range finding studies prior to testing each compound. Four groups of two mice each are subjected to the induction procedure, including shaving, tape stripping, and application of the test material, and then the ears exposed as during challenge. At lease 4 concentrations are evaluated in the range study. Minimally irritating or nonirritating concentrations are selected for the induction application. The highest nonirritating concentration is used at challenge.

Seven days after the last topical induction application, challenge is performed by applying 20 μl of the test material in vehicle to one ear of each animal (test and control) and 10 μl of the vehicle to the opposite ear. Ear thickness is measured before application of the challenge dose and 24 and 48 h after application. Animals are lightly anesthetized with ether, and the thickness of both pineas is measured with an Oditest engineer's micrometer. Positive respondents are defined as animals in which the ear dosed with the test material shows at least a 2–3 fold greater increase in thickness than the vehicle-treated control ear. The control group should not show greater than a 10 % increase in ear thickness for the test to be considered valid.

If the control groups show more than a 10 % increase, the study should be repeated using lower doses. The percentage of respondents is calculated. The degree of ear swelling should also be calculated by dividing the thickness of the ear to which the test material was applied by the thickness of the vehicle treated control ear. Measurements from all animals in the test group are included.

EVALUATION

Gad and co-workers (1986, 1887) presented validation studies of 72 compounds and reported a false-negative rate of only 2 % and no false-positives when comparing MEST to GPMT data on the same materials. The incidence of sensitization in MEST was consistently lower than that produced by GPMT. Similar findings were reported for comparisons between the Buehler assay and MEST in the same paper. More recent publications have confirmed that the incidence of positive response in MEST is consistently lower than that in GMPT, and weak and moderate sensitizers are not identified correctly (Cornacoff et al. 1988 and Dunn et al. 1990).

CRITICAL ASSESSMENT OF THE METHOD

Although numerous approaches to developing a predictive sensitization assay in mice have been proposed, MEST is one of only two assays sufficiently developed to warrant consideration as standardized assays (LLNA being the other). The MEST has been accepted by the Environmental Protection Agency for registration of chemicals under the Toxic Substances Control Act (TSCA).

MODIFICATIONS OF THE METHOD

The type of micrometer used may affect interpretation of the test results. When Van Loveren et al. (1984) compared the use of spring-loaded caliper-type instruments with screw and friction thimble micrometers, the spring-loaded instruments were found to be the best. This should be taken into account for future modifications of the MEST. In addition, the use of anesthetic can be eliminated by operator training and prior handling of animals.

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I.P.4.3
The Vitamin A Enhancement Test (VAET)

PURPOSE AND RATIONALE

VAET is another assay performed in mice. In this test, the reactivity of the immune system is heightened by maintaining animals on a diet with high doses of vitamin A for a preconditioning period, throughout induction and challenge. Challenge is topical and is assessed by measuring ear thickness (Table 2).

PROCEDURE

Miller and her colleagues (1984, 1986) decreased the dose of strong sensitizers required to induce sensitization by maintaining the animals on diet supplemented with high levels of vitamin A acetate. Ear thickness measurement was selected for use in the predictive assay. Principal features of the test included a preconditioning period of the diet of 28 days, 6 exposures to the shaved abdomen and thorax during the 12-day induction period, and challenge 4 days later. Results from the test group were compared statistically to that of controls, indicating sensitization. The minimally irritating concentration was used for induction, and the highest nonirritating does was used at challenge (determined by dose response in separate groups of mice). The vehicle was selected based on non irritancy and solubility of the test substance.

EVALUATION

VAET enhancement test is used for evaluation of ingredients of consumer products in mice.

CRITICAL ASSESSMENT OF THE METHOD

VAET enhancement test has been used for the evaluation of ingredients of consumer products but has not been used by a sufficient number of laboratories to be considered standard.

MODIFICATIONS OF THE METHOD

The long conditioning period required prior to the study is one of the limitations of this assay. The test was never widely adopted or submitted to formal validation procedures. General comments regarding choice of micrometer for MEST also apply to VAET.

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I.P.5
Human Sensitization Assays

PURPOSE AND RATIONALE

Human Sensitization Assays allow chemicals to be tested for their ability to induce contact hypersensitivities on the skin of human volunteers with obtained informed consent. Human studies should only be undertaken for a new compound after the results of predictive tests in animals for such compound are available. If a compound contains significantly increased levels of common ingredients, it should also undergo predictive tests in animals prior to humans.

Generally, materials identified as sensitizers in animals are not tested on humans. However, if the potential benefit of the material warrants, a small group of human subjects may be tested with materials inducing sensitization in animals. Such situations should be reviewed by an Institutional Review Board. Test subjects should be informed of the increased risks, and the number of subjects used should be limited (additional subjects can be exposed if members of a small group do not respond).

Table 2 Principal features of human sensitization assays

Feature	Complete	Shelanski/ Shalanski	RIPT Draize	Griffith–Voss–Stotts	Modified Draize	Human Maximization
Number of subjects	200	200	200	200	100–200	25
Induction						
Exposure site	Upper arm	Upper arm, same site	Upper arm or back; naïve site for each exposure	Upper arm same site	Upper arm or back, same site	Upper arm or lower back same site
Number of exposures	1	15	10	9	10	5
Duration of exposures	24–72 h	24 h	24 h	24 h	48–72 h	48 h
Frequency of exposures	–	3 per week	3 per week	3 per week	3 per week	24 h between patches
Evaluation schedule	At removal, 24, 48 h	At removal	At removal	48–72 h	30 minutes are removal	Before each application
Miscellaneous	4-week usage period	Fatiguing index		Pilot group	Continuous exposure	SLS/irritation as adjuvant
Rest period duration		14–21 days	10–14 days	14 days	14 days	14 days
Challenge						
Exposure site	Upper arm	Upper arm	Upper arm	Upper arm	Upper arm or back	Lower back, upper arm
Duration of exposure	24–72 h	48 h	48 h	24 h	72 h	SLS 1 h; 48 h
Evaluation of schedule	At removal, 24, 48 h	At removal	At removal	48 and 96 h	At removal, 24 h	At removal, 24, 48 h
Miscellaneous			Naïve test site	Original & naïve sites	Naïve test site; may use two 48-h exposures	At removal, 24, 48 h Sensitization Index

RIPT – Repeat Insult Patch Tests
FCA – Freund's complete adjuvant; SLS – sodium lauryl sulfate; TS – test substance; V – vehicle; id – interdermal.
Reproduced from Maibach and Patrick (2001) with permission.

PROCEDURE

Subjects are randomly selected with some indicated precautions. Tests should not be performed on scar tissue. Subjects should not be tested with materials to which they are known to be allergic (demonstrated by diagnostic patch test or in previous predictive assays).

There are 4 basic predictive human sensitization tests in current use: (1) single induction/ single challenge patch tests; (2) repeated insult patch tests (RIPT); (3) RIPT with continuous exposure (modified Draize); and (4) the maximization test. Principal features of human sensitization assays are summarized in Table 2.

All methods described use customized patches. For assays other than maximization, usually 150–200 subjects are tested.

EVALUATION

Repeat Insult Patch Test (RIPT)

RIPT has four major variations that are commonly used: (1) the Draize human sensitization test (1955, 1959); (2) the Shelanski/Shelanski test (1953); (3) the Voss/Griffith test (1969, 1976); and (4) Marzill/Maibach modification.

- In the Draize human sensitization test, an occlusive patch containing the test material is applied to the upper armor upper back of 200 volunteers. The patch remains in place for 24 h and is then removed. The test site is evaluated for erythema and edema at patch removal. Twenty-four hours after the removal of the 1st patch, a 2nd patch test is applied. This

process is repeated until 10 patches are applied. For convenience, the test may be run on a Monday-Friday schedule, with subjects removing their own patches Saturday (72 h between Friday and Monday applications). Ten to 14 days after application of the last induction patches, subjects are challenged via a patch applied to a new site. Duration of contact is 24 h; sites are visually evaluated at removal of the patch. The results are compared and the incidence of sensitization is reported.

- The Shelanski/Shelanski test employs occlusive patches in contact with the skin of the upper arm for 24 h in a similar fashion to the Draize RIPT described above. However, the patches are placed on the same test site each time with a total of 15 sets applied during induction. The test site is evaluated before application of a new patch to the site. If inflammation has developed, the patch is placed on an adjacent uninflamed site. Two to 3 weeks after the induction period, subjects are challenged by application of a patch to be remained in place for 48 h. Test sites are evaluated for erythema and edema at patch removal and the incidence of positive responses is reported.
- Voss/Griffith RIPT test differs only minimally from Draize RIPT. Many investigators apply patches to the same site during induction and refer to the procedure as a Draize RIPT. The value of multiple grades at challenge is widely recognized and used. Multiple test materials are tested simultaneously in all of RIPT for better efficiency and economy. Although the distinctions between Draize and Voss/Griffith procedures have blurred with common usage, the Shelanski/Shelanski test, with 5 to 6 more induction applications, remains distinct.
- Marzill/Maibach modification: this modification simplifies compliance – in that the laboratory (rather than the individual) removes the patch. This is applied Monday, Wednesday, and Friday for 3 weeks, with readings performed approximately one hour post removal. Elicitation is for 48 h – with readings at 49 and 96 h – in concordance with the international Contact Dermatitis research Group (ICDRG) guidelines. (Lachapelle JM, Maibach HI (2003) Patch Testing and Prick Testing. A Practical Guide. Springer, New York).

Human Maximization Test

The Human Maximization Test was designed and later modified by Kligman et al. (1959, 1966). This test uses irritancy as an adjuvant. Irritating compounds are tested during induction at a concentration that produces a moderate erythema within 48 h. If materials are nonirritating, the test site is pre tested with a 24-h patch of 5 % sodium lauryl sulfate (SLS). Additional pretreatment SLS patches may be applied before each patch application until a brisk erythema is achieved. Petrolatum is the appropriate vehicle; the induction concentrations should be at least 5 times higher than use levels. Often, custom-made Webril/Blenderm patches or Duhring Chambers are used. Patches are applied to outer arm or lower back, and up to 4 different materials can be tested at once. Careful tape wrapping should ensure occlusion; bandage sprays may be used to assure sealing of the test site.

Five sets of patches are worn on the same site for 48 h each, with a 24 h rest period between removal and reapplication. Following a 2 week rest period, an SLS provocative patch is applied to prepare the skin for challenge. A patch saturated with a 2.5–5.0 solution of SLS is applied to previously untreated sites on the lower back. SLS concentration is based on the season and on individual subject response. The SLS patch is removed after 1 hour, and a patch containing the test material is applied. A control site is patched with SLS (1 h) and petrolatum (48 h) to aid in interpretation of the results. The patch is removed 48 h after application. Test sites are evaluated at removal, and then reexamined 24 and 48 h after patch removal. The number of subjects developing a positive response is reported and a sensitization index based on percentage of subjects responding is assigned to the test material. In common practice, the human maximization procedure is performed on either the outer upper arm or the back.

Modified Draize Human Sensitization Test

The RIPT procedure was modified to provide for continuous patch exposure. Materials are applied to the outer upper arm each Monday, Wednesday, and Friday until 9 patches have been applied during a 3-week period. Patches remain in place until approximately 30 min before application of a fresh patch. This brief rest period allows some clearing of responses to tape and facilitates grading. Fresh patches are applied to the same site unless moderate inflammation has developed; the patches are placed on adjacent non inflamed skin if inflammation becomes pronounced. This produces a continuous exposure of 504 to 552 h compared to a total exposure period of 216 to 240 h for RIPT of comparable induction applications. In addition, induction concentration was increased to levels above usage exposure. Two weeks

after induction, subjects are challenged by exposure of a new site to a patch of 72 h duration at a nonirrigating concentration. Test sites are evaluated at patch removal and 24 h after removal.).

CRITICAL ASSESSMENT OF THE METHOD

Results of RIPT, modified Draize test, and human maximization tests have been accepted as valid by regulatory agencies; however, some sponsors routinely use one of the methods described and defend its use as the "standard of the industry". FDA reviewed details of sensitization procedures and developed a guidance document (1999) for evaluating skin sensitization to chemicals in natural rubber products.

MODIFICATIONS OF THE METHOD

Stotts (1980) presented detailed examples of proper interpretation of human repeat insult patch tests. Sensitization is characterized by challenge reactions stronger than reactions early in the induction phase, by persistence of responses through delayed readings, by delayed appearance of a response, or by weak responses in a few subjects when the material has not produced irritation in the panel.

Jordan and King (1977) proposed modifying the challenge procedure to two consecutive 48-h patch periods. The modified Draize test has recently been selected as the test of choice for chemicals in natural rubber products by the FDA. Tests for the transdermal products are currently being evaluated by the FDA.

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I.P.6 In Vitro Assays for Allergic Contact Dermatitis

In spite of decades of extensive investigations, an in vitro assay for identification of contact allergens has not been validated.

I.P.6.1 Irritation Tests in Animals

I.P.6.1.1 Draize-Type Tests

PURPOSE AND RATIONALE

All Draize-type tests evaluate corrosion and irritation by using albino rabbits as subjects (Table 3). The Federal Hazardous Substance Act (FHSA) adopted one modification as a standard procedure to this method (1997).

Further description of the test see Chapters I.H (Peripheral Nervous System) and I.O (Ocular Toxicity Tests).

PROCEDURE

The backs of 6 albino rabbits are clipped free of hair. Each material is tested on two 1-in square sites on the same animal; one site is intact and one is abraded in such a way that the stratum corneum is open but no bleeding produced. Abrasion can be performed using the tip of a hypodermic needle drawn across the skin repeatedly or commercial instruments such as the

Berkeley Scarifier as described by Haley et al. (1974). Materials are tested undiluted, and 0.5 ml liquid, or 0.5 g solid or semisolid material is applied. Each test site is covered with two layers of 1-in square surgical gauze secured in place with tape. The entire trunk of the animal is then wrapped with rubberized cloth or other occlusive impervious material to retard evaporation of the substances and hold the patches in one position. The wrappings are removed 24 h after application and the test sites are evaluated for erythema and edema using a prescribed scale. Evaluations of abraded and intact sites are recorded separately. Test sites are evaluated again 48 h later (72 h after application) using the same scale.

When severe reactions, which may not be reversible, are noted, test sites are observed for a longer period. Delayed evaluations usually are made on days 7 and 14; however, evaluations have been made as late as 35 days after application. Environmental Protection Agencies (EPA) bases interpretation on 7-day observation. The basic exposure procedures for skin irritation/corrosion of Organization for Economic Co-operation and Development (OECD) guidelines have been further modified to test for corrosion during shorter periods. Under a directive of the European Economic Community, a shorter, 3-min exposure was added. The United Nations' recommendation for the Transport of Dangerous Goods is based on exposure times of 4 h, 1 h, and 3 min, with the recommendation that the 1-h exposure be conducted first. Evaluations are made 1, 24, 48, and 72 h, and 7 days after dosing.

EVALUATION

The Draize test provides tremendous value in warning consumers, workers and manufacturers of potential dangers associated with specific chemicals so that appropriate precautions can be taken. Although vesiculation, ulceration, and severe eschar formations are not included in the Draize Scoring Scales, all Draize-type tests are used to evaluate corrosion as well as irritation.

CRITICAL ASSESSMENT OF THE METHOD

The Draize method has generally erred on the side of safety in that it over predicts the severity of skin damage produced by chemicals, thus producing a safety factor for those exposed. Some investigators report repeatedly that the test is not sensitive enough to separate mild from moderate irritants. Although Draize-type tests will be replaced by in vitro assays some time in the future, we have no validated in vitro substitute at present.

MODIFICATIONS OF THE METHOD

Numerous modifications to the Draize procedures have been proposed to improve its prediction of human experiences, including changing the species tested, reducing exposure period, using fewer animals, and testing one intact skin only. Mezei et al. (1966, 1970) have supplemented visual evaluation with other techniques, but these additions have not been considered for the standard method. Several governmental bodies have used their own modification of the Drake procedure for regulatory decisions. The Consumer Product Safety Commission (CPSC), Department of Transportation (DOT), EPA and OECD guidelines are contrasted to the original Draize methods in Table 3. Cruzan et al. (1986) proposed a composite test that meets requirements of major agencies.

Summaries and evaluations of the scores vary. Draize reported values for individual animals at each time point, and then averaged the 24-h and 72-h evaluations for intact and abraded sites separately. He also calculated a primary irritation index (PII) that was the average of the intact and abraded sites. Agents producing PII of 2 were considered only mildly irritating, 2–5 moderately irritating, and more than 5 severely irritating. The primary irritation calculated for FHSA is essentially the PII of Draize. A minimum PII of 5 defines an irritant by CPSC standards. The method of the National Institute Of Occupational Safety and Health (NIOSH) does not combine responses of abraded sites and includes probable effects on normal and damaged skin in their evaluation.

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Table 3 Comparison of skin irritation test based on the Draize method

Feature	Draize	FHSA	DOT	FIFRA	OECD[a]
Number of animals	3[b]	6	6	6	3
Abrasion	Abraded and intact	Abraded and intact	Intact	2 abraded and 2 intact	Intact
Dose liquids	0.5 ml undiluted	0.5 undiluted	0.5 ml	0.5 ml undiluted	0.5 ml
Dose solids	0.5 g	0.5 g in solvent	0.5 g	0.5 g in moistened	0.5 g in moistened
Wrapping materials	Gauze and rubberized cloth	Impervious material			Semiocclusive
Length of exposure	24 h	24 h	4 h	4 h	4 h
Evaluated at[c]	24, 72 h	24, 72 h	4, 48 h	0.5, 1, 24, 48, 72 h	0.5, 1, 24, 48, 72 h
Treatment at removal	Not specified	Not specified	Skin washed	Skin wiped, not washed	Skin washed
Excluded from testing				Materials pH < 2 or > 11.5	Materials pH < 2 or > 11.5

[a] Although other species are acceptable, the abino rabbit is the preferred species.
[b] Draize tested four materials on six rabbits. Three abraded and three intact sites with each materials.
[c] Times listed are after removals for FIFRA and OECD. Time listed for Draize, FHSA, and DOT are after application of the test material.
DOT– Department of Transportation; FHSA – Federal Hazardous Substance Act; FIFRA – Federal Insecticide, Fungicide, and Rodenticide Act; OECD – Organization for Economic Cooperation and Development.
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I.P.6.1.2 Non-Draize Animal Studies

PURPOSE AND RATIONALE

These are animal assays that evaluate the ability of chemicals to produce cumulative irritation. Many such tests have been described in literature, but only a few have been studied extensively enough to mention. Even those used more often are not as well standardized as Draize-type tests, and many variables have been introduced by multiple investigators.

PROCEDURE

1. Repeat application patch tests – diluted materials are applied to the same site each day for 15–21 days using the guinea pig or rabbit most commonly (Phillips et al. 1972). Various patches are procedures as a control. The degrees of inflammation produced by the materials in a single assay are compared. Test sites are evaluated for erythema and edema using the Draize-type tests or more descriptive scales developed by the investigator. The data from cumulative irritancy assays in rabbits have been used to predict reactions in man. Other investigators have used multiple applications with shorter periods of time to evaluate materials (Justice et al. 1961).
2. A 5-day dermal irritation test – performed in rabbits to compare various consumer products (MacMillan et al. 1975). Animals' backs are shaved and 0.5 ml of test materials is spread over a 5×4.5 cm skin area. The test sites are protected from grooming by placing the animal in a leather harness or Elizabethan collar. After 4 hours, sites are cleaned and graded using the Draize scoring system. The procedure is repeated every day for 5 days. The authors showed good agreement between the assay and 21-day human patch tests of liquid detergents, after bath colognes and hair preparations; the technique was less satisfactory for other types of materials.
3. The guinea pig immersion assay – used to evaluate the irritancy of aqueous detergent solutions. Ten guinea pigs are placed in restraining devices that

are immersed in a 40°C test solution for 4 h. The apparatus is designed to maintain the guinea pig's head above the solution. Immersion is repeated daily for 3 treatments. Twenty-four hours after the final immersion, the flank is shaved and the skin is evaluated for erythema, edema, and fissures. A photographic grading scale for this assay was presented in MacMillan et al. (1975). Only materials of limited toxic potential are suitable for this assay because systemic absorption of a lethal dose is possible. Concentration of test materials varies somewhat but is usually below 10 % to limit systemic toxicity of the agents. A second group of animals is usually tested with a reference material as a control for the material of interest.

4. An open application procedure in guinea pigs – uses microscopic examination of skin biopsies of sites treated with weak irritants to rank materials. Biopsies are taken after 3 daily applications of 10 % of solvent or 5 % aqueous test solutions to 1 cm^2 areas of the shaved flank. Sites are reevaluated visually for erythema and edema, and microscopically, 3 μm histological are stained with May-Grunward-Giemsa under oil immersion for epidermal thickness and dermal infiltration. A composite score reflecting the microscopic evaluation, number of applications before development of visible response, the epidermal thickness, and the cellular response is used to rank chemicals. This method provides information on pathogenesis of the response to each chemical but the extensive processing may limit its application to special studies.
5. Uttley and Van Abbey (1973) mouse ear test – undiluted shampoos were applied to one ear daily for 4 days. The degree of inflammation was quantified visually as vessel dilation, erythema and edema. A reference material was tested on another group of mice and the two were compared. The use of anesthetic may be a confounding factor in this assay as it may alter inflammation development.
6. Finkelstein and colleagues (1963, 1965) test – distinguishes between mild and moderate irritants in an acute exposure setting. Test sites are pretreated with an irritant. Trypan blue is injected to enhance visualization of the response by increasing test sensitivity. The technique was performed in anesthetized rabbits, rats or guinea pigs. A circular area of the shaved abdomen was painted with a 20 % solution of formaldehyde and then was allowed to dry for 5 min. This was repeated three times and then 1-in cotton flanned pads saturated with test material were applied to each site. A control substance of known irritancy was tested in each study. Pads were secured in place and then the entire trunk was wrapped in polyethylene. A solution of trypan blue was injected into subcutaneous tissue away from the dosage sites. The dye was absorbed and served as a marker for plasma leakage because it spontaneously binds Albumin. After 16 h, patches were removed and the degree of bluing at each site was evaluated on a 0 to 100 % scale.
7. Repeat Animal Patch (RAP) test – compares irritation potential of surfactants. Solutions were applied to the clipped back of immobilized albino mice with a saturated cotton-tipped applicator. The test site was covered with a rubber dam to prevent evaporation. This process was repeated seven times at intervals of 10 min. The skins were evaluated microscopically for epidermal erosion.
8. Brown (1971) – both open and closed exposures are used to rank surfactants for skin irritation potential. Tests ranged from 6-h patch exposures each day for 3 consecutive days in rabbits to daily open application to the skin of rabbits, guinea pigs, or hairless mice for up to 4.5 weeks. Good agreement among the test methods was not obtained, and none of the methods gained wide acceptance, although they are similar to techniques developed by others later.

CRITICAL ASSESSMENT OF THE METHOD

These are used largely for the developments of products that are better tolerated by consumers and industrial workers.

MODIFICATIONS OF THE METHOD

Maloney and Teal (1988) used ear thickness to quantify inflammatory changes produced by n-alkanes applied to ears of mice. They dosed animals twice per day for 4 days in order to produce inflammation. More recently, dithranol-induced skin irritation and the modulating effects of different pharmacologic agents, like the corticosteroids and lipoxygenase and cyclooxygenase inhibitor studies, were studies using the mouse ear model (Viluksela 1991).

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I.P.7 Human Irritation Tests

I.P.7.1 Single-Application Irritation Patch Tests

PURPOSE AND RATIONALE

Many forms of patch tests exist. Duration of patch exposure has varied between 1 and 72 h. The single-application patch procedure outlined by the NAS (1977) incorporates important aspects of assays used by many investigators. The procedure is similar to FHSA tests in rabbits.

PROCEDURE

Commercial patches, chambers, gauze squares, or cotton bandage material, such as Webril, applied to either intrascapular region of the back or to the dorsal surface of the upper arms are expected to produce equivalent reactions. Patches are secured in place with surgical tape without wrapping the trunk of the arm. For new volatile materials, a relatively nonocclusive tape, such as Micropore, Dermical, or Scanpore, should be used. Increasing the degree of occlusion with occlusive tapes such as Blendem or chamber devices such as the Duhring or Hilltop Chamber generally increases the severity of responses. A 4-hour exposure period was suggested by the NAS panel; however, it is desirable to test new materials and volatiles for shorter periods – 30 min to 1 hour – and many investigators apply materials intended for skin contact for 24 to 48 hour periods. Subjects should remove patches immediately if any unusual discomfort develops. After the period of exposure, the patches should be removed, the area cleaned with eater to remove any residue, and the test site marked by study personnel.

EVALUATION

Responses are evaluated 30 min to 1 h after patch removal (to allow hydration and pressure effects to subside), and again 24 h after the patch is removed. Persistent reactions may be evaluated for 3 to 4 days. The Draize scales can be used to evaluate erythema and edema; however, the integrated scales ranging from 4 to 16 points are preferred. Such integrated scales are able to score popular, vesicular or bullous responses in addition to erythema/edema evaluation. Up to 10 materials can be tested simultaneously on each subject. The position that the materials are placed on the skin (i.e., upper right back, lower left back, etc.) should be systematically varied within each study since skin reactivity varies by body region.

CRITICAL ASSESSMENT OF THE METHOD

The method has been used and modified by many authors, e.g. Wooding and Updyke (1967); Griffith et al. (1969). Regulatory agencies do not routinely require testing in men, however, human tests are preferred to animal tests in some cases secondary to uncertainties of interspecies extrapolation.

It is possible to conduct predictive irritation assays in man as only a small area of the skin needs to be tested (provided that systemic toxicity is low and informed consent is obtained). Patch test responses generally heal rapidly, within a week or so. More severe reactions should be evaluated periodically over a longer period to determine how the inflammatory response is resolved. Some subjects may develop changes in pigmentation level at the test site following severe responses. It

is prudent to prearrange for medical consultation whenever human clinical tests are undertaken.

MODIFICATIONS OF THE METHOD

Wooding and Updyke (1967) investigated the effects of modifying some test parameters on intensity of the response, and the intensity of inflammation has been shown to increase after patch removal in some cases (Rietschell 1982). A standardized procedure for evaluating the irritation potential of new chemicals in man as a replacement for the Draize rabbit test has been proposed. The method has been tested in several different laboratories and results seem to be reproducible. The classification of irritancy is based on comparison of the length of exposure to the chemical being tested versus the length of exposure producing irritation following application of a 20 % solution of sodium laurel sulfate (SLS). Chemicals producing irritation after shorter exposures than used for SLS are considered irritants. Each test subject is exposed to the undiluted test material in occlusive chambers (Hill Top Chambers) and to a 20 % solution of sodium laurel sulfate. Length of exposure begins with a 15-min exposure with evaluation at removal and at 24 and 48 h. If no response is observed, then another set of patches are applied and worn for 30 min. This process of patching, evaluation, and patching for a longer exposure interval is repeated until the subject responds to the SLS exposure or until a 4-h exposure has been completed. The test was developed for chemicals but may have use for evaluating consumer products as well. For most household chemicals and cosmetics, this cycle can probably be shortened considerably.

Nixon et al. (1975) have used a 4-hour FHSA-type procedure (including abrasion) to evaluate a range of household products.

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I.P.7.2 Repeat Application Irritation Patch Tests

PURPOSE AND RATIONALE

Inflammation produced late in the induction phase of sensitization tests without positive responses at challenge is cumulative irritation. The HRIPT for skin allergy was modified to evaluate skin irritation. As with single-application patch tests, many investigators developed their own version of the repeat application patch test.

Kligman and Wooding (1967) applied the Litchfield and Wilcoxon probit analysis to cumulative irritation testing with calculation of IT 50 and ID 50 values. Their early work forms the basis for the 21-day cumulative irritation assay, which is currently widely used.

The cumulative irritation assay was developed to compare antiperspirants, deodorants, and bath oils to provide guidance for product development.

PROCEDURE

A 1-in square of Webril is saturated with liquid, or up to 0.5 g of viscous substances, and applied to the surface of the pad to be applied to the skin. The patch is applied to the upper back and sealed in place with occlusive tape. The patch is removed after 24 h, the area is evaluated and a fresh patch applied. The procedure is repeated daily for up to 21 days. Lanman et al. (1968) increased the sensitivity of the assay by increasing the number of test subjects from 10 to 24.

EVALUATION

The ID 50, as described by Kligman and Wooding (1967), was used to evaluate and statistically compare test materials.

CRITICAL ASSESSMENT OF THE METHOD

Repeated-application patch tests on intact skin fail to predict some adverse reactions due to repeated application of materials to damaged skin (i.e., acne, shaved underarms, or sensitive areas such as the face).

The chamber scarification test was developed to evaluate materials that would normally be applied to damaged skin. Light-skinned whites who developed severe erythema with edema and vesicles following a 24-h exposure to 5 % sodium lauryl sulfate in Duhring Chambers applied to the inner forearm are preselected as subjects. Six to eight 10 mm^2 areas on mid-volar are scarified with 8 crisscross scratches made with a 30-gauge needle. Four scratches are parallel, with another four at right angles.

Although bar soaps produce erythema when tested by conventional patch test techniques, the typical clinical response is dryness and flaking with occasional erythema and fissuring. Frosch and Kligman (1979) developed the soap chamber test to compare the "chapping" potential of bar soaps.

MODIFICATIONS OF THE METHOD

Modifications of the cumulative irritation assay have been reported. Intensity of response has been evaluated using other evaluation schemes, the interval between application of fresh patches has been varied, and other methods of data evaluation have been proposed.

The newer chamber devices have replaced Webril with occlusive tape in some laboratories. Some investigators currently use cumulative scores to compare test materials and do not calculate IT50.

Kligman and Wooding performed their studies on surfactants in 10 days. Lanman et al. (1968) needed 21 applications to discriminate between baby lotions.

Finkelstein et al. (1963, 1965) described tests using either a 5–6 or a 17–18 h exposure each day for 4 days. Test sites were evaluated 1 h after patch removal. Modifications of this procedure have also been used to evaluate shaving creams and toilet soaps by Smiles and Pollack (1977).

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I.P.7.3 Exaggerated Exposure Irritation Tests

PURPOSE AND RATIONALE

Patch tests have been useful in detecting differences in the irritation potential of some materials; however, in some cases predicted differences were not apparent when the materials are used by consumers. Exaggerated exposure tests have been developed to bridge the gap between responses occurring during product use and patch tests.

PROCEDURE

One of the oldest nonpatch irritancy tests still in use is the arm immersion technique (Kooyman and Snyder 1942) in which the relative irritancy of two soap or detergent products is compared. Soap solutions of up to 8 % are prepared in troughs. Temperature is to be maintained at 105°F while subjects immerse one hand and arm to just above the elbow in one test solution and the other arm in a solution containing a second product. The period of exposure varied between 10 and 15 min 3 times each day for 5 days or until observable irritation is produced on both arms.

EVALUATION

In most volunteers, the first sign of irritation was erythema of the anticubital surface of the arm, published by Justice et al. (1961). Later the hands developed dryness and cracking, and, as a result, separate assays on the anticubitalarea and the hands have been developed. Published methods compare two products of the antecubital washing test but dosing regimes vary somewhat. Investigators have used two or three washing procedures per day, and some specify that lather is allowed to remain

on the skin for a brief period. Erythema and edema are evaluated as end points in all studies.

At least two types of hand immersion procedures have been used. In small studies (i.e., 10 subjects), relatively concentrated solutions (up to 2 %) of two materials are tested. Up to 4 hands dishwashing products have been compared at near use concentrations in studies on 64 subjects using a Latin square dosing pattern by Bannan (1975). Exposure conditions have varied from two or three 10–15 min immersions each day (Griffith 1969a) to a single 30-min exposure each day (Bannan 1975). Grading scales for this type of assay focus on scaling and cracking as well as erythema.

CRITICAL ASSESSMENT OF THE METHOD

Evaluation of skin condition before and after use in the home has also been used to compare the irritation potential of various products. These tests represent skin tolerance studies, as either irritation or allergy could be detected.

MODIFICATIONS OF THE METHOD

The clinical method published by Johnson et al. (1953) has been varied to include tests of bar soaps, laundry soaps and detergents, and dishwashing detergents. The method is a double-blind crossover study with usage periods of 2 weeks, as published by Carter et al. (1965). Skin condition is evaluated by a dermatologist before the study and after use of each product. Magnification of the area is used to facilitate grading using a 0 to 10 scale. More than 300 housewives are tested per product and up to eight materials can be evaluated simultaneously using a Latin square design. The principles used in conducting this type of large-scale usage study have been applied to laundry powders for diapers and bar soaps used in infants (Griffith et al. 1969) and to fabric softeners in adults (Weaver 1976).

Special emphasis should be placed on statistical design of clinical trials of this type to assure validity of the study, as discussed by Allen (1978).

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Chapter I.Q
Magnetic Resonance Imaging in Pharmaceutical Safety Assessment

Paul Hockings

I.Q.1 Introduction

ICI (now AstraZeneca) and Sandoz (now Novartis) introduced the first **Magnetic Resonance Imaging (MRI)** scanners into the pharmaceutical industry over twenty years ago. Most major pharmaceutical companies have since invested in in-house MRI for the evaluation of preclinical drug efficacy and most are now using MRI in clinical trials at extramural centres. MRI has been successful in the pharmaceutical industry for the same reasons that it is popular in clinical practice; it is a non-invasive imaging technique with superb soft tissue contrast capable of delivering quantitative 3D information on organ anatomy and function (Beckmann et al. 2004; Maronpot et al. 2004). Because it is non-invasive aside from the need to anaesthetise animals to immobilise them during image acquisition, animals can be imaged on multiple occasions and studies can be designed so that each animal serves as its own control increasing the statistical power of experiments and allowing group sizes to be reduced. However, despite penetration into preclinical and clinical drug efficacy studies, there are relatively few reports of the use of MRI in drug safety studies. Toxicology accounts for approximately one third of attrition in development and is thus a major cost in the pharmaceutical industry. MRI is a powerful tool that could potentially be used to reduce attrition in the late pipeline where it is most expensive. It is important to understand why MRI has not been more widely used in the drug safety arena before describing in detail a few of the MRI assays appropriate for preclinical safety studies (which includes both toxicity and safety pharmacology studies).

There are three types of safety pharmacology studies conducted in the pharmaceutical industry, 1) single dose core portfolio preclinical safety studies conducted to **Good Laboratory Practise (GLP)**; 2) supplemental studies of compound specific effects after chronic dosing that are conducted when results from the core battery of tests raise concern; and 3) 'frontloading' safety studies conducted in the drug discovery function with the aim of designing safety liabilities out of the lead compound series.

The first type of study forms part of the legally required activities towards the registration of a pharmaceutical product. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) safety pharmacology guidelines recommend the use of unanaesthetised animals, which is incompatible with the standard MRI experiment in which animals are anaesthetised to prevent motion interfering with image quality. It is certainly feasible to habituate conscious animals to the MRI environment, however in practice the results may not warrant the effort involved. In addition, it is unlikely that MRI assays will replace conventional endpoints or shorten the study duration. Thus there is little incentive to routinely incorporate MRI assays in the core package.

The second type of study is investigational studies conducted when results from the core battery of tests raise concern. In almost all cases the pharmaceutical industry prepares a comprehensive package of studies for the regulatory authorities that includes the examination of compound specific effects of candidate drugs after chronic dosing. The chronic dosing regime encourages the design of imaging experiments in which each animal acts as its own control, increasing statistical power with smaller group sizes, and allowing longitudinal studies without the need to kill groups of animals at each timepoint; two factors that separately and combined, offer dramatic sparing of laboratory animals. In these investigational studies there are no guidelines against anaesthesia although clearly

one must consider the impact of anaesthesia on each individual experiment. One of the most significant obstacles in incorporating MRI assays into investigational safety studies is that these studies are often on the critical path for drug development and therefore there is an urgency that leaves little time to develop and evaluate sophisticated new assays. Thus MRI is most appropriate to investigate adverse events that recur regularly in safety assessment departments so that the appropriate validation work with positive and negative controls can be in place before the technique is needed in earnest.

The third type of study is not regulatory studies conducted within dedicated safety assessment functions. It is now widely recognised that the pharmaceutical industry can no longer afford to start safety evaluation only after candidate selection, knowing that many candidates will quickly fail due to safety issues. The pressure is on to reduce attrition in the late pipeline by introducing safety screens in the early pipeline when it is still possible to design known safety liabilities out of the lead series. In the future we expect to see increased numbers of these early pipeline, non-GLP safety pharmacology studies of pre-candidates conducted in the drug discovery functions for purely internal decision-making purposes. These 'frontloading' studies are likely to be the most amenable to MRI as the drug project lifetime is sufficient to discover and develop the appropriate MRI assays.

Good Laboratory Practice is often considered a major hurdle in the use of MRI in safety assessment studies. GLP ensures that the data produced from non-clinical studies are of high quality, reliable and valid. Since regulators use these data to authorise clinical trials and marketing of the end product, it is important that they are correctly recorded and reproducible. An experienced, multi-disciplined and dedicated function is needed to ensure that such work is in compliance with legal requirements. The current generation of preclinical MRI scanners are not equipped with GLP software tools that would guarantee consistent spectrometer operation or data transfer in compliance with GLP. In principle there is no reason why collaboration between MRI scanner manufacturers and the pharmaceutical industry could not produce GLP compliant MRI assays however the burden of GLP documentation makes compliance for complex and innovative assays impractical. In practice, regulatory agencies do accept investigatory studies not to GLP if the work is critical to a scientifically based risk assessment and has been conducted to an acceptable standard. In this case there is still a definite advantage if protocols, data acquisition, transfer, archival, staff records and so on are in accord with GLP principles.

Despite the obstacles mentioned above the advantages of non-invasive imaging techniques to drug safety studies are obvious. It is possible to design longitudinal studies in which the same animal is studied at baseline and then at several timepoints while on study. Changes in individual animals can be quantitated and compared with baseline measurements either in simple percentage terms or, for example, using more sophisticated linear mixed effect models (Brown et al. 1999), leading to a reduction in group size and obviating the need to sacrifice animals at each timepoint. Baseline data can be used either to select or de-select animals to be included in a study or as a basis for randomisation between groups. And, of course, at the end of the study the animal is still available for other, complimentary, analysis techniques. In general, the readout time for MRI endpoints is faster than that for histology leading to faster management go/no-go decisions. Some biomarkers are only amenable via MRI, e.g. quantitation of intramyocellular lipid (IMCL) is straightforward with MR spectroscopy (MRS) but time-consuming with traditional microdissection techniques.

Further, the **imaging biomarkers** identified in preclinical safety assessment studies can also be used in clinical drug safety studies, as MRI is widely available and safe to use in volunteer studies. This can be an advantage for the preclinical safety assessment function as it provides feedback on predictability of animal models to human disease using the same endpoint. Clearly, one would not run MRI on all clinical safety studies but in those cases where there is no cheaper, simpler safety biomarker available and there is doubt about the degree of risk posed in man, for example, because of species differences or because the effect size in the placebo group is expected to be very high.

The conclusion is perhaps best put in a recent posting on the FDA website, "Imaging technologies provide powerful insights into the distribution, binding, and other biological effects of pharmaceuticals. As part of its Critical Path initiative, FDA has joined the National Cancer Institute (NCI), the pharmaceutical industry, and academia in a number of activities that will facilitate the development of new imaging agents and the use of medical imaging during product development. We believe that with a little effort on the part of all of us, imaging agents and technologies can contribute important biomarkers and surrogate endpoints during disease progression and contribute

to the development of new therapies to treat disease" (http://www.fda.gov/cder/regulatory/medImaging/default.htm).

I.Q.2 Liver Volume Measurement

PURPOSE AND RATIONALE

Liver hypertrophy is a frequent side effect in drug development caused by a wide variety of compounds. Because it is often the first indication of the hepatocarcinogenic potential of a drug candidate, liver weight is routinely monitored in safety assessment studies (Ou et al. 2001; Shoda et al. 2000). This is necessarily a terminal procedure, so that longitudinal evaluation of hypertrophy must involve serial kills of groups of animals at the time points of interest. Assuming that the compound administered does not significantly change liver density, liver volume changes should be at least as sensitive as liver weight changes. Non-invasive serial MRI measurements of liver volume can reduce animal usage by following the same groups of animals over the time points of interest. In addition, the ability to measure difference from baseline instead of a single time point liver volume usually increases the precision of treatment measurements and the resulting increase in statistical power can be used to reduce group sizes.

PROCEDURE

Rats are anaesthetized with isoflurane (1–1.5 %, 0.6–1 l/min) and then MR images are acquired. Data acquisition is synchronized with the respiratory cycle of the free breathing rats using a fibre optic respiratory monitor (Wilson et al. 1993). A segmented 3D fat suppressed inversion recovery snapshot readout sequence is used to determine liver volumes. The FOV (field of view) is 5 cm and a 128 × 96 × 96 matrix is acquired. Other parameters are SW (sweep width) 100 kHz, TE (echo time) 3.3 ms, 16 phase encoding steps per segment, and a TI (inversion recovery time) of 950 ms. Spectrometer triggering is set such that data acquisition occurs during the expiratory phase of the respiratory cycle. The TR (repetition time) is approx. 3 s allowing each scan to be completed in 30 min.

EVALUATION

Images are evaluated with AnalyzeAVW (Biomedical Imaging Resource, MN, USA) running on a Silicon Graphics O2 computer (Silicon Graphics, CA, USA). Liver volume is determined by manual segmentation of each slice using the ROI tool (Figure 1). Surface reconstructions of the liver are generated with the AnalyzeAVW Volume Renderer. To improve liver segmentation at later time points, follow-up scans can be registered to the baseline scans (Hajnal et al. 1995).

MODIFICATION OF THE METHOD

Cockman et al. (1993) used a multislice spin-echo method and reported that respiratory triggering increased the accuracy of rat liver volume measurements. Hockings et al. (2002) and Hockings, Changani et al. (2003a) reported rat liver volumes obtained with the respiratory triggered segmented 3D fat suppressed inversion recovery snapshot readout sequence described above at both 7 Tesla and 2 Tesla and reported a correlation coefficient between in vivo MRI liver volume and post-mortem liver wet weight of 0.96 and 0.99 respectively. Tang et al. (2002) used a non-respiratory triggered multislice spin-echo method in rats and reported a correlation coefficient of 0.9 against liver wet weight with a systematic overestimation of MRI liver volume. The coefficient of variability of MRI precision was 2.3 % and operator reliability for segmentation 2.9 %. Garbow, Kataoka et al. (2004a) measured liver volume in mice with MRI at 4.7 Tesla using an intraperitoneal injection of contrast reagent to increase contrast between liver and surrounding organs. The correlation coefficient between MRI volume and wet weight was 0.94.

CRITICAL ASSESSMENT OF THE METHOD

The correlation between MRI liver volume and liver weight has been established by a number of groups using a variety of MRI methods indicating the robustness of the technique. Its advantage over the direct measurement of liver weight is the dramatic sparing of animals as groups of animals no longer need to be sacrificed at each time point and because the ability to make within animal comparisons leads to greater precision and a reduction in group sizes. Hockings et al. (2002) reported a reduction in animal usage from 120 to 6 with the same level of precision. In order to measure liver volume with precision it is necessary to produce good contrast between liver and surrounding tissues such as intercostal muscle, fat, spleen, stomach wall and kidney. This can be done through judicious optimisation of the MRI pulse sequence and timings. In addition, some researchers have used fat suppression pulses to null the signal from fat to enhance contrast to surrounding organs. Image acquisition normally takes several minutes so motion from breathing and peristalsis in the GI tract can produce artefacts and blurring of the images. Fast imaging, averaging, breath holding or respiratory triggering strategies can reduce

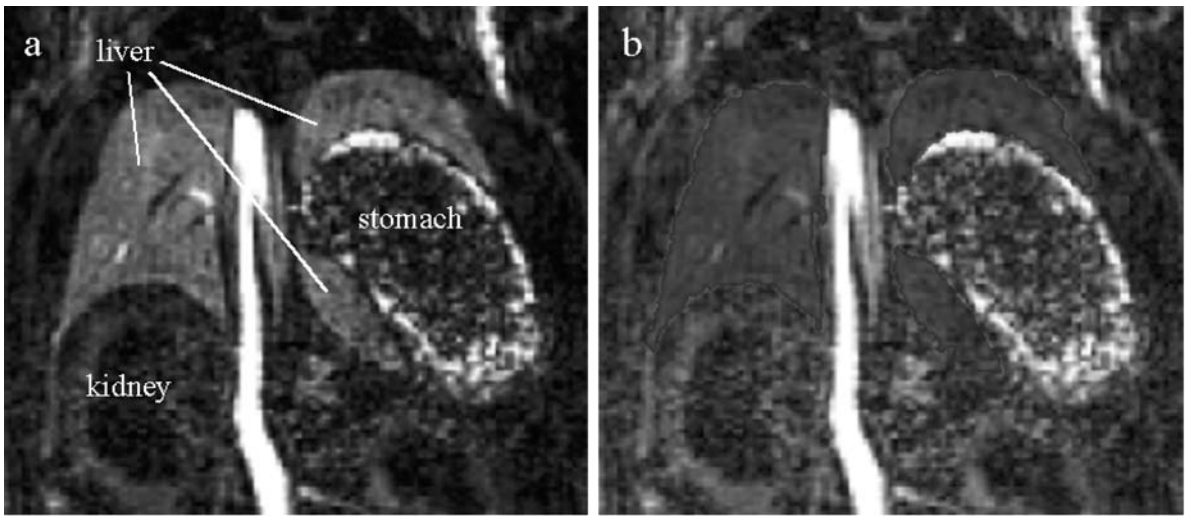

Fig. 1. MRI coronal section through a rat liver showing good contrast from surrounding tissues (a), and image segmentation of the liver (b)

motion artefacts from respiration. The respiratory triggering strategy synchronizes data acquisition to the respiratory cycle and is the most widely applied strategy for preclinical liver volume determination. Peristaltic motility can be reduced by overnight starvation or the application of spasmolytic such as buscopan however neither approach is usually necessary.

One possible confound for this experiment is that liver weight changes by up to 15 % during the day as glycogen levels drop (Latour et al. 1999) and so care must be taken in longitudinal studies that animals are always imaged at the same time of day to reduce within animal variance. In addition, care must be exercised with the choice of anaesthetic as anaesthetics such as halothane are hepatotoxic and may influence the outcome of the study when there are several imaging sessions.

I.Q.3 Cardiac Hypertrophy

PURPOSE AND RATIONALE

Measurement of **cardiac function** and morphology is a key part of the preclinical evaluation of experimental medicinal compounds. Blood pressure, heart rate, and electrocardiogram evaluation are part of the core portfolio of safety pharmacology studies carried out in conscious telemetry dogs. If results from the core battery of tests raise concern then supplemental studies are conducted to measure endpoints such as left ventricular pressure, pulmonary arterial pressure, heart rate variability, baroreflex, cardiac output, ventricular contractility and vascular resistance. However, many of these endpoints involve invasive surgery and so are only appropriate for acute single time point studies. To date there have been relatively few pre-clinical studies using MRI to measure cardiovascular function, especially in the dog which is a large animal species widely used in toxicology. MRI can be used to determine myocardial volume, wall thickness, left ventricular (LV) and right ventricular (RV) end-diastolic and end-systolic lumen volumes (EDV and ESV respectively). These parameters can be subsequently used to derive functional indices such as wall stress, degree of eccentric hypertrophy, stroke volume (SV), cardiac output (CO) and ejection fraction (EF). MRI studies are particularly suited to chronic dosing regimes with multiple imaging timepoints in the same animals.

PROCEDURE

Adult male beagle dogs (Harlan U.K.) weighing between 9 and 14 kg are used. On days prior to scanning food is withheld from approximately 4 p.m. Dogs are anaesthetised with a bolus intravenous dose of propofol (approx. 10 mg/kg) followed by propofol (32–42 mg/kg/h) maintenance anaesthesia and ventilated with medical air via an endotracheal tube. The dorsal metatarsal or femoral artery is cannulated for blood pressure measurements and to enable sampling of arterial blood for monitoring blood gases to ensure adequate ventilation. ECG, capnography, pulse oximetry, body temperature and arterial blood pressure are monitored throughout the scanning sessions on a Bruker Maglife C (Wissembourg, France). Body temperature is maintained with the aid of a thermostatically controlled heating blanket.

MRI scanning is performed in a 1 m bore, 2T Bruker Medspec (Ettlingen, Germany) using a 28 cm transmit/receive birdcage resonator. ECG triggered segmented gradient echo cine images are acquired during the expiration phase of the respiratory cycle as measured directly from the ventilator. An average of 16 frames per heart cine traverses approximately 80 % of the cardiac cycle starting from end-diastole. Other relevant imaging parameters are: Gradient echo flip angle 20°, TE 3 ms, TR 8 ms, 1 to 3 averages, SW 100 kHz, image matrix 128 × 128, in-plane field of view 200 mm, 4 phase-encoding steps per frame and linear traverse of k-space. Hence, the time resolution per cine frame is 32 ms. Each individual slice cine is acquired in about one to one and a half minutes depending on heart rate, so each set of multislice cines takes about 15 to 20 minutes.

To obtain true short axis views, scout imaging commenced with a mid-ventricular coronal slice allowing the vertical long axis (VLA) to be located by aligning another scout through the apex and mid-mitral valve, thus allowing for the leftward angle of the heart. From the VLA, the downward inclination of the heart is allowed for by taking a further scout lining up the apex and mid mitral valve to generate the horizontal long axis plane (HLA). The scouts are acquired at end diastole (0 ms delay after the QRS wave) so that the atrioventricular (AV) ring, which descends apically in systole, is in its most basal position. The first short axis cine is then placed just forward of the AV ring on the HLA image, to cover the most basal portions of the right and left ventricle. Approximately 15 contiguous 5 mm thick segmented gradient echo cines with no interslice gap are then sequentially acquired moving towards the apex, and including the apical tip. In this way, the entire ventricle is imaged.

EVALUATION

Frames corresponding to end diastole and end systole are identified from each cine sequence and regions-of-interest (ROI) drawn around the left ventricular (LV) epi- and endocardial borders using ParaVision software (Bruker). The area of the ROIs is summed and multiplied by the inter-slice distance (5 mm) to calculate the end diastolic and end systolic volumes (EDV and ESV) of the whole ventricle and lumen. Other cardiac parameters are calculated as follows:

$$\text{Stroke Volume} : \text{SV} = \text{EDV}_{\text{Lumen}} - \text{ESV}_{\text{Lumen}}$$

$$\text{Cardiac Output} : \text{CO} = \text{SV} \times \text{HeartRate}$$

$$\text{Ejection Fraction} : \text{EF} = (\text{SV}/\text{EDV}_{\text{Lumen}}) \times 100$$

Left ventricle myocardial mass at end systole is calculated as:

$$\text{Mass}_{\text{LV}} = (\text{ESV}_{\text{Ventricle}} - \text{ESV}_{\text{Lumen}}) \times \text{D}$$

where D is the density of the myocardium (1.05 g/ml) (Hoffmann et al. 2001).

Left ventricle myocardial wall thickness in diastole is calculated from the epi- and endocardial areas at the slice where the epicardial area is maximum as follows:

$$\text{LV wall thickness} = \sqrt{\frac{\text{Area}_{\text{LV}}}{\pi}} - \sqrt{\frac{\text{Area}_{\text{Lumen}}}{\pi}}$$

The two ROIs used are assumed to be concentric and circular.

MODIFICATION OF THE METHOD

Markiewicz et al. (1987) examined eight pentobarbital anaesthetised dogs and reported that cardiac output and stroke volume measured by ECG triggered MRI correlated significantly with thermodilution measurements ($r = 0.73$ and 0.93 respectively). Shapiro et al. (1989) also used ECG triggered MRI in dogs subjected to myocardial infarction and found excellent correlation between MRI derived myocardial mass and wet weight ($r = 0.97$) and that MRI derived myocardial mass measured in systole and diastole correlated closely ($r = 0.95$). Bambach et al. (1991) examined carbon monoxide induced ventricular hypertrophy in rats using scan averaging instead of triggering to reduce artefacts from cardiac motion. They found that the mean outside diameter of the left ventricle plus interventricular septum (LV + S) showed a strong correlation with the duration of CO ($r = 0.73$, $p < 0.01$) and to the haematocrit ($r = 0.72$, $p < 0.05$). Rudin et al. (1991) used a dual respiratory gated and ECG triggered approach in two models of cardiac hypertrophy in rats. The correlation coefficient between LV mass determined by MRI and post mortem LV weight was 0.99 and LV volume, SV and EF in control animals showed statistically significant differences from cardiac hypertrophy animals. Siri et al. (1997) applied ECG triggered MRI to murine hearts and found LV mass determined by MRI correlated well with LV weight ($r = 0.87$). This data demonstrated the dependence of LV mass estimates in the mouse on the geometric model of the heart used and show that MRI provides more accurate estimates of LV mass in mice than does two dimensional directed M-mode echocardiography. Slawson et al. (1998) used a dual respiratory and cardiac gated MR sequence in mice and obtained a correlation coefficient of 0.99 between MRI

and post-mortem heart weight. Hockings, Busza et al. (2003a) used the method described above to measure dobutamine and minoxidil induced changes in cardiac function in dogs. They showed good correlation between cardiac output measured by MRI and cardiac output measured by thermodilution ($r = 0.94$) and that MRI could reliably detect acute changes in cardiac output induced by dobutamine infusion ($p = 0.01$) in small groups of animals ($n = 7$). Furthermore, they showed that MRI could detect LV enlargement induced by chronic administration of minoxidil and that the increase in EDV without an accompanying change in LV wall thickness indicated a preload induced hypertrophy. Interestingly, the MRI technique was able to detect small amounts of pericardial effusion.

CRITICAL ASSESSMENT OF THE METHOD

MRI has become the gold standard imaging technique for the study of the human heart. The main advantages are that it is non-invasive, has pronounced contrast between myocardium and blood, and good temporal resolution allowing images to be acquired at any phase of the cardiac cycle. Thus it is an accurate technique for measuring ventricular volumes independent of geometric assumptions, although clearly the precision with which myocardial geometry can be characterised depends on the number of image slices acquired through the heart and on the in-plane resolution. Image acquisition during end diastole and end systole allows the calculation of functional parameters such as stroke volume, ejection fraction and cardiac output. One of the most important factors in the acquisition of artefact free images is the quality of the MRI system's ECG and respiratory triggering. Cardiac exams in the clinic are usually conducted using breathhold rather than with respiratory gating because of the difficulty of obtaining a regular breathing cycle in conscious volunteers and patients. However, in anaesthetised animals breathing irregularities are not usually a significant problem and complications due to the increase in heart rate with hypercapnia during breathhold usually outweigh the time penalty involved in waiting for the respiratory gate. The studies described above indicate that combined respiratory gating and ECG triggering improve the precision of measurements.

Alternatives to MRI include echo cardiography to measure LV wall thickness, lumen volume and cardiac output (Coatney 2001; Collins et al. 2003; de Simone et al. 1990; Zhou et al. 2004), dye-dilution techniques such as bolus thermodilution to measure cardiac output (Siren et al. 1990), and implanted pressure transducers and flow probes to measure left ventricular pressure and blood flow parameters. Like MRI, echocardiography is non-invasive and has the further advantages that it provides low cost, real-time images with structural, functional and haemodynamic information. Functional information is usually acquired in M-mode and hence it is necessary to make geometrical assumptions that may not be applicable if heart morphology changes. Ultrasound is also operator dependant relying on the knowledge and expertise of a well-trained sonographer. Both dye-dilution and implanted pressure and flow probes are invasive techniques.

When planning functional studies it is important to consider that most anaesthetics cause cardiac and respiratory depression. For chronic studies it may only be important to ensure that the depth of anaesthesia is reproducible from imaging session to imaging session, however for acute studies it is necessary to consider interactions between the anaesthetic and the test substance. The complexity of cardiac structure and function needs to be understood to devise a well-planned imaging protocol.

I.Q.4 Hepatic Steatosis

PURPOSE AND RATIONALE

Hepatic steatosis is a side effect associated with a number of classes of compounds including some metal compounds, cytostatic drugs, antibiotics, and estrogens. In some cases drug-induced hepatic steatosis patients can present with a rapid evolution of severe hepatic failure, lactic acidosis and ultimately death (Diehl 1999). The absence of predictable correlation between abnormalities in liver enzymes and histologic lesions led Clark et al. (2002) to conclude that localised **Magnetic Resonance Spectroscopy (MRS)** was the best non-invasive way to quantify liver fat in patients. This approach was favoured because it avoids the risks associated with invasive liver biopsy. Lee et al. (1984) demonstrated that MRI can detect fatty infiltration of the liver clinically and Longo et al. (1993) that MRS is a reliable non-invasive method, comparable to computerized tomography (CT), for quantifying clinical liver steatosis in humans. Recently Szczepaniak et al. (2005) used localised MRS to show a strikingly high prevalence of hepatic steatosis in the US population.

For twenty years localised MRS has been used in medicine and biomedical research to obtain non-invasive biochemical information from living tissue (Koretsky et al. 1992). The spectra obtained possess the very valuable property that the intensity of a given peak is proportional to the number of nuclei contribut-

ing to that peak provided that certain experimental precautions are taken. This allows a quantitative determination of a substance if there is an appropriate internal or external reference. In the case of localised in vivo 1H spectroscopy the water signal is usually chosen as internal standard as the proportion of body water to ash and protein is relatively invariant. Single voxel localised MRS allows spectra to be obtained with spatial resolutions down to 15 microlitres in some circumstances allowing localisation of a volume of interest entirely within the liver in animals as small as mice (Figure 2).

PROCEDURE

Isoflurane anaesthetized rats were scanned in a Medspec S200 (2T 1m bore) MR scanner (Bruker, Ettlingen, Germany) using a 10 cm inner-diameter transmit/receive radiofrequency birdcage-design resonator (Spin Systems, Brisbane, Australia). MRI and MRS acquisition was synchronized with the respiratory cycle to minimize artefacts (Schwarz et al. 2000; Wilson et al. 1993). Scout multislice spin-echo images through the liver were used to determine voxel placement. Localized 1H PRESS spectra (Bottomley 1987) (TE/TR 20/2000 ms, NS 128) were obtained from a 5 × 5 × 5 mm cube in the right lateral lobe adjacent to the portal vein and well removed from the surface of the liver and distinct hyperintense fatty deposits.

EVALUATION

Quantification was accomplished by simulating the water signal (which was used as a chemical shift reference) at 4.7 ppm, and the fat signals at 2.1, 1.3 and 0.9 ppm, with a 60:40 Gaussian:Lorenzian lineshape using the Bruker XWINNMR package. Without knowing the average lipid chain length and degree of unsaturation, it is impossible to calculate a valid molar fat:water ratio, so the relative fat content is expressed as the percentage of the sum of the fitted peak areas of the three fat peaks to the fitted water peak area.

MODIFICATION OF THE METHOD

Hazle et al. (1991) used MRS to follow the time course of ethanol induced liver steatosis in rats. Spectra were acquired without respiratory triggering and lipid signal was normalised to signal from an external reference sample. Correlation between MRS normalised lipid signal and biochemically determined lipids was moderate (r = 0.52). Ling et al. (1992) used respiratory triggered MRS to examine the same model and were able to show that a 5.5-fold increase in lipid signal on treatment was matched by ex vivo analysis although a correlation coefficient was not given. Szczepaniak et al. (1999) used two animal models to show a close correlation between hepatic triglyceride measured by in vivo MRS and liver biopsy (r = 0.93). These researchers converted the MRS fat:water signal ratio to micromoles triglyceride/gram wet tissue by correcting for NMR relaxation and triglyceride proton density relative to water. Daubioul et al. (2002) used non-triggered localised MRS to show a reduction in hepatic steatosis in Zucker rats fed a dietary supplement of nondigestable carbohydrates. The spectra presented showed artefacts consistent with respiratory motion during acquisition. Hockings, Changani et al. (2003a) used the method described above to measure the MRS fat:water ratio in the livers of Zucker rats. They found a good correlation between MRS fat:water ratio and the fractional volume of intrahepatic fat determined by histology (r = 0.89) and were able to show that rosiglitazone treatment reduced liver fat content. Kuhlmann et al. (2003) reported similar findings in Zucker Diabetic rats treated with rosiglitazone. Liver lipid levels in mice were examined by Garbow, Lin et al. (2004). They reported that respiratory triggered acquisition of spectra was important to remove the deleterious effects of respiratory motion and that the variation in MRS lipid content across the liver was typically less than 10 %. The correlation coefficient between in vivo MRS and ex vivo wet chemistry lipid measurements was 0.95. Zhang et al. (2004) reported the use of a respiratory triggered 3D three-point Dixon MRI method to determine liver fat:water ratio in rats treated with a microsomal transfer protein inhibitor known to produce hepatic steatosis. They reported a high level of reproducibility in *in vivo* measurements and were able to detect drug-induced steatosis but the correlation coefficient against liver triglyceride and information on spatial inhomogeneity of lipid accumulation in the liver was not given.

CRITICAL ASSESSMENT OF THE METHOD

A number of both clinical and preclinical studies have shown a robust correlation between liver fat:water signal ratio measured by in vivo localised MRS and ex vivo analysis. Most groups have used a short echo time PRESS sequence with respiratory triggering to reduce motion artefacts and water as an internal standard. Both liver biopsy and single voxel localised MRS are hampered by sampling errors if fatty infiltrations are inhomogeneously distributed in the liver. In the clinical setting alternative MRI or spectroscopic imaging techniques have been used to measure lipid content

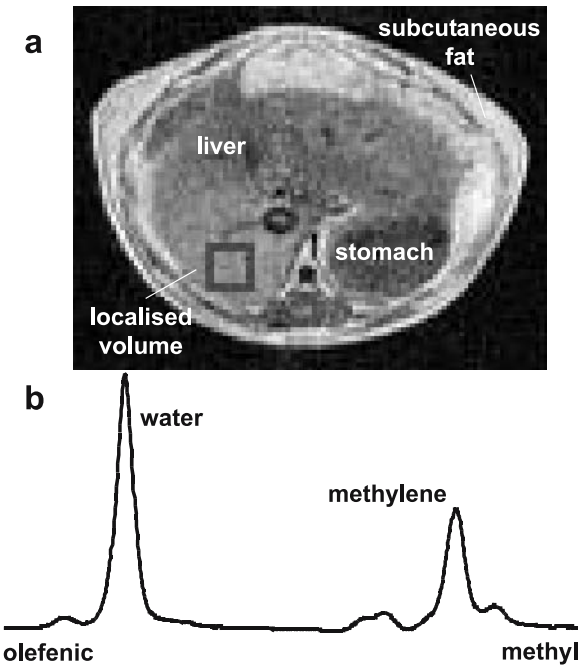

Fig. 2. a) Axial view through the liver of a Café diet mouse showing the position of the 3×3×3 mm localised MRS voxel in the right lateral lobe of the liver, and b) the resultant in vivo localized PRESS spectrum (acquisition parameters: Bruker Biospec 4.7T 40 cm magnet; repetition time approx. 3000 ms (dependant on respiration rate); echo time 6.8 ms; number of averages 64) (Abdel Bidar, AstraZeneca; personal communication).

across the entire liver where there is a risk of fatty infiltrations. Preclinically, Ling et al. (1992) have shown that fat is distributed homogeneously throughout the liver in rats with ethanol induced hepatic steatosis and Garbow, Lin et al. (2004b) reported similar findings for wild-type and two transgenic strains of mice on low fat or high fat diets. Most researchers avoided the problem of potential inhomogeneous lipid distribution by selecting one region of the liver and always returning to the same region in serial timepoint studies. For preclinical studies it is clearly possible to kill groups of animals at each timepoint, but, particularly when the within group variability is large in comparison to the measurement precision, the introduction of a non-invasive technology can result in a dramatic sparing of animals.

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Section II
Safety Pharmacokinetics

Chapter II.A
Introduction

Jochen Maas

The present volume of this book focuses on the impact of pharmacokinetics and metabolism in the safety of drugs. Pharmacokinetics per se is the study of absorption, distribution, metabolism and excretion of drugs or – in other words – describes the exposure and elimination of a potential drug in the body. Some of the readers may now ask "where is the connection to safety?" In general, and with only a few exceptions (for instance, locally acting drugs in the gastrointestinal tract), exposure is a conditio sine qua non both for the efficacy of a drug and for its toxicity. Pharmacodynamic effects and safety-related effects depend directly on the exposure and therefore on the pharmacokinetics of compounds. Consequently, a textbook about safety assays has to include pharmacokinetic assays as well.

In the last 15 years, drug discovery and development has changed rapidly. In silico models, combinatorial chemistry, high-throughput screening and various-omics approaches have been introduced widely. And the impact of pharmacokinetics and pharmacokinetic assays has considerably increased: Whereas 15 years ago pharmacokinetic data were regarded as "nice to have" in earlier phases of the discovery process, those data became more and more important and reached a status as a selection criterion for compounds already in the late 90s. Today we are in a situation where druggability parameters (and PK-parameters are the most important ones) and efficacy parameters are balanced out from the beginning of the discovery and development process: this multidimensional compound optimization is currently replacing the unidimensional, only efficacy-driven approach of the past.

First results of this early consideration of pharmacokinetic parameters are already visible: the exposure-related attrition of compounds in the preclinical and clinical stage of development decreased considerably in many companies. Nevertheless, the attrition rates based on toxicological reasons remains high, resulting in many approaches to decrease it. All of them require information about the exposure, the earlier the better. And information about exposure requires pharmacokinetic assays as they are summarized in this part of the book.

But we have to consider an "assay" in an extended way as well. A simple technological description of the assay per se normally will be not sufficient to explain the specific pharmacokinetic and toxicological behavior of drugs. Besides the technology part, it is often very important to be familiar with the studies to be conducted to create pharmacokinetic data. Therefore we also decided to include typical study designs in animal and human PK-studies in this assay compilation. Since the clinical part of safety studies is often underrepresented in pre-clinically oriented textbooks and since the design of human studies is often the game winning activity for a marketing success of a drug, a detailed chapter about studies to determine the human exposure was regarded as mandatory.

In some cases it was regarded as useful for the complete understanding of the assays to include a general introduction into the method.

The first section deals with physicochemical properties of compounds, which could influence their pharmacokinetic behavior tremendously. In particular, solubility and lipophilicity impact the penetration across membranes resulting in different pharmacokinetics. The next sections are focused on the four big pillars of pharmacokinetics: absorption, distribution, metabolism and excretion. Each of those is subdivided into specialized topics explaining more specific and more detailed aspects. Since many patients take some drugs simultaneously, drug-drug interactions is an important topic to be considered both under a pharmacokinetic and a toxicological point of view.

The tendency to predict the pharmacokinetic situation in humans as early as possible required one section about the earliest possibility, the in-silico modeling. The relationship between pharmacokinetics

and pharmacological/toxicological efficacy and the extrapolation of single data to the population are covered in specific sections. Those sections contribute – together with the chapter about the design of clinical studies – to our ambition to cover a comprehensive overview of pharmacokinetic approaches.

I would like to thank the authors for their efforts in bringing this book to completion. No doubt the rich practical and theoretical experience of the authors expressed in this volume will be of great value for all readers, experienced or junior, and this volume will be a treasure in many laboratories.

Chapter II.B
Physicochemical Properties

Heiko Tietgen

PURPOSE AND RATIONALE

Characterization of physicochemical properties attains growing interest in the pharmaceutical research arena. It is one of the key challenges to develop a pharmaceutically active ingredient into a drug which combines biological activity with an appropriate physicochemical profile. Poor solubility in aqueous media is one of the major hurdles in the drug development process. Many promising drug candidates have failed simply due to inadequate solubility.

A poor solubility can be overcome if a compound has an appropriately high permeability through a cell membrane. Passive permeability through a membrane has been correlated with the lipophilicity of a compound in many cases (Lipinsky). Generally speaking, high lipophilicity will ease passive cell permeation but may result, in general, in poor soluble compounds. Finding a compromise here belongs to the art of drug discovery.

Another important physicochemical parameter is the pKa, which describes the ionisation state of a compound at a given pH. The ionisation state of a compound in the different components of the gastro intestinal system (stomach, jejunum, ileum and colon) is crucial for the understanding of drug absorption (Dressman). Ionised compounds generally have better solubility, but passive permeation through the membrane is limited (Comer).

All these parameters have been long investigated in drug discovery but were part of the later stage of the process. Nowadays the characterisation of physicochemical properties has also moved into the lead finding phase of drug discovery (Kibbey). Thus screening assays had to be developed which were able to give comparably accurate data and a throughput to characterize hundreds of compounds a day (Kerns).

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II.B.1
Solubility Assays

PURPOSE AND RATIONALE

Solubility assays are gaining growing attention in drug discovery, because many pharmaceutically active compounds can be adjusted to in vivo testing merely with co-solvents. Furthermore, in vitro assays may also lead to false results, simply for precipitation of a compound in the assay media. Solubility assays vary in one main point: they are either performed from solids or stock solutions. A nomenclature has been established in the literature which tries to distinguish between these methods. Determinations from stock solutions are often called “kinetic solubility” whereas “thermodynamic solubility” stands for solubility of solids (Kerns). Thermodynamic solubility takes the crystal lattice forces into account. Batch to batch variations, polymorphism

of investigated compounds are neglected in early stage of discovery but may become a key focus at a later stage. Ritonavir is a prominent example (Law).

Stock solutions – already prepared by central logistic centres – have the advantage of standard start conditions, which are also similar to conditions found in biological assays. They allow a high throughput because the time delaying sample preparation is already done.

II.B.1.1 Determination of Solubility by Hyphenated HPLC Methods

PURPOSE AND RATIONALE

The range of solubility assays has to be < 0.01 mg/ml up to > 10 mg/ml in some cases. In vivo tests (oral or intravenous) need sufficient solubility of a compound in physiological or buffered solution. "True" data are also needed in compound optimization, where solubility is one of the parameters to be improved. Classification in poor, medium or good solubility is often not sufficient. Hyphenated HPLC methods have a long tradition in quantification and characterization of compounds in analytical sciences. Many reviews and handbooks cover this field (Unger, Lunn). The detection of the compounds is done with UV, mass spectroscopy, light scattering or by nitrogen detection. Additionally to the quantification of the solubility, impurities and side products are detected. Furthermore, buffer instability can be determined.

PROCEDURE

As described in the general introduction for solubility assays, there is a difference between kinetic and thermodynamic solubility. Thermodynamic solubility needs solid material of good quality whereas kinetic solubility relies on organic stock solutions. The sample preparation is therefore different (Kibbey).

The procedure for determining solubility follows standard quantification methods with hyphenated HPLC. 0.5–1 mg of solid is weighed in a micro-vial and 250 µl buffer is poured over the solid. The vial is shaken for 24 hours at 25 °C. After shaking the suspension is centrifuged and the supernatant is filtered. An aliquot (1–10 µl) of the supernatant is injected in a HPLC system. Eluents are generally acetonitrile and water containing 0.5 % trifluoric acid or 0.5 % formic acid. Both eluents are mixed via gradient device from 5 % acetonitrile to 95 % acetonitrile content. Separation is obtained on a reversed phase column. The same aliquot of a standard is injected in a following run if one point calibration is done (Pan). Greater accuracy but longer evaluation time is needed if a five point calibration is done covering a range between 0.1 µg/ml up to 2000 µg/ml. Gradient time is set either to have higher throughput (fast gradient) or better chromatographic resolution.

The quantification is done via a calibration curve where the compound is weighed as standard and dissolved in an appropriate organic solvent (in most cases acetonitrile/water). The throughput of this method is 20 compounds a day.

If the standard stock solution is delivered from a centralized stock solution the throughput can be increased significantly. Solubility obtained from organic stock solutions leads to kinetic solubility data (see also chapter 1.2).

An aliquot of 30 µL is transferred in 250 µl buffer. The solution is shaken for 24 hours at 25 °C. If precipitation occurs the sample is centrifuged and filtrated. The following procedure is the same as for the solubility from solids described above. The achieved throughput is mainly limited by the gradient time. Pan et al. investigated the effect of filtration on the quantification of solubility. They recommend poly(tetrafluoroethylene) (PTFE) as filter material of choice. In their compound set they found 98 % recovery after filtration.

Quantification of the compounds is done via different detection modes as:

1. UV or photodiode array (PDA)
2. Mass spectroscopy (MS)
3. Nitrogen detection (CLND)
4. Light scattering (ELS)

and combinations of them (Guttman). Evaluation of the compound structure before quantification is necessary, because not all compounds have for instance a chromophor (UV-detection) or nitrogen (CLND).

EVALUATION

High throughput methods use one point calibration. The quantification of the solubility (c_{sol}) is done via the peak areas under the curves (AUC) of standard and measurement. For one point calibration:

$$c_{sol} = \frac{AUC_{sol}}{AUC_{st}} * c_{st}.$$

Identification of the compound is done either via the retention time and/or the mass of the peak, which is to be quantified. If the chromatographic resolution is not good enough to separate impurities or degradation product the measurement has to be repeated

with longer gradient times or longer reversed phase columns.

CRITICAL ASSESSMENT OF THE METHOD

In early stage of drug discovery the main variance of solubility data are in most cases linked to batch-to-batch variations of a synthesized compound. Most batch-to-batch variations are related with different side-product profile or purity, and differences in the crystallinity. In extreme cases, oily, amorphous or crystalline batches may be obtained for one compound. These effects superimpose errors coming from the experimental conditions.

Very poorly soluble and very well soluble compounds will give an AUC far different from the standard injection. This may leave the linear range of the calibration. They are therefore quantified far more accurately with 5-point calibration, which covers a wide concentration range. Very well soluble compounds should be diluted to reach the linear range of the $AUC/c_{analyte}$ curve.

Very poorly soluble compounds are in most cases lipophilic. They tend to stick to the used filter systems or used plastic devices, which leads to compound loss. Thus very poorly soluble compounds may be underestimated due to the sample preparation (Pan).

Each detection system has its own limitations. Compounds with no chromophor are not UV active, for instance, and may be quantified with ELS. A short analysis of the compound class is therefore needed to prepare and interpret the solubility experiments. Direct quantification of the sample without any external calibration is possible if a nitrogen detector is used. Throughput is higher and a standard injection is not necessary.

If a compound with high solubility exceeds the buffer capacity it may change the pH of the buffer system. This is not realized without any pH control, which could be difficult to control in a standardized and miniaturized environment. This methodology fails for compounds without nitrogen or with nitrogen-nitrogen double bonds.

MODIFICATIONS OF THE METHOD

To achieve high quality data with low sample amount, a commercial pSOL instrument (pION, Woburn, MA) can be used. It delivers with an pH metric approach a solubility pH profile with 100 μg of solid compound. The throughput is 4–6 compounds a day.

TREGA describes a protocol for pH adjusted measurements (TREGA) They need very accurate data for their idea and a computational program to simulate drug absorption in the gastro intestinal system. Five times 30–50 mg of compound are weighed in 10 ml vials. The vials are filled with 8 ml of buffer covering a pH range between 2 and 8. The vials are shaken for 8 hours and the pH is controlled. If necessary, the pH is adjusted with HCl and NaOH. After pH adjustment the compounds are shaken for additional 2 hours. The pH adjustment is done until saturation solubility is achieved and the pH is stable. The experiment is tedious and reduces the throughput to a couple of compounds a day.

Kerns et al. reported a method whereby solid compounds are weighed into a Mini-Uni-Prep™ filter chamber. 0.45 aqueous buffer is added and the chamber is shaken for 24 hours at room temperature. After automatic filtration the supernatants are directly injected in a HPLC instrument. The throughput is reported to be 50 compounds a day per HPLC instrument.

Dressman reviewed the methodologies used for simulating bio fluids. These methodologies focus on the solubility behavior and dissolution rate in solvents, which mimic the fluids in the gastro intestinal system like Fessif and Fassif. These dissolution tests were invented for better prediction the in vivo performance of drug products. They are barely comparable with solubilities in buffered aqueous media and have a limited throughput of a couple of compounds a day.

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II.B.1.2 Highthroughput Solubility Assays

PURPOSE AND RATIONALE

In vitro testing of drug candidates and combinatorial chemistry lead to an increase of lipophilic compounds with poor solubility (Lipinsky). Poor solubility itself may lead to poor oral availability of a potential drug. The growing demand for solubility data in lead phase of drug discovery is answered by a variety of simple solubility assays, which allow the classification of a compound without real quantification (see previous section). One of the easiest way to detect saturation in a solvent is the turbidity of the solution if precipitation occurs. The turbidity caused by precipitation of a poorly soluble compound can be detected by a couple of detection methods (Van de Hulst, Hongve). Lipinsky describes the first methodology, which use UV as detection method and is able to screen hundreds of compounds a day with one instrument.

PROCEDURE

Lipinsky dissolved compounds in DMSO at a concentration of 10 μg/ml. Complete dissolution is controlled by eye. One micro liter of this solution is added into a cuvette containing 2.5 ml pH 7 phosphate buffer. Mixing of the system is controlled via an integrated mixing device. The temperature is kept constant between 22 °C–25 °C. Stepwise one micro liter portions are added to the mixing chamber. After each step a equilibrium time of 5 minute is allowed before turbidity is analysed. These steps are repeated up to 14 times covering a range of < 5 μg/ml to > 65 μg/ml. If precipitation occurs the addition of compound is stopped after further two consecutive additions. The volume percent aqueous DMSO does not exceed 0.67 %.

EVALUATION

Precipitated particles lead to an increase in UV absorbance due to light scattering. Lipinsky used a diode array UV (Hewlett Packard HP8452) at 600–820 nm for their experiment. UV absorbance (y-axis) vs. μL DMSO plots (x-axis) is used to detect the precipitation point. A strong increase in the slope of the curve indicates precipitation. Precipitation defines the maximum solubility level in this experiment. The method allows a classification between poor, moderate and good soluble compounds. Poorly soluble compounds have in this scheme a solubility less than 10 μg/ml, whereas good solubility is defined as a solubility higher than 65 μg/ml.

CRITICAL ASSESSMENT OF THE METHOD

The method has been used and modified by various authors (Bevan, Green) and is today a standard solubility assay in industry. Advantages are the simplicity of the experiment and the low sample consumption. Impurities of the samples (which are common in the early drug discovery) are not detected and could lead to false positives if the mixture has a solubility better than the pure material. False negatives are observed if impurities have lower solubility as main material. Growing DMSO content alter the solubulizing properties of the aqueous media. Up to 5 % DMSO content are seen as appropriate for a solubility ranking. It does reflect also the situation in many biological assays where the DMSO content is up to 5 % (Lipinsky). However, the quality of the data allows a ranking of compounds in poor/medium/good soluble compounds.

The method underestimates compounds with slow dissolution rate. To minimize the risk of a wrong assessment control measurements with other methods (preferably HPLC-methods) are recommended. This could be done with one or two members of a compound class.

MODIFICATIONS OF THE METHOD

Bevan introduced a high-throughput alteration of the method. He uses 10 mM DMSO stock solutions for a precipitation assay. In many biological assays 10 mM DMSO stock solutions are the standard stock solution, from which aliquots are added to the various assays. 10 mM DMSO stock solutions are readily available and the sample preparation can be minimized.

The assay itself is performed in a 96 well plate. DMSO stock solution is diluted 20 fold in PBS (0.01 M, pH 7.4). The diluted solution is again diluted 10 times with 5 % DMSO/95 % PBS across the 96 well plate in order to keep 5 % DMSO content stable. The solubility assay is performed with 2 replicates for each compound, thus 8 compounds can be measured per well. Detection of the precipitation is done with a helium/neon laser nephelometer (BMG Labtechnologies, Offenburg, Germany). The instrument lases at 632.8 nm, whereby the laser beam passes the well in a vertical and concentric path. The instrument detects just linear light from the source. A clear solution gives no signal.

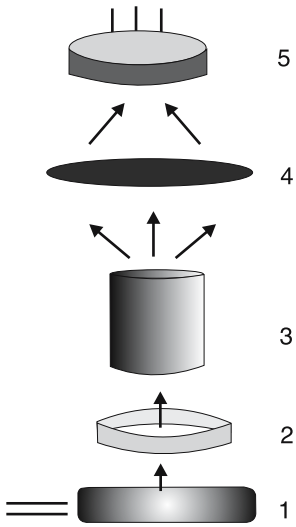

Fig. 1. Principle of nephelometric detection.

Precipitation leads to light scattering which is detected. Following solubility classifications are used: poor soluble (< 10 mg/ml); medium solubility (10–100 mg/ml); and good solubility (> 100 mg/ml).

The methodology is now also adopted for 384 well plate format (Green). The data obtained by this method have good correlation with data obtained for 96 well plate format. The batch-to-batch variability is 5 %. The quality of the well-plates has strong influence on the data obtained. Even microscopic scratches may pretend precipitation. Pan et al. investigated different well plate types. Their recommendation is to use Costar clear bottom white plates. Clear polypropylene CLINIPLATE (Labsystems) are also of good quality but provide less signal enhancement.

Avdeef introduced an alternative approach. Aliquots of DMSO stock solutions are pipetted robotically into incubation well plate containing an aqueous buffer. The concentration of the test compound should be between 50–150 μM in order to keep the DMSO content below 0.5 %. After a time of incubation, the plate is filtered and the solved compound is quantified with an UV plate reader. The method is fast, robust and reported to be reliable (Kerns). 200–300 compounds can be measured per day. Additionally, pH solubility profiles can be set up easily (Kibbey).

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II.B.2 Determination of pKa

PURPOSE AND RATIONALE

The pKa is dissociation constant for a given media. It is a parameter which indicates the ionization state at a given pH and describes the acidity of a compound in a special medium. In drug discovery it is a valuable parameter for SARs but also helps in the interpretation of pH-solubility profiles. For an acid:

pKa = –log10 Ka (Ka is ionization constant)

$HA = H^+ + A^- Ka = [H^+][A^-]/[HA]$

$pKa = pH + \log [HA]/[A^-]$

In practice the pKa is the pH where 50 % of the compound is ionized.

Molecules can have more than one pKa depending on the number of ionizable centers. In pharmaceutical research the ionization state between pH 2 (stomach) and 8 (pH in colon) is most interesting. A review by Taylor covers the effects of the different protonization/ionization forms of a molecule on pharmacokinetic and pharmacodynamic properties. Molecules are less soluble in aqueous media but more permeable through membranes in their neutral form.

Today most pKas are determined by classical titration with UV (Albert) or potentiometric (Sirius GLpKa) detection. These methods could be coupled with multiwavelength spectrophotometer detection (Sirius, D-PAS). Tam et al. compared both methodologies and found good correlation between them.

PROCEDURE

The classical potentiometric approach (Sirius, GLpKa) can be coupled with multiwavelength detection, thus gaining enhanced sensitivity with decreased sample consumption.

Multiwavelength spectrophotometer detection is based on the change of the ionization state of a molecule. A change of the ionization state leads to a change of the electron density, which directly leads to an increase or decrease of UV absorbance. The titration is performed with KOH or HCl across a pH range of 1.8–12.2. HCl and KOH solutions are prepared for the titration according Avdeef. The aqueous medium for compound solutions can be chosen individually. Ionic strength of 0.15 potassium chloride is recommended. The D-PAS uses an automated titration system coupled with fibre optics dip-probe, an UV light source and a photodiode detector. Spectral changes, which arise during the titration, are captured at concentrations down to 10^{-5} M to 10^{-6} M.

The titration intervals can be set individually via the instrument software. The compound is solved in the measurement cell at a concentration of 10–100 μM, whereby the pH adjustment by automated titration of the system has an accuracy of 0,02 pH units. Tam suggests in their report titration steps of 0.1 pH units and to perform 25–35 pH readings and absorption spectra measurements during each titration.

Poorly soluble compounds can be measured with co-solvents as methanol, octanol or DMSO at different concentrations. Their true water solubility can be extrapolated by the Yasuda–Shedlovsky method.

EVALUATION

The instrument software computes the operational pH readings to the pH value by an multiparameter equation reported by Avdeef. The pKa values are calculated from the pH values and the absorption data of different wavelength. The calculation is done by target factor analysis (Allen).

The Yasuda–Shedlovsky method

$$pKa + \log [H_2O] = (A/\epsilon) + B$$

is used for the extrapolation of molecules with poor aqueous solubility measured within aqueous buffer containing different percentages of organic co-solvent. ϵ stands for the dielectric constant of the co-solvent mixture, $[H_2O]$ for the molar concentration of water, whereas A and B represent slope and intercept of the plot. $pKa + [H_2O]$ is plotted against $1/\epsilon$.

CRITICAL ASSESSMENT OF THE METHOD

The method shows very good correlation to pKas obtained by classical titration (Allen) or the literature (Lide). Weak UV absorbance also limits the determination of pKas far away from a chromophor. A range of 3 bonds is considered as tolerable (Tam). Poor UV absorbance may be enhanced by raising the analyte concentration. Poor solubility of a compound may prevent an increase of the compound concentration. In this case the use of co-solvents is recommended.

Furthermore, it has to be considered that side products or hydrolysis of the analyte in the pH profile can give rise to false positives. Thus a quality control step beforehand is recommended.

MODIFICATIONS OF THE METHOD

Comer et al. developed a high-throughput pKa assay (SGA profiler, Sirius Analytical) which is able to measure the pKas of up to 200 compounds between pH2 and pH12. Compounds are dissolved in DMSO to give 10 mM stock solutions. 5–20 μl of the stock solution is transferred into a deep well plate and is

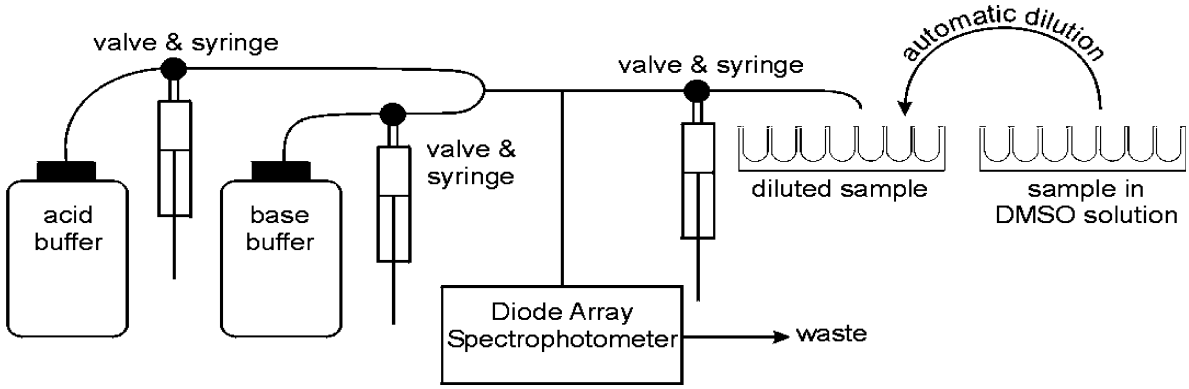

Fig. 2. Highthroughput pKa determination.

diluted with 2 ml of water. Up to 96 compounds can be measured per plate. The dilution is injected in a flow pH gradient, which is produced by mixing two buffer solutions containing mixtures of weak bases and acids. These mixtures have low absorbance in the UV above 250 nm. The flow gradient passes a diode array spectrometer covering 280–800 nm.

Evaluation is done with the instrument software. The software calculates the change in UV absorbance at 30 different wavelengths in order to calculate the pKa. Since the pH gradient of the combined mixtures is linear over a wide range the pH is a linear function of time. The pKas are calculated from the change of UV absorbance at multiple wavelengths as a function of time.

Since most compounds in drug discovery have a chromophor and are nitrogen containing up to 70 % of all samples can be taken into account for a measurement. The sample consumption is very low. Poor solubility of an analyte in the used buffer system is major hurdle of the method. If a compound precipitate during the measurement dramatic UV changes are observed. If the compound is not soluble in water at all it will precipitate in the mother plate. Thus no UV change will be observed due to lack of compound. This will lead to false negative results.

In order to measure a wider range of compounds, the traditional pH titrimetic methods (Jander) are still of good use. In the pH metric method, the sample is titrated in a pH range of choice with acid or base. The titration is monitored with a pH electrode. The pKas can be calculated from the shape of the titration curve. In order to get good resolution small pH intervals are titrated thus limiting the throughput of the method and leading to an increase in sample consumption. For validation and reference measurement this methods is still the method of choice.

Small sample consumption combined with data of high accuracy can be obtained with capillary electrophoresis. Miller et al. demonstrated that pH dependent capillary electrophoresis can be used for the titration of pKas. The work based on earlier contributions of Gluck et al. Analogous to the pH metric method, capillary electrophoresis is performed at a variety of different pHs. The mobility of the sample is measured and can be plotted in a pH/mobility curve. pKas are derived from the shape of the curve. Jia showed that capillary electrophoresis can be used in medium throughput for a broad range of pharmaceuticals.

There is now one commercial system available (MCE2000, Combisep) which uses parallel capillary synthesis. It is reported (Strasburg) that up to 96 individual CE separations can be performed simultaneously (Kibbey).

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II.B.3 Lipophilicity

PURPOSE AND RATIONALE

Lipophilicity of a compound is associated with many physicochemical and physiological properties. Lipophilicity is described in most cases as partition between two phases (hydrophilic and hydrophobic). Hansch was one of the first to examine the relevance of partition coefficients for the explanation of structural activity relationships.

The partition coefficient (P) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents. In the case of n-octanol and water:

$$P_{ow} = c_{\text{n-octanol}}/c_{\text{water}}.$$

The Partition Coefficient itself is a constant. It is defined as the ratio of concentration of compound in aqueous phase to the concentration in an immiscible solvent, *as the neutral molecule*. The partition coefficient (P) therefore is the quotient of two concentrations and is usually given in the form of its logarithm to base 10 (log P). The Log P will vary according to the conditions under which it is measured and the choice of partitioning solvent.

However, log D is the log distribution coefficient at a particular pH. It takes into account the equilibrium between ionised and not ionised species with the two solvents.

Distribution Coefficient D = [Unionised](o)/[Unionised](aq) + [Ionised](aq)

Log D = log10 (Distribution Coefficient)

Log D at pH 7.4 is often quoted to give an indication of the lipophilicity of a drug at the pH of blood plasma. There are meanwhile 100 000 log P and log D values available thus making it to a valuable tool for comparison.

II.B.3.1 Lipophilicity by Octanol/Aqueous Shake Flask

PURPOSE AND RATIONALE

In order to determine a partition coefficient, equilibrium between all interacting components of the system must be achieved, and the concentrations of the substances dissolved in the two phases must be determined. Octanol and water are standard solvents for the equilibrium experiments. They are readily available and allow equilibrium experiments with good repeatability and reproducibility.

PROCEDURE

Two large stock bottles (2 l) of high purity analytical grade n-octanol or water are saturated with a sufficient quantity of the other solvent. Both solvents are shaken for 24 hours on a mechanical shaker. They are allowed to stand for two days to permit the phases to separate. In order to determine log P, a buffer system is chosen which has $>$ 2 pKa units above the pKa of the compound if the compound is a base. For acidic compound the pKa has to be $<$ 2 pKa units below. Neutral compounds can be examined in water.

In order to be determine log $D_{7.4}$ an appropriate phosphate buffer has to be chosen. After shaking for 4 hours two equal volumes of the phases are placed in a centrifugation tube. An aliquot of the test compound is added. The centrifuge tube is rotated 100 rotations in five minutes 180 ° about the transverse axis. In order to separate the phases the samples are centrifuged at room temperature. They stand for at least one hour to ensure phase separation and constant temperature. An aliquot of each solvent compartment is then and quantified with an appropriate method. Examples of analytical methods are:

1. photometric methods
2. gas chromatography
3. high-performance liquid chromatography.

EVALUATION

For the determination of the partition coefficient, it is necessary to determine the concentrations of the test substance in both phases. The total quantity of substance present in both phases should be calculated and compared with the quantity of the substance originally introduced.

The log P and log D values are calculated from the ratio of their presence in the two components:

$$P_{ow} = c_{\text{n-octanol}}/c_{\text{water}}.$$

CRITICAL ASSESSMENT OF THE METHOD

The measuring range of the method is determined by the limit of detection of the analytical procedure. This should permit the assessment of values of log Pow in the range of –2 to 4 (occasionally, when conditions apply, this range may be extended to log Pow up to 5) when the concentration of the solute in either phase is not more than 0,01 mol per liter.

The test temperature should be kept constant (±1 °C) and lie in the range of 20 to 25 °C. The method is restricted to samples of high purity and where plenty of compounds are available.

The throughput of the methods is limited due to the sample preparation and tedious experimental set-up. It gives high quality data, which can be used for cross validation of methods and reference data.

MODIFICATIONS OF THE METHOD

Hitzel reported a high-throughput variation of the method. They adopted the shake flask experiment to well plate format. Octanol, buffer and compounds are transferred in the well plate by a liquid handling system (Beckman Biomek 2000). The plate is sealed and shaken on reciprocal shaker for 30 minutes. The plate is centrifuged afterward and aliquots of the aqueous and organic phase are analysed via fast gradient high throughput chromatography. The correlation of the method with manual shake flask experiments in Eppendorf vials was excellent according to the authors.

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II.B.3.2 Lipophilicity by Partition Chromatography

PURPOSE AND RATIONALE

Partition P of a compound between octanol phase (lipophilic) and water (hydrophilic) is one of the most common used parameter to express lipophilicity. The logarithmic value of P $\log P$ is the most common lipophilicity parameter. Strictly spoken $\log P$ is the partition parameter of the neutral compound. Thus in most cases the $\log D$ is the measured parameter of choice (partition of neutral and charged molecule between buffered solution at a given pH and octanol). Besides shake flask experiments (see section 3.1) chromatography is one of the major methods to determine lipophilicity data. Chromatography is readily available and easy to use. However, one gets no exact partition coefficient but you find good correlation with shake flask measurements. The throughput of chromatographic methods is far higher than the traditional shake flask experiments (Valko).

PROCEDURE

The partition of the compound between stationary phase (in most cases octadecane bonded on silica) and mobile phase is used as surrogate parameter for lipophilicity. The retention time of the analyte correlates with the lipophilicity of the compound.

In order to get direct octanol water partitioning, it is essential to immobilize octanol at the stationary phase (Mirrless, Unger). The coating is achieved with repeated injections of pure octanol with a buffered eluent saturated with octanol. The octanol-coated columns have length of 1 cm (for $\log D\ 0 \rightarrow 4.5$) and 25 cm (for $\log D$ 0–1.5). The mobile phase is octanol saturated 10 mM phosphate/KCl buffer (pH 7.4). Saturation of the buffer with octanol is achieved by shaking one liter of octanol with 20 ml of octanol. The resulting octanol saturated buffer is filtered. For high $\log D$ determination up to 30 % methanol are added.

20 µl of a 10 mM compound solution in DMSO are injected in the HPLC. The 1 cm column is used for high $\log D$ with a flow rate of 10 ml/minutes, whereas the compounds with low lipophilicity ($\log D < 1.5$) are characterized with the 25 cm column at a flow rate of 2 ml/min. In order to avoid octanol loss on the column due to the DMSO, the direction of injections is reversed with every sample.

EVALUATION

The capacity value k′(retention time – column dead time) is used for the calculation of the $\log D$. For this purpose a standard set of compounds with known $\log D_{7.4}$ is used for calibration of the system.

$\text{Log}\ D_{7.4}$ for the standards is plotted against $\log k'$. The linear equation

$$\log D_{7.4} = a \log k' + b$$

is used for the determination of $\log D_{7.4}$. In order to determine $\log P$ a buffer system is chosen which has > 2 pKa units above the pKa of the compound if the compound is a base. For acidic compound the pKa has to be < 2 pKa units below.

CRITICAL ASSESSMENT OF THE METHOD

The method gives high quality data with very good correlation with traditional shake flask (Slater) derived $\log D$s. The method is not suited for compounds with very low lipophilicity $\log D < -1$, because for those no retention on the stationary phase is achieved. The other limitation are compounds with a lipophilicity > 4.5.

Due to the long retention time the peaks become no longer detectable. Furthermore, the method is limited to compounds with chromophors. The throughput of the method is about 20–40 compounds a day.

MODIFICATIONS OF THE METHOD

An isocratic octanol-saturated 0.02 MOPS buffer in water is used by Minick et al. for chromatographic extrapolation of log D. They use an octyl (C8) bonded stationary phase and vary the methanol content of the buffer. The methanol free log k′is derived via extrapolation. This methodology is well suited for compounds with high lipophilicity but also needs a chromophor due to the UV detection of the instrument set-up.

Lombardo et al. use also different octanol concentration in their isocratic method. They use an LC-ABZ column (Pagliara). This method is capable to cover a wide lipophilicity range with good correlation with log P_{ow}.

Alternative detection methods such as mass spectrometry, ELS or nitrogen detector are in most cases inappropriate due to the octanol saturated aqueous media. An alternative is the use of standard HPLC reversed phase gradient systems (Valko). These systems do not represent true octanol water partitions, but are also good lipophilicity parameters by themselves.

Valko et al. have developed chromatographic methods which are based on established reversed phase methods with acetonitrile water gradients. The lipophilicity is characterized as a so-called chromatographic hydrophobicity index (CHI), which approximates the percentage of acetonitrile necessary for equal distribution between mobile and stationary phase. The method is able to provide hundreds of CHIs parallel to the quality control of compounds via RP/HPLC.

The next section focuses on alternative partition coefficients to P_{ow}.

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Chapter II.C
In-Silico ADME Modeling

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INTRODUCTION

There is increasing pressure on research efficiency and cost in the pharmaceutical industry which has resulted in a paradigm shift to bring promising molecules earlier to the market (Wess 2002; Lawrence 2002). Continuously increasing research expenses are mainly attributed to attrition in late stage development often caused by inadequate understanding of pharmacokinetic behavior of drugs (Prentis et al. 1988; Kennedy 1997; Drews 2000). The conversion of biologically active molecules into effective and safe pharmaceuticals adds substantial value to the drug discovery process. Consequently, the improvement of a compound profile towards a clinical candidate is one of the essential skills in integrated drug discovery teams. Those candidate requirements include multiple parameters including potency and efficacy, selectivity against related proteins or "antitargets", favorable physicochemical, and pharmacokinetic properties leading to the required bioavailability after oral administration and an acceptable half-life of the final candidate. A simultaneous optimization of multiple parameters in carefully planned iterations is therefore required to arrive at molecules with suitable properties and profiles.

The requirement for shortening the pharmaceutical discovery process caused the integration of pharmacokinetic and drug development efforts into earlier discovery to rapidly focus on molecules with increased optimisation potential. It is mandatory to initiate time-consuming optimisation programs only for those chemical series which show a potential to be convertible into drug molecules.

Within the drug discovery process, in-silico approaches to identify or improve chemical structures are utilized in the lead generation and optimization phase. First, lead structures are identified representing a prototypical chemical structure that demonstrate activity and selectivity in a pharmacologically or biochemically relevant assay (Wess et al. 2001; Bleicher et al. 2003). In this phase, mainly general in-silico models are applied. The following lead optimization phase is the most challenging part of drug research (Wess et al. 2001; Wess 2002). Its ultimate object is to turn an efficacious molecule into an effective and safe drug. This is accomplished by structural variations to improve the molecule such that its profile of characteristics fulfils defined criteria for the respective therapeutic application. During this phase, the ADME (absorption, distribution, metabolism, excretion) as well as the toxicity properties of the drug candidate are being optimised. These activities are mainly supported by local in-silico models. The following stages of the value chain deal with the early and the late stage clinical development and finally the launching of a new drug. Although the clinical development is also supported by numerous in-silico tools to predict and/or describe the pharmacokinetic behavior of the drug candidate, a detailed description of these tools is beyond the scope of this chapter.

The introduction of several new technologies like protein crystallography, ADME assays, medicinal

chemistry automation and others have added not only new opportunities but also more complexity to the lead optimisation phase. Consequently, it is vital for success to conceive lead optimisation as simultaneous multidimensional optimisation of compound properties rather than addressing one parameter at a time. Traditionally, binding affinity is first optimised, while ADME parameters are addressed later in this process (Oprea 2002; Zamora et al. 2003). This approach, however, showed only limited success as optimising for affinity only can result in chemical classes, where further improvement becomes impossible. The efficiency of the drug discovery process is expected to improve if both aspects are considered simultaneously. Beside high biological activity in vitro, it is of great importance to simultaneously ensure sufficiently high drug concentrations at the target site in vivo by seeking for favorable ADME properties. To this end, various in vitro assays have been introduced to monitor compounds as early as possible for their ADME characteristics. However, all these techniques require the synthesis of the compound to be tested. To save time and chemical resources, it is desirable to introduce in-silico tools into the drug development process allowing the reliable assessment of compound properties prior to synthesis and subsequently the drawing up of a priority list of the most promising compounds to be synthesized. Thus, facing the requirements of modern drug development, rational approaches to alter molecules being originally directed by quantitative structure-activity relationship (QSAR) and structure-based design become more and more a tight interplay between multiple disciplines: medicinal chemistry, structural biology, pharmacology and pharmacokinetics.

This overview discusses selected computational methods and strategies towards the generation and application of in-silico models in a drug discovery project context. In-silico models can be classified in general and local, chemotype-specific models. This grouping broadly reflects the range and diversity of chemotypes incorporated in the training set for establishing the model. The underlying assumption is that general models might find wider applicability to novel structures for prediction while local models are focussed towards a particular class. Thus, general models might be of interest in earlier phases of a discovery project (library design, hit exploration) without many experimental ADME information on a particular chemotype. Local models on the other hand are based on previous knowledge for one series and are more useful in the lead optimisation phase. Frequently, experimental data for one particular ADME property is then available to build a model. Then, this information can be turned into a more focused model with, of course, less wide applicability, but the resulting model certainly will help more to direct further synthesis efforts.

It is beyond the scope of this contribution to provide an exhaustive list of software tools, descriptors, models, and approaches to address multiple ADME issues. We rather discuss a selected list of approaches which have shown to be valuable to us and others in several drug discovery projects. The entire field, the methods, and descriptors are changing rapidly and the interested reader thus is referred to recent review articles for this purpose (Clark et al. 2000; Clark 2001; Matter et al. 2001; van de Waterbeemd et al. 2003). The herein discussed computational approaches can be classified into three categories: a) simple descriptors like PSA (polar surface area), often computed from the two-dimensional molecular structure; b) alignment-free descriptors capturing global information from three-dimensional molecular representations like VolSurf or GRIND descriptors; and c) 3D-descriptors, which depend on the alignment of molecules to obtain spatially resolved information on regions affecting the interesting property, like Catalyst, CoMFA and related 3D-QSAR approaches.

In the following sections, this overview provides examples for both general and local models to illustrate different approaches for building models on structurally diverse or related training sets. Datasets for intestinal human absorption and human serum albumin binding will be discussed in detail while models for other relevant ADME properties have also been obtained. In addition, two local models will also be discussed addressing intestinal absorption from both an in vitro and an in vivo perspective. Those models, however, do not stand alone, but are used in combination with complementary models tailored for affinity and selectivity in the frame of multidimensional lead optimization.

It has to be pointed out that prediction failures of general ADME models are often related to two major sources namely the quality of experimental data used to derive the model and the interpretation of the final model. These problems are discussed in depth by Stouch et al. (2003). Some models fail as they were built from data collected from different sources and laboratories. Although this might work for some robust standardized ADME assay, it could produce incomparable data for others. Such problems have been reported for example for Caco-2 assays from different laboratories. Even if the experimental

data for the training set were collected in a single laboratory another potential source of errors is present, if such a model then is transferred to another place where a slightly different assay is utilized to monitor the predictivity of the model. Furthermore, it is very ambitious to derive a truly general model for a wide range of chemotypes. Often the limited coverage of training set chemical space plus the presence of novel compound classes to be predicted in research projects which are very dissimilar to any training molecule might also significantly limit the scope of a particular model. Finally, a classification model should not be used to rank-order compounds, but only to filter undesirable compounds. Any other application would lead to an over-interpretation of the model's precision. Furthermore, it has to be emphasized here that adding more compounds with additional experimental data to an existing general model does not guarantee that its predictivity will increase. This is because the addition of new compounds might introduce new modes of interaction and/or new reaction mechanisms.

Although the ADME space is of lower dimensionality that means there are less descriptors building the structure-property model, ADME properties in practice are more difficult to predict than biological receptor affinities as corresponding experimental screens are often multi-mechanism rather than single-mechanism systems (Lipinski 2000). In contrast, biological assays for the majority of pharmacological targets are typical single mechanism systems for which computational models to correlate structural descriptors are easier to develop and resulting predictions tend to be more robust. Computational models based on experimental data combining multiple underlying biological mechanisms tend to get worse if more data for more diverse molecules are integrated into the training set. This is mainly due to the fact that the increase of assay data relates to an increase of underlying mechanisms, on which those data have been obtained and the noise level rises for each individual mechanistic component (Lipinski 2000). For smaller, structurally and thus probably mechanistically homogeneous data sets, acceptable correlations are obtained, while the ability of descriptors to capture a more diverse experimental data set is limited. Although the same descriptors might still have statistical significance and thus explain trends for inhomogeneous data, their predictivity is often too low to be useful for lead optimization purposes. This only allows to implement validated filters based on property distributions. Hence, it is mandatory to use high quality single mechanism ADME experimental data for building single mechanism predictive models (Lipinski 2000). Alternatively, the assumption might be valid that congeneric series behave similar in multiple mechanism assays, so that a local model for this series is primarily capturing the main trend of this series.

Poor intestinal absorption of a lead structure for example can be due to insufficient physicochemical properties or poor membrane permeation, but also due to the net result of efflux mediated by transporter proteins including the multi-drug resistance protein MDR1 (P-gp) or multi-drug resistance related proteins (MRP's) situated in the intestinal membrane. Cell-lines with only one single efflux transporter are currently engineered for in vitro permeability assays to get suitable data for reliable QSAR models. In addition, efforts to gain deeper insight into P-gp and other ATP-binding cassette (ABC) transporters on a structural basis are ongoing (Litman et al. 2001; Rosenberg et al. 2003). These examples might serve to illustrate the complexity when dealing with different ADME assays and diverse molecules. Hence, single mechanism data are essential for successful lead optimization.

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II.C.1 Computational Approaches

II.C.1.1 Polar Surface Area (PSA)

PURPOSE AND RATIONALE

As for most drugs, the preferred route of administration is per os, many efforts have been made to identify physicochemical properties that favor intestinal absorption as first step to oral bioavailability. As reliable quantitative predictions for many ADME properties are still not fully developed and undergo constant modifications, many of the existing computational approaches provide only a qualitative ranking rather than a quantitative prediction. However, quantitative predictions are possible with regard to passive drug permeability across the intestinal epithelium and the endothelium of the blood-brain barrier (Pickett et al. 2000; Artursson et al. 2003). Among the various strategies to predict intestinal absorption in-silico, the utilization of molecular surface areas is a very attractive method due to its speed and simplicity. Molecular surface areas have been used for more than 30 years in the modeling of solvation and partitioning processes (Hermann 1972; Pearlman 1980). More recently, approaches to predict ADME properties have been developed making use of a quantity derived from the molecular surface known as the polar surface area (PSA). Recent reviews highlight the importance of this descriptor (Artursson et al. 2003) to understand passive transport problems across membranes.

PROCEDURE

The PSA of a molecule is defined as van der Waals surface arising from all oxygen and nitrogen atoms including attached hydrogens. From this definition it becomes obvious that PSA should be related to the capacity of a compound to form hydrogen bonds which is seen as key physicochemical determinant of passive intestinal absorption.

There are three main methods to calculate PSA values. The dynamic polar surface area PSA_d is calculated from a Boltzmann-weighted average from a set of low-energy conformers (Palm et al. 1996; van de Waterbeemd et al. 1996, Palm et al. 1997; Krarup et al. 1998). To this end, all low-energy conformations from a conformational analysis with a certain energy cut-off (for example ΔE 2.5 kcal/mol, depending on the force field) are taken into account. The probability of a molecule to adopt a certain conformation is calculated from a normalized Boltzmann distribution (Palm et al. 1996; Lipkowitz et al. 1989). The chosen energy cut-off value of 2.5 kcal/mol ensures that all conformations that contribute more than 1 % to the conformational space at 37 °C are considered upon computing the PSA value.

The polar surface area of a single conformer PSA_s is calculated from the global minimum conformation of the molecule (van de Waterbeemd et al. 1996, Clark 1999a). Alternatively the 3D structure of the molecule could be generated by 2D/3D converters like CONCORD (Pearlman 1987; Balducci et al.), or CORINA (Sadowski et al. 1992,1994), followed by energy minimization. Finally, the PSA of the minimized conformation is calculated for example by MOLVOL (Dodd and Theodorou 1991) or other approaches.

While these descriptors above rely on the 3D molecular structure the topological polar surface area TPSA is calculated by simply summing up tabulated surface contributions of polar fragments rapidly obtained from SMILES representations (Daylight Chemical Information System Inc.) of 2D chemical structures (Ertl et al. 2000). For that purpose, a set of fragments comprising 43 polar atom types was defined. The contributions of these atom types were determined by least-squares fitting to the single conformer 3D PSA (PSA_s) of a large subset of drug-like structures from the World Drug Index (World Drug Index Database). From this database, all molecules with apparent valence errors, molecular weights outside the interval of 100–800, and molecules not having at least one oxygen, nitrogen, sulphur, or phosphorus atom were removed. The remaining set used for the least-squares fitting consisted of 34810 reasonably drug-like molecules. A comparison of PSA_s and PSA_d data of β-adrenoreceptor blocking drugs revealed that the correlations between the surface properties of the

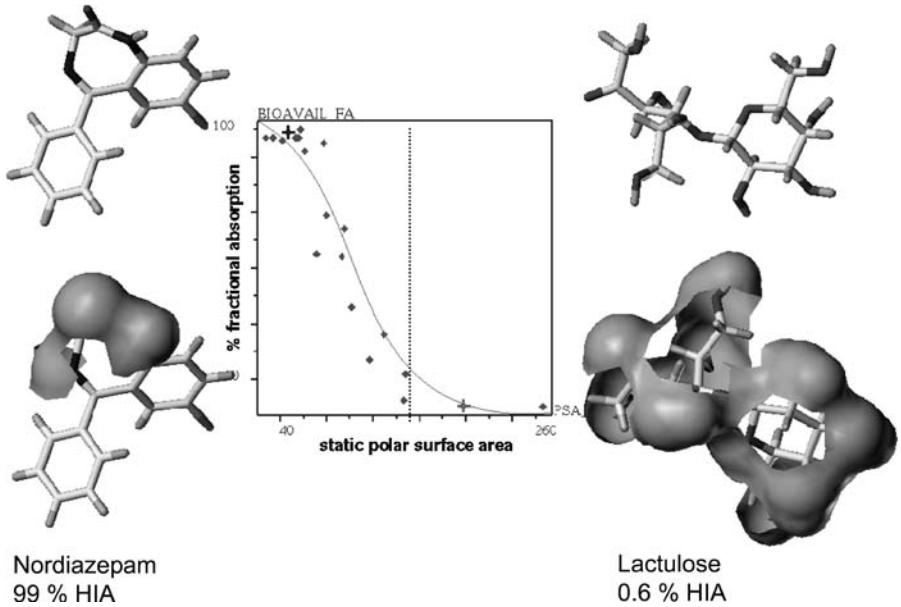

Fig. 1. Sigmoidal correlation between human fractional dose absorption and PSA_d for 20 drugs (data from Palm et al. 1997). The influence of the polar surface area on fractional absorption is illustrated by comparing PSA regions for the orally available nordiazepam (*left*) to the non-available lactulose (*right*).

global minimum conformations and permeability were only slightly poorer than correlations obtained using the dynamic properties (Palm et al. 1996). Thus, the much faster to calculate PSA_s data gave only slightly poorer results than the time consuming dynamic PSA_d data. Furthermore, the fastest TPSA data showed a very good correlation with PSA_s ($r^2 = 0.99$) and PSA_d ($r^2 = 0.98$) data. Finally, the TPSA method has proven its applicability to predict not only intestinal absorption but also blood-brain barrier penetration and Caco-2 cell permeability (Ertl et al. 2000) providing results of the same quality as with PSA_s and PSA_d data.

EVALUATION

Two of the major physicochemical determinants of passive absorption are lipophilicity and hydrogen bonding potential. As mentioned, PSA is a good predictor for hydrogen bonding potential (Palm et al. 1997) while it is not directly related to lipophilicity. This was shown by Clark (1999a) considering a series of aliphatic alcohols. In this series, PSA is constant, while the lipophilicity increases with increasing carbon chain length. Thus, one could expect that a combination of lipophilicity data and PSA data should result in an even better predictor to guide drug design toward orally available compounds.

CRITICAL ASSESSMENT OF THE METHOD

Using small datasets it was initially shown that the dynamic PSA is strongly correlated with the membrane permeability of a series of β-adrenoreceptor blocking agents (Palm et al. 1996). Subsequently, it was found that PSA_d is able to predict the absorption after oral administration to humans of 20 structurally diverse drug molecules (Palm et al. 1997). The resulting sigmoidal correlation between fractional dose absorption and PSA_s for 20 drug molecules is given in Figure 1. From these results it was concluded that drugs with complete oral absorption should have a PSA_d value of < 60 Å^2 while > 140 Å^2 is generally seen as suitable threshold for compounds with unacceptably low absorption. For molecules with a higher PSA value, a poor absorption is very likely, if no active transport mechanisms are involved during the intestinal passage. However, the lower PSA values are necessary but not a sufficient criterion for good absorption. For example highly hydrophobic compounds may have a low PSA value but due to their low solubility their intestinal absorption is low. Therefore, it is important not to use PSA naively as a guide to absorption but to couple it to other information and existing medicinal chemistry knowledge.

These findings were confirmed by Kelder et al. (1999) calculating the PSAvalues for approximately 2000 orally available drug-like molecules that have

reached at least clinical phase II. They concluded that orally active drugs that are transported passively by the transcellular route should not display a PSA > 120 Å^2. In addition, oral drugs tailored to brain penetration were shown to have PSA values of < 70 Å^2. A very similar finding was reported by Clark (1999b) who derived a simple quantitative structure-activity relationship (QSAR) for brain penetration from a combination of logP and PSA. The resulting interpretation that smaller PSA values are more favorable for brain penetration is reasonable as it is known that the endothelial cell monolayer of the BBB forms a much tighter barrier than the intestinal epithelial cell barrier (Artursson et al. 1991; Pardridge 1996).

MODIFICATIONS OF THE METHOD

It has been estimated that the scatter in the correlation between PSA and transcellular membrane permeability is likely to increase with more chemotypes from different therapeutic classes to be incorporated. Hence, to improve the predictivity, the PSA was combined with other predictive molecular descriptors such as calculated logP (Clark 1999b, Egan et al. 2000) and number of rotatable bonds (Veber et al. 2002).

In addition to the PSA, a non-polar surface area (NPSA) can be computed by taking the total surface area minus the PSA into account. A linear combination of PSA and NPSA was reported to give satisfactory models for analogue series like dipeptides and endothelin receptor antagonists, where PSA alone was not a satisfactory predictor of passive membrane permeability (Stenberg et al. 1999a,b). Collectively, these results suggested that a more general model of passive membrane permeability should incorporate different molecular descriptors capturing information about polarity and lipophilicity.

This led to the concept of fragmentation of the total molecular surface area in combination with multivariate analysis (Stenberg et al. 2001) towards predictive models of drug permeability for more complex datasets. Permeability models were established based on so-called "partitioned total surface area (PTSA)" descriptors. Each of the PTSA descriptors corresponds to the surface of a certain atom type, differentiated by hybridisation, which results in individual descriptors for e.g. sp^3, sp^2, and sp carbon atoms. The resulting permeability model based on 19 descriptors finally consisted of oxygen, nitrogen and polar hydrogen surfaces, while the main contribution for prediction of Caco-2 permeability was attributed to PSA. In addition some more lipophilic contributions were seen as relevant to obtain a significant PLS model.

This work was expanded towards a classification of drug molecules into the established biopharmaceutical classification system (BCS) on the basis of PTSA descriptors alone, which included separate models for solubility and permeability (Bergström et al. 2003). The PLS models reported for solubility and permeability resulted in a correct biopharmaceutical classification for as many as 85 % of the studied compounds, while an external FDA standard drug test set was correctly classified with 77 % accuracy.

While models to understand a single chemotype were reported to include only a few informative descriptors from this surface-area family for larger and more diverse datasets, multivariate analysis of multiple descriptors might be more useful.

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II.C.1.2 Alignment-Free 3D Descriptors (VolSurf)

PURPOSE AND RATIONALE

As described above, molecular surface areas were successful to predict important ADME properties like passive membrane permeability and intestinal absorption. This concept was taken further towards the development of a set of descriptors capturing a wide range of physicochemical relevant properties extracted from molecular interaction fields of three-dimensional (3D) structures. To this end, Cruciani et al. developed a new tool called VolSurf (Cruciani et al. 2000a, Cruciani et al. 2000b, Cruciani et al. 2003) integrating enhanced molecular descriptors with multivariate statistical tools for the analysis of physicochemical and pharmacokinetic data. VolSurf transforms the information present in 3D-molecular interaction fields into a limited set of descriptors. Those have been shown to carry relevant information related to ADME properties like polarity, hydrogen bonding, lipophilicity, size, polarizability, and others. As these descriptors are easy to calculate, to understand, and to interpret from a chemistry point of view, they provide further design guidelines for chemical optimization after a linear model has been established.

PROCEDURE

The interaction of drug molecules with biological membranes is a three-dimensional recognition that is mediated by surface properties. Information about surface properties of drug molecules and other solutes can be extracted from 3D molecular interaction fields. In VolSurf, the information from calculated molecular interaction fields is extracted and compressed in a few informative descriptors. Those descriptors capture various aspects of physicochemical molecular properties and thus can be successfully correlated to pharmacokinetic properties. To this end, the 3D grid maps of interaction energies between drug molecules with particular probe atoms are calculated using the GRID force field (Goodford 1985). This advanced force field is able to calculate energetically favorable interaction sites for a variety of chemically relevant probe atoms and functional groups around a molecule in a given 3D conformation. As the information contained in 3D molecular fields is related to the interacting molecular partners, the amount of information captured in those interaction fields is generally superior to global descriptors or those computed from only the 2D chemical structure.

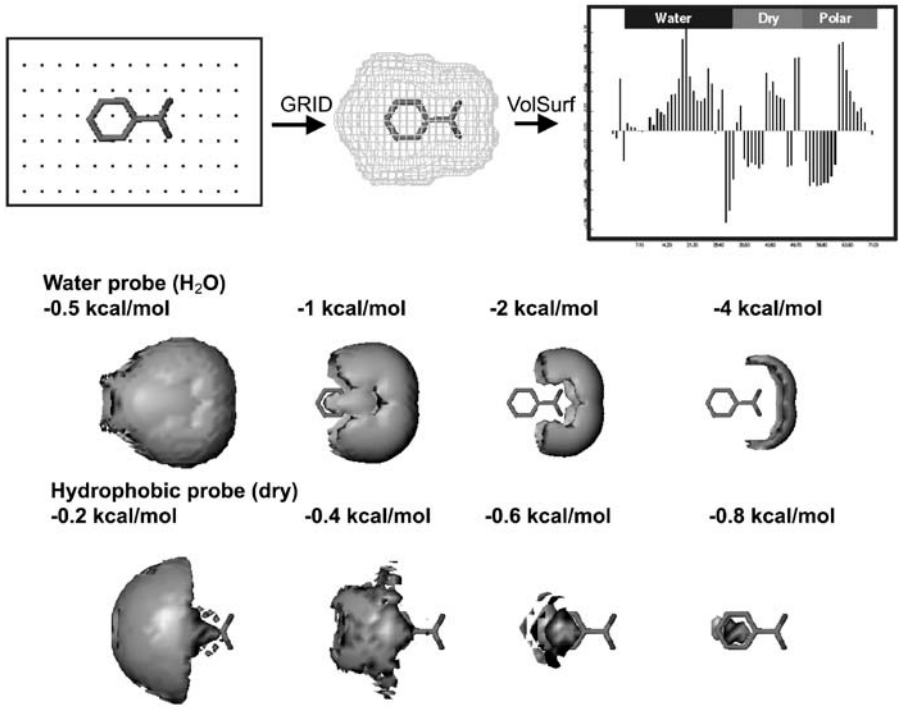

Fig. 2. Computation of Volsurf descriptors (Cruciani et al. 2000a) derived from GRID molecular interaction fields. For any molecule, interactions with GRID water and dry probes at different energy levels are used for contouring. Those levels serve to compute vectors of 72 volume, size, and surface related descriptors.

In most cases, the GRID water, the hydrophobic (DRY), and the carbonyl oxygen (O) probes have been utilized for computing molecular interaction fields which are then analyzed using VolSurf to extract meaningful descriptors. GRID uses a potential based on the total energy of interactions (Lennard-Jones, H-binding, and electrostatic terms) between a target molecule and a probe and can be used to characterize putative polar and hydrophobic interaction sites around target molecules. The water probe simulates solvation/desolvation processes while the DRY and the O probes encounter drug-membrane interactions. This treatment of hydrophobic interactions is done at each grid point by a combination of the following terms $E_{Entropy} + E_{Lennard\text{-}Jones} - E_{H\text{-}bonding}$. $E_{Entropy}$ is the ideal entropic component of the hydrophobic effect in aqueous environment, $E_{Lennard\text{-}Jones}$ accounts for induction and dispersion interaction occurring between any pair of molecules and $E_{H\text{-}bonding}$ subtracts the effect of hydrogen-bonding contributions between water and polar parts of the solute surface.

Each drug molecule is characterized by its potential hydrogen bonding, polar and hydrophobic interactions, starting from its three-dimensional structure, but without the necessity to bring different molecules into an alignment by atom-based or field-based superposition methods.

Subsequently, VolSurf extracts information present in these 3D molecular fields and transforms it into a limited number of descriptors, as shown in Figure 2. These descriptors capture relevant molecular properties which are related to ADME and pharmacokinetic properties like polarity, hydrogen bonding, lipophilicity, size, shape, polarizability, and others. In particular the size and shape of hydrophilic and hydrophobic molecular regions at different interaction energy levels and the balance between these regions is extracted by VolSurf. Other descriptors for quantitative ADME models are the amphiphilic moments and critical packing parameters. A detailed account of these descriptors and the approach to extract shape and surface information is given by Cruciani et al. (2000a).

The originality of VolSurf resides in the fact that surfaces, volumes, and other descriptors can be obtained from 3D molecular fields without complex algorithms of trigonometric projections, recursive generations, and tessellations. In total, the analysis of molecular recognition is achieved by image analysis approaches while the image compression step involves chemical knowledge by selecting an appropriate parametrization according

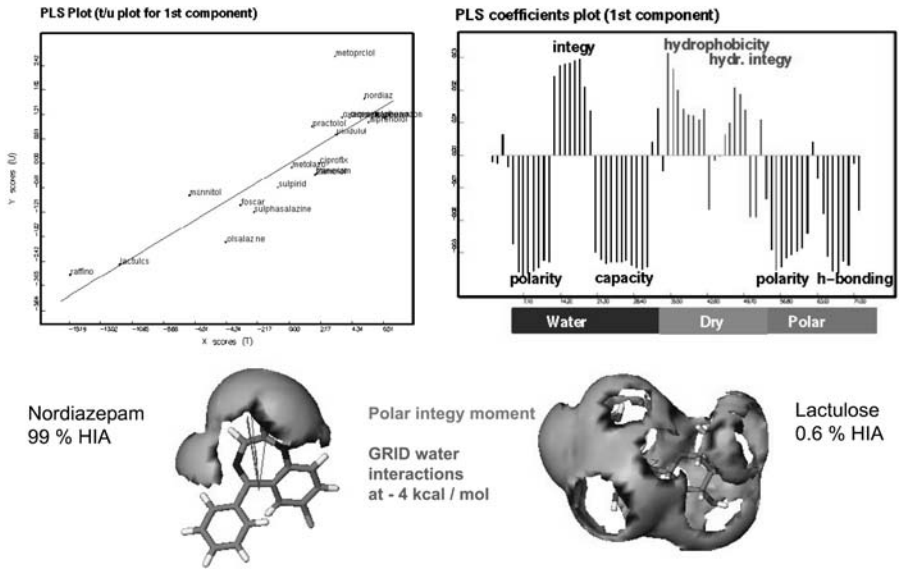

Fig. 3. Correlation of VolSurf descriptors with human intestinal absorption using multivariate statistics (PLS) based on 20 drug molecules as reported by Guba et al. (2000). The PLS plot (u1 versus t1) and the corresponding PLS coefficient plot is shown. Different interaction pattern with the GRID water probe are displayed for the orally available nordiazepam (*left*) versus the large area for the non-available lactulose (*right*).

to the 3D map of interest. The resulting set of descriptors thus have a clear chemical meaning.

EVALUATION

The descriptors generated by VolSurf have been successfully correlated using multivariate statistical techniques like partial least-squares projection to latent structures (PLS) in literature and in internal studies with bioavailability, blood-brain partitioning, membrane transport and other relevant ADME and pharmacokinetic properties (Alifrangis et al. 2000; Crivori et al. 2000; Cruciani et al. 2000c; Guba and Cruciani, 2000; Cruciani et al. 2003). One earlier examples is the study by Guba and Cruciani to unravel essential molecular features correlated to human intestinal absorption of 20 drug molecules (Guba et al. 2000). The correlation of VolSurf descriptors for 20 diverse drugs to this pharmacokinetic parameter is shown in Figure 3. The interpretation of PLS models for factors influencing permeability and absorption are in agreement with earlier, qualitative findings based on global molecular properties represented by descriptors like logP and others. However, due to the nature of the descriptors this approach allows to better understand physicochemical requirements for a pharmacokinetic effect and to use it for design, for example the balance between lipophilic and hydrophilic parts in combination with size, volume, and other effects. A special emphasis in the analysis of VolSurf models can be placed on the interpretation after multivariate statistics, because any rational design to improve molecular properties depends on the understanding of how molecular features influence physicochemical and ADME properties.

The successful use of VolSurf was also reported for predicting absorption properties (Cruciani et al. 2000c) from drug permeability data of 55 molecules compounds (Irvine et al. 1999) in Caco-2 cells (human intestinal epithelial cell line derived from a colorectal carcinoma) and MDCK (Madin-Darby canine kidney) cell monolayers. In this interesting case it was shown that models including counterions for charged molecules clearly show significantly better statistical quality and overall performance. The final model was also able to correctly predict to a great extend the relative ranking of molecules from another Caco-2 permeability study by Yazdanian et al. (1998).

This model recently has been extended (Cruciani et al. 2003) to a dataset of 450 carefully selected Caco-2 data from the literature excluding compounds showing high efflux rates which might indicate active transport by P-glycoprotein (P-gp) through the intestinal membrane. This attempt was done to compile a dataset of molecules having a similar mechanism of transport. However, as a quantitative comparison of Caco-2 data from different laboratories is nearly impossible due to experimental uncertainties and variations of conditions, the molecules were classified in low and high permeable compounds. The use of partial least squares discriminant analysis (DA-PLS) resulted in

a statistically significant model after crossvalidation, which allows to discriminate in the 2D PLS score plot between compounds with low and high permeability, while compounds at the interface of both classes could not be predicted with high certainty. Classification models like this are valuable for earlier phases of the lead optimization process, as external compounds can be projected in the chemical space represented by such a model in order to classify Caco-2 permeability potential for novel, untested molecules. Of course, the prediction accuracy of classification models is lower and results are only meant to be used in a qualitative sense for design and decision making.

The suitability of VolSurf to describe the interaction of compounds with membranes was reported by Alifrangis et al. (2000). They established a structure-property model for membrane-partitioning for 20 peptides with experimental data tested in two chromatographic systems with phospholipids as stationary phase, immobilized artificial membranes (IAM) and immobilized membrane chromatography (ILC). The relationship between these measures and the sets of calculated descriptors derived from molecular surface area, MolSurf, and VolSurf were analysed using PLS, showing that the VolSurf derived model was significantly better than both other models. Especially the VolSurf critical packing descriptor, developed to describe the interaction of amphiphilic molecules with membranes, was found to be important to explain the peptide membrane partitioning ability.

In addition to the above mentioned properties of VolSurf, no alignment of the compounds is necessary prior to the calculation of the molecular interaction fields and the following generation of the VolSurf descriptors. Furthermore, this approach is often hardly influenced by conformational sampling and averaging (Mannhold et al. 1999; Crivori et al. 2000; Cruciani et al. 2000a, Guba and Cruciani 2000). This is probably due to the peculiarity of the GRID force field which allows for the conformational flexibility of external groups, hydrogens, and lone pairs. In general, the automated protocol consisting in a simple 2D-to-3D structure conversion followed by energy minimization produces good results, without the need of molecular dynamic sampling or Boltzmann averaging. This makes VolSurf descriptors computationally efficient and well suited for fast quantitative structure-property relationship studies, especially when dealing with a large number of compounds.

Volsurf descriptors were also successfully used to build a classification model for predicting blood-brain barrier (BBB) permeability for drug-like molecules (Cruciani et al. 2000c, Crivori et al. 2000). Based on 72 descriptors, a discriminant PLS analysis was performed to build a model for qualitative experimental BBB permeation data for 110 molecules. Another 120 molecules served for model validation by external prediction. For racemic or diastereomeric compounds, all stereoisomers were used to generate descriptors based on the reasonable assumption of negligible stereoselectivity in passive membrane permeation. Based on both datasets and combined discriminant PLS, a new model with 229 molecules was derived. It correctly predicts 90 % of the BBB data and the interpretation of the PLS coefficients provides insights to drug design, pharmacological profiling and screening. Descriptors encoding polarity such as the hydrophilic water-accessible surface regions, capacity factors (volume / total surface at distinct energy levels, i.e. number of polar interactions per surface unit), and hydrogen bonding are inversely correlated with BBB permeability. Hence, BBB permeability decreases with increasing polar surface in agreement with the PSA model discussed above. Some more quantitative information can be extracted, such as the influence of charge distribution and electron lone-pairs. The influence of capacity factors means that although diffuse polar regions are tolerable, dense and localized polar regions are detrimental for BBB permeation. All descriptors encoding hydrophobic interactions show less influence, but are directly correlated with BBB permeation. While size and shape of the molecules have no influence, the critical packing and hydrophilic-lipophilic balance are important showing that a balance of nearly all descriptors are relevant for BB permeation. Utilizing all molecules from another dataset (Luco 1999) for external prediction the VolSurf model was able to correctly predict the BBB permeability range for more than 75 % of the compounds (Cruciani et al. 2000c), which is encouraging, as BBB permeation is not only dependent on passive diffusion, but also on active transport and metabolism.

Other important applications include the generation of a model to predict thermodynamic water solubility (Cruciani et al. 2003). This model is based on consistent solubility data from literature plus additional measurements for 970 compounds. Its quality allows to differentiate between very poorly/poorly/medium/highly and very highly soluble molecules while exact rankings within individual classes are not possible. However, given the different factors influencing experimental thermodynamic solubility data, it is not likely that significantly improved models for this key property in pharmaceutical sciences can be derived.

VolSurf was also used in a study to obtain information about metabolic stability in human liver preparations (Cruciani et al. 2003). A training set of 500 compounds with data from metabolic stability testing of the compounds in human CYP3A4 cDNA-expressed microsomal preparations was used to built a model using principal component analysis (PCA). Although this statistical approach did not incorporate any experimental information about compound stability, a grouping of metabolically instable molecules in one focussed region in the final model was observed upon inspection of the PCA score plots. Recently this model has been improved (Crivori et al. 2004) by adding other GRID derived descriptors in addition to VolSurf. These novel descriptors named Grid-Independent Descriptors (GRIND, Pastor et al. 2000) are implemented in the program Almond (see below). On an extended training set of 1800 compounds tested in one laboratory at a pharmaceutical company, a classification model using discriminant PLS was established and validated using different test sets. This extended model correctly predicted 75 % internal compounds and 85 % public drugs and revealed an overall precision of 86 % correct selection of metabolically stable compounds.

CRITICAL ASSESSMENT OF THE METHOD

From many published and internal applications of VolSurf (see below), the assumption that the derived 3D molecular descriptors capture relevant information about physicochemical and pharmacokinetic properties of drug molecules is underscored. Other studies have shown that the method is not significantly depending on the chosen conformation, as long as there is a consistent approach to generate them. Often molecular 2D/3D converters like CONCORD (Pearlman 1987) and CORINA (Sadowski et al. 1992; Sadowski et al. 1994) with subsequent energy minimization might be applied for this purpose. Hence, these descriptors are extending pioneering concepts like PSA and logP by capturing similar information like these global molecular descriptors in a series of carefully chosen descriptors. In combination with multivariate statistical approaches, the resulting models then allow a more quantitative understanding of factors influencing the physicochemical or ADME property of interest which in combination with intrinsic chemical interpretability provide important guidelines for further compound optimization.

The suitability of these descriptors for database mining was investigated in a comparative analysis (Cruciani et al. 2002). While 2D descriptors like Unity fingerprints and MACCS keys were shown to outperform VolSurf descriptors and logP in studies related to pharmacodynamics (clustering behavior on chemically homogeneous series with different pharmacological effect) VolSurf shows the most realistic clustering for pharmacokinetic aspects exemplified by solubility and blood-brain partitioning behavior.

MODIFICATIONS OF THE METHOD

While VolSurf have been extensively used to model pharmacokinetic properties it has only recently been shown to also capture relevant information to describe protein-ligand interactions (Zamora et al. 2003). Steric, hydrophobic, and hydrogen-bonding interactions captured by these descriptors were also shown to be useful for describing ligand binding affinities to various proteins. Significant PLS models for both a diverse set of protein-ligand complexes and a congeneric series of ligands to a single binding site were reported which open the road towards a simultaneous optimization of ligand binding affinity and pharmacokinetic properties using a framework of consistent descriptors.

Additional shape descriptors in an extended VolSurf approach have been applied to model biological activity of a series of quinolones using a quantitative structure-activity relationship (QSAR) approach (Cianchetta et al. 2004). The molecular shape function used to augment the VolSurf approach is constructed from probe-drug interaction energies from the GRID force field. It can be seen as a combination of steric vector descriptors with one extreme in the center of mass of a molecule and with the other located in the molecular surface. These extended VolSurf descriptors now might allow for a simultaneous optimization of affinity and pharmacokinetic properties using multivariate statistical analysis using two or more dependent variables (i.e. biological properties) to model. Any resulting model then might allow to identify favorable descriptor combinations for both properties under investigation and thus enable a more focused lead optimization by concentration on those regions in chemistry space, which result in acceptable properties simultaneously.

Another modification of the VolSurf approach to analyze molecular interaction fields was described by Pastor et al. (2000). The same information previously been used to compute VolSurf descriptors now served to compute GRIND descriptors. In contrast to VolSurf, these descriptors capture information about pharmacophoric groups and their distances within individual molecules. These descriptors represent favorable interaction energy regions where groups

of a potential protein would interact favorably with the ligand. Hence, this set of descriptors is ideally suited to model ADME properties related to molecular recognition events between a protein and a series of ligands like for example inhibition of or substrate binding to cytochrome P450 isoforms (Afzelius et al. 2002; Afzelius et al. 2004) as well as binding to transporters like P-gp, Pep-T1 and others.

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II.C.1.3 3D-QSAR (Comparative Molecular Field Analysis, CoMFA)

PURPOSE AND RATIONALE

The relationship between chemical properties like solvent partitioning and biological activity was first discovered over a century ago (Meyer 1899) while the field of quantitative structure activity relationship (QSAR) was opened by Hansch and Fujita (1964) with statistical correlations of global descriptors like logP, molar refractivity, and shape to biological activity of congeneric series. Numerous successful applications of QSAR over the following four decades highlighted the fundamental relationship between chemical structure and biological activity, which allows to estimate the activity of untested molecules based on a validated "QSAR equation". However, the identification of important global descriptors often does not lead to a design of new compounds, as these descriptors do not capture specific, spatially resolved information about molecular recognition events. The approaches discussed in previous sections are describing prop-

erties like passive permeability which are related to fundamental physicochemical behavior. But the understanding of structural requirements for protein-ligand recognition in ADME like binding and inhibition of drug molecules to the family of cytochrome P450 enzymes or specific transporters like P-gp or Pep-T1 requires a more specific description of molecules and their putative modes of interaction with their macromolecular partners. The idea that three-dimensional molecular interaction fields (Goodford 1985) can be combined with multivariate statistics like PLS (Wold et al. 1984; Dunn et al. 1984) led to the first widely used approach for 3D-QSAR named Comparative Molecular Field Analysis (CoMFA) (Cramer et al. 1988b; Clark et al. 1990). Statistical relationships are derived between molecular property fields of aligned compounds and biological activities. Electrostatic and steric interaction energies are computed between each ligand and a probe atom located on predefined grid points for CoMFA. These fields have been successfully replaced or augmented by alternative approaches to compute molecular interaction fields, for example various probes from the GRID force field (Goodford 1985; Baroni et al. 1993), lipophilic fields (Kellogg et al. 1991), or the comparative molecular similarity index analysis (CoMSIA, Klebe et al. 1994a).

PROCEDURE

The first step of a 3D-QSAR study is to satisfactorily superimpose all molecules as this critically translates to the overall success of the investigation. There are multiple approaches towards a reasonable alignment including the use of X-ray crystallography for template molecules (Waller et al. 1993), docking into experimentally determined binding sites or homology models, an automated pharmacophore-based alignment (Martin et al. 1993; Greene et al. 1994; Sprague et al. 1995; Jones et al. 1995), field fit similarity methods (Clark et al. 1990; Klebe et al. 1993), flexible molecular alignments (Klebe et al. 1994b, Lemmen et al. 1998), and manual alignment strategies based on a mixture of conformational searching and chemical intuition. While sometimes a simple alignment onto a common core produces a chemically meaningful model this only allows to interpret the influence of different substitution properties to biological activity. The real value of 3D-QSAR, however, is seen in the fact that not only models for congeneric series can be established, but that different series could be merged into a single model, given that a reliable alignment of differing core structures could be obtained. This step is mainly driven by the identification of putative pharmacophoric points, namely those molecular features that are likely to interact with the protein binding site. The 3D-QSAR statistical models might then allow to discriminate between different alignment hypotheses simply on the basis of the comparison of the predictivity of both alternative models. Although the dependency of grid-based 3D-QSAR methods of the alignment is often seen as disadvantage, a careful and stepwise alignment procedure might produce deeper insights than might be extracted from quantitative information about pharmacophore hypotheses (Kellogg et al. 2003).

After superposition, steric and electrostatic interaction energies between a probe atom and every molecule are calculated at surrounding points of a predefined grid box. The nature of the probe is depending on the property to be mapped. Typically, this is based on a volume-dependent lattice with 1 or 2 Å grid spacing, a positively charged carbon atom and a distance-dependent dielectric constant. The magnitude of the regions are typically defined to extend the superimposed conformers by 4.0 Å along the principal axes. The maximum field values have to be truncated for mathematical reasons at cutoff values, often 30 kcal/mol for steric and +/– 30 kcal/mol for electrostatic energies. For points "*inside*" a molecule (steric energy above the cutoff), no electrostatic energy is computed, those values were set to the mean of the related column. Consequently, these maps can be perceived as 3D profiles, which might correspond to binding site recognition requirements in terms of steric, electrostatic, and hydrophobic complementarity.

In CoMSIA, molecular interaction fields are replaced by fields based on similarity indices between probe atoms and each molecule. The same alignment can be used to compute steric, electrostatic, and hydrophobic similarity index fields for CoMSIA. Hydrophobicity in this context is based on Crippen's partial atomic hydrophobicities (Ghose and Crippen 1986). The advantage of CoMSIA fields is that no singularities occur at atomic positions due to a Gaussian-type distance dependence of the physicochemical properties, thus, no arbitrary cutoffs are required. Similarity indices (Kearsley and Smith 1990) are typically computed using a probe with charge + 1, a radius of + 1, a hydrophobicity of + 1, and an attenuation factor α of 0.3 for the Gaussian-type distance dependence.

Equal weights for different CoMFA or CoMSIA fields were assigned using the CoMFA_STD scaling or block scaling option while columns, i.e. grid points with no or little variations are often rejected prior to

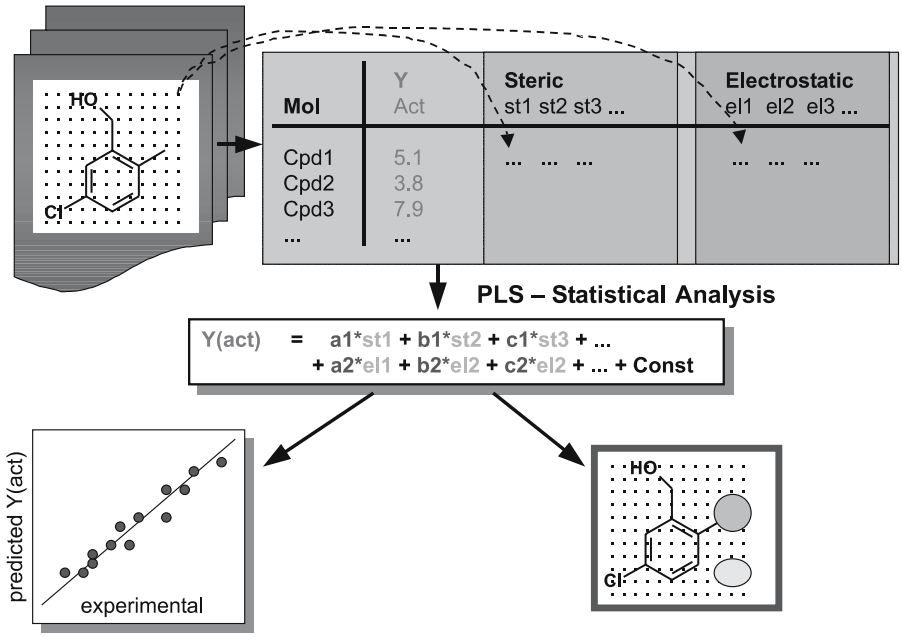

Fig. 4. The CoMFA process consists of individual steps (adapted from Cramer et al. 1988). First, interaction energies are computed between all molecules in alignment and a probe atom on a regular grid. This information is stored in a particular format in order to allow for multivariate statistical analysis. The PLS method then produces a statistical model which can be interpreted and used for prediction.

statistical analysis. Then PLS is used as multivariate statistical approach to derive a linear relationship for highly underdetermined matrices while crossvalidation (Wold 1978) is used to check for consistency and predictiveness. A faster approach to crossvalidation has been implemented in SAMPLS (Sheridan et al. 1994). An overview of the CoMFA method is provided in Figure 4.

The resulting std*coeff (standard deviation * PLS coefficient) contour maps from 3D-QSAR models enhance the understanding of electrostatic, hydrophobic, and steric requirements for ligand binding guiding the design of novel molecules to those regions where variations altering steric or electrostatic fields reveal a significant correlation to biological properties.

EVALUATION

A survey on 3D-QSAR literature (Oprea 2004) reported more than 1100 entries in the Chemical Abstracts database on CoMFA, 3D-QSAR, and related keywords. For CoMFA alone, 586 publications between 1988 and 2001 demonstrate its wide distribution and applicability. As the number of potential targets in drug discovery is steadily increasing, it is likely that 3D-QSAR models and methodologies will continue to be developed in the future. Successful applications were not only reported to understand target related affinity but also for some ADME relevant targets like transporters and cytochrome P450 isoforms. For example a 3D-QSAR model for peptidic substrates of the mammalian H^+/Peptide cotransporter PepT1 (Gebauer et al. 2003) showed a high degree of internal consistency and was able to correctly predict a test set of 19 peptide derivatives. In another example, structural features for the P-glycoprotein (P-gp) mediated transport of a series of glucocorticoids were recently identified by means of CoMFA and CoMSIA models (Yates et al. 2003). Further applications in the area of ADME modeling include the generation of 3D-QSAR models for cytochrome P450 2C9 inhibitors (Rao et al. 2000) and 2D6 substractes (Haji-Momenian et al. 2003) to predict potential drug–drug interactions.

CRITICAL ASSESSMENT OF THE METHOD

As for all statistical approaches, there is a need for robust validation to ensure that the resulting models are not misleading due to chance correlation and that these models show predictive power to novel molecules. Hence, the successful application depends on the chosen validation strategy using internal validation and evaluation using an external test set. Furthermore, the choice of a set of appropriate descriptors leading to directly interpretable 3D-QSAR models is crucial for interpretation and discussions with medicinal chemists.

The use of 3D-QSAR is based on a series of particular approximations (Oprea 2004) as given in the following. The ligand and not a metabolite nor a covalently linked inhibitor must be responsible for the biological effect. Ligands should also be modeled in their presumable "bioactive" conformation. No significant conformational changes should occur upon binding to the target and the binding site should be the same for all ligands. Kinetic effects are neglected which requires the system to be in equilibrium. The entropic contributions to the free energy of binding should be comparable across the entire ligand series while solvent effects, temperature, diffusion, transport, pH, and salt concentrations known to contribute to the binding energy are neglected.

The alignment rule significantly impacts results and predictivity of the final 3D-QSAR models. Hence, the use of consistent alignment rules for novel molecules that are less similar to the training site molecules is one of the main prerequisites of this approach. It has clearly be recognized that the definition of a justified alignment rule matching a user-defined pharmacophore is important to avoid wrong conclusions and errors in CoMFA studies (Kubinyi 1998). Whenever possible, experimentally determined alignment should be given preference in 3D-QSAR. Despite those limitations, the arsenal of 3D-QSAR methods is an important tool in the daily practice of drug discovery programs, especially in the absence of experimental 3D-structure of macromolecular receptors or ADME antitarget in complex with representative ligands.

MODIFICATIONS OF THE METHOD

In the meantime, other approaches have been reported to compute molecular interaction fields and other descriptors on a regular grid, like different probes from the GRID force field (Goodford 1985; Baroni et al. 1993), lipophilic fields (Kellogg et al. 1991), comparative molecular similarity index analysis including hydrophobic and hydrogen-bonding fields (CoMSIA, Klebe et al. 1994a). Further additions to the family of 3D-QSAR methods include 4D-QSAR (Dunn et al. 1998), CoMPASS (Jain et al. 1994), receptor surface models (Hahn and Rogers 1998), the pseudoreceptor approach (Gurrath et al. 1998) and comparative molecular surface analysis (CoMSA, Polanski et al. 2002). As there is still no convincing and transferable solution of the alignment problem available, further research led to the development of alignment-free 3D descriptors like for example GRIND (see above; Pastor et al. 2000). Furthermore, the use of predictive and validated CATALYST pharmacophoric models (Greene et al. 1994; Sprague et al. 1995) has also been found wide application to understand structure-activity relationships. This approach has also been successfully applied to ADME relevant targets like human cytochrome P450 datasets (Ekins et al. 2001) to understand structural requirements of their active sites on the basis of inhibitor and substrate series in the absence of crystal structures for these human enzymes.

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II.C.2 Examples for General and Local ADME Models

This section will provide an overview on general and local ADME models from our group to illustrate our approach for building predictive models on structurally diverse training sets. We will discuss two datasets for human intestinal absorption and human serum albumin binding more in detail as examples for general models. Significant models for other relevant ADME properties have also been obtained. The main application of these models is seen in earlier phases of the drug discovery process related to library design and lead identification when no or little information on analogs is available. In addition, we will also show the generation of local, chemotype specific models by means of two examples namely the prediction of permeability for a series of thrombin inhibitors and the prediction of systemic exposure in rabbits following oral administration of MMP-8 inhibitors. Both models exemplify the typical situation in lead optimization where a collection of analogs has already been experimentally profiled. Those models, however, do not stand alone but are used in combination with appropriate QSAR models tailored for affinity and selectivity for individual projects in the frame of multidimensional lead optimization.

II.C.2.1 Correlating 3D Structure to Human Intestinal Absorption

PURPOSE AND RATIONALE

The knowledge of the extent of human intestinal absorption is an important factor in the design of novel

drug candidates. Several in-silico models to predict human oral absorption have been reported in the literature (Agatonovich-Kustrin et al. 2001, Deretey et al. 2002; Egan et al. 2000; Fu et al. 2001; Klopman et al. 2002; Norinder and Österberg 2001, Oprea and Gottfries 1999; Raevsky et al. 2000; Sugawara et al. 1998; Zhao et al. 2001). Simple models are based on only few physicochemically meaningful descriptors like logP, logD, or polar surface area (PSA). Consistenly, all these models are only applicable if the compounds are passively absorbed. In case of absorption via active transporters or if efflux is involved prediction of absorption is still not successful. This is a typical example where the attempt to predict data related to multiple underlying mechanisms do not produce significant models.

Prediction of systemic exposure or bioavailability from molecular structure is much more difficult, since these parameters depend on solubility, absorption, and first-pass clearance. To this end, Yoshida and Topliss (2000) generated a QSAR model by applying "fuzzy adaptive least squares" using logD at pH 7.4 and 6.5 as physicochemical properties and the presence of key functional groups as structural descriptor. They achieved a classification of drugs into one of four bioavailability categories with an overall accuracy of 60 % (Yoshida and Topliss 2000). Another recent approach uses neural networks and a collection of chemical descriptors to arrive at a model with good predictability (Turner et al. 2004).

While the majority of published models are based on a limited number of drug molecules, especially the study of Zhao et al. (2001) provides the most extensive compilation from available literature data and a statistical model derived from those using Abraham descriptors. We used this carefully selected dataset to build a quantitative model for human intestinal absorption employing VolSurf descriptors (see Cruciani et al. 2000).

PROCEDURE

A dataset of 169 drugs and drug-like molecules was extracted from the compilation of literature data from Zhao et al. (2001). These molecules are considered to have reliable data about human intestinal absorption. Biological data were used as percent human intestinal absorption (% HIA) for statistical analysis. All molecules were treated as neutral and converted into their 3D structures using CORINA (Sadowski et al. 1992). The molecules were not aligned to each other. After 3D conversion, no further energy minimization was employed. Molecular interaction fields were computed using the GRID force field in VolSurf employing the water, dry and carbonyl oxygen probes. Subsequently a series of 72 VolSurf descriptors was computed from analysis of these three molecular interaction fields. These descriptors encode the following information (Cruciani et al. 2000):

1. Total volume (at an energy level of 0.25 kcal/mol)
2. Total surface (at an energy level of 0.25 kcal/mol)
3. Rugosity as ratio between total volume/total surface
4. Globularity
5. Volumes of interactions with GRID water probe at eight energy levels (– 0.2 to – 6.0 kcal/mol)
6. Integy moments as distance from the center of mass to the center of interaction with the water probe eight energy levels
7. Capacity factors as ratio of water volumes of interactions to total surface at eight energy levels
8. Local interaction energy minima and distances
9. Volumes of interactions with GRID dry probe at eight energy levels (– 0.2 to – 1.6 kcal/mol)
10. Integy moments as distance from the center of mass to the center of interactions with the dry probe at eight energy levels
11. Hydrophilic versus lipophilic balance (from –3, –4 kcal/mol water probe to –0.6, –0.8 kcal/mol dry probe)
12. Amphiphilic moment as vector from hydrophobic to hydrophilic domain center
13. Critical packing parameter defined as the volume of the hydrophobic part divided by the product of the surface of the hydrophilic part times the length of the hydrophobic part
14. Volumes of interactions with GRID carbonyl oxygen probe at eight energy levels (–0.2 to –6.0 kcal/mol) to model hydrogen bonding capacity.

All descriptors were normalized using autoscaling and subjected to multivariate statistical analysis using PLS. From initial models encompassing all 72 descriptors, a set of 40 informative descriptors was selected using fractional factorial design (FFD) implemented in VolSurf (Baroni et al. 1993; Cruciani et al. 2000).

EVALUATION

This procedure led to a predictive PLS model for 40 VolSurf descriptors and 4 relevant PLS components with an crossvalidated r^2(cv) value after leave-one-out crossvalidation of 0.662 and a conventional r^2 value

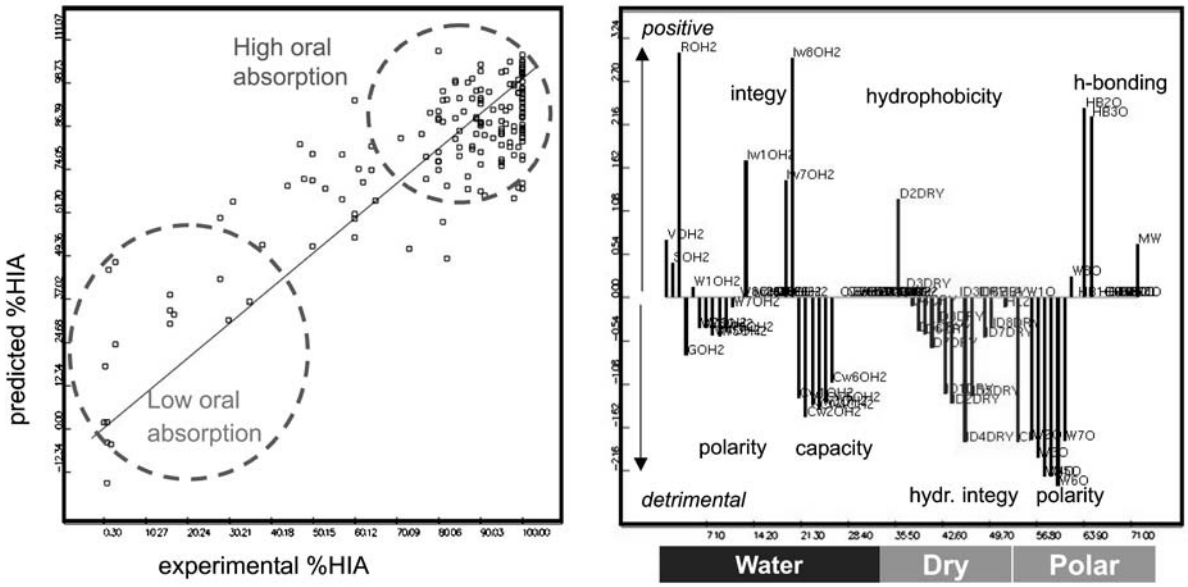

Fig. 5. Correlation of VolSurf descriptors with human intestinal absorption for 169 drug molecules. *Left*: Predicted versus experimental %HIA (human intestinal absorption) from final 4-component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of human intestinal absorption.

of 0.709[1]. Further statistical validation using leave-two-out and leave-multiple-groups-out crossvalidation procedures further underscores the significance of the final model. The overall statistical quality of this model is similar to the model reported by Zhao et al. (2001; model 5; r^2: 0.74; r^2(cv): 0.72). This model also favorably compares to a previously reported VolSurf model for human intestinal absorption based on 20 drug molecules only. This earlier model with one PLS component showed an r^2(cv) of 0.726 and a conventional r^2value of 0.782 for 72 descriptors without variable selection (Guba and Cruciani 2000; cf. Figure 3) while the structural range of molecules in the extended data set from Zhao et al. (2001) certainly encompasses more chemotypes and relevant functional groups.

The correlation of VolSurf descriptors to the human intestinal absorption for 169 drugs is shown in Figure 5 from the final 4-component PLS model. On the left panel the plot of experimental versus predicted % HIA is displayed. Although the data points do not fall onto a straight line, the model clearly is able to discriminate between compounds with high, medium or low intestinal absorption. The analysis of PLS coefficients from this model allows for a chemical interpretation of the importance of individual descriptors contributing to the model as shown in Figure 5 on the right panel. Factors like rugosity (ROH2), integy moment derived from water interactions (Iw*OH2), hydrophobic contributions at lower energy levels (D2DRY), and hydrogen bonding (HB*O) are positively correlated to human intestinal absorption. Thus, if a chemical modification of the underlying structure increases any of these descriptors it is likely that the intestinal absorption will be positively affected. In contrast, the increase of polarity from both water and carbonyl O probes (W*OH2, W*O), the associated capacity factor (Cw*OH2), and the hydrophobic integy moment are detrimental for intestinal absorption. These conclusions for factors influencing permeability and thus intestinal absorption are in agreement with earlier findings pointing to the positive impact of hydrophobicity, integy moment, shape, and hydrogen bonding while polarity derived using the GRID water and carbonyl O probes as well as the capacity factors from polar interactions on the entire surface are detrimental. Previous work also suggests H-bond donors, acceptors, and polar surface contributions as important to model human intestinal absorption (Clark 1999; Wessel et al. 1998; Palm et al. 1997). However, due to the nature of the VolSurf descriptors in combination with multivariate statistical analysis, this approach led to an enhanced understanding and quantitative modeling of physicochemical requirements influencing this pharmacokinetic observable. In particular, the balance

[1] r^2(cv) also known as q^2 indicates crossvalidated r^2, that means each compound is removed once from the model, then, a submodel is generated and the removed compound is predicted using the submodel. The subsequent analysis of all predictions produces r^2(cv) (Wold 1978; Cramer et al. 1988).

between lipophilic and hydrophilic parts in combination with size, volume, and other effects guides the design of new compounds.

CRITICAL ASSESSMENT OF THE METHOD

VolSurf descriptors are able to predict absorption for a diverse set of drugs. The presented model is derived using a consistent frame of relevant chemically interpretable descriptors, which find applications in different local and general models. However, absorption is not only controlled by passive membrane permeability. There are other factors influencing in vivo human absorption namely the in vivo dissolution rate in small intestinal fluid and the dose used for the human study. Furthermore, active transport or efflux mechanisms are difficult to rule out but can only be partially monitored by in vitro experiments. These important pieces of information should be known before any QSAR analysis is attempted on human absorption. This lack of consistent information throughout the literature is difficult to overcome, in particular for human studies. Hence, this study for the dataset from Zhao et al. (2001) provides a reasonable attempt to address these problems to carefully selecting members of the final dataset.

Another problem for this and related datasets is the biased distribution of the biological observable. There are too many compounds with high absorption as this information gets primarily published after a successful drug discovery program. Compounds with no or low absorption by design or as failures are much harder to find in the public domain. Furthermore, this model is built within a limited chemical space which certainly does not reflect the chemical diversity in typical corporate libraries within the pharmaceutical industry. Although individual members of the training set are quite diverse to each other, the global coverage of chemical space is limited.

This collection of limiting factors clearly influences the quality of the model and its predictive power. In view of these sources of inconsistencies and errors in determining the intestinal absorption in humans, it is impressive that the quality of the model is as good as it is. In particular this model was shown to be of particular use as general model in earlier phase for classification of molecules. Any significant improvement is likely not to come from statistical or computational methods but from more consistent data on a broader collection of drug-like molecules preferably by an in vitro system eliminating some sources of experimental errors.

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II.C.2.2 Correlating 3D Structure to Human Serum Albumin Binding

PURPOSE AND RATIONALE

Problems related to poor systemic exposure of drug molecules are also related to their volume of distribution, which is indirectly related to plasma protein binding. Human plasma contains more than 60 different proteins of which the major components are serum albumin (HSA, 60 %) and glycosylated proteins like α-acid glycoprotein (AGP). Human serum albumin (66 kDa) is the most abundant protein in blood plasma with concentrations of 0.53–0.75 mM. Eighteen different variants arising from single amino acid mutations have been identified, accounting for different protein binding. Allelic variation makes data consistency difficult and hence modeling of the resultant data less reliable (Kariv et al. 2002). Considering the high concentration of albumin and the wide range of effective concentrations of therapeutic drugs from nanomolar to millimolar, the free concentration for a therapeutic effect can be significantly reduced for drugs with high binding to plasma proteins, independent of the fact that the affinity of drugs to their target protein is often higher than for plasma proteins. A total of eight hydrophobic binding sites for fatty acids have been identified while for drug-like compounds two high affinity binding sites were postulated in subdomains IIA and IIIA. The analysis of HSA X-ray structures (Bhattacharya et al. 2000; Petitpas et al. 2001) also showed that increased ligand concentrations led to binding to all known binding sites with different affinities and different pharmacological relevance.

There have been only a limited number of attempts to understand the molecular factors influencing drug binding to HSA (Colmenarejo et al. 2001; Colmenarejo et al. 2003; Kratochwil et al. 2002; Saiakhov et al. 2000; Hajduk et al. 2003). Although is has been generally discussed that plasma protein binding is only related to ligand lipophilicity, a study by Kratochwil et al. (2002) demonstrated that lipophilicity is rather poorly correlated to HSA binding for a diverse set of molecules, in contrast to congeneric series, where lipophilicity is often found to be the dominant factor. This suggests that specific molecular recognition elements and other physicochemical properties are also involved in the binding event. This led these authors to derive a model based on pharmacophoric similarity for 138 drug molecules and a comprehensive list of pharmacologically relevant first association constants to HSA from the literature.

Colmenarejo et al. (2001, 2003) reported another significant and predictive computational model to predict binding to HSA based on a series of 95 diverse drug-like molecules, again aiming to cover a large region of the chemical space. A set of consistent experimental data from a single laboratory were generated for this study using high performance affinity chromatography (HPAC) with HSA-immobilized stationary phases. We decided to use this homogeneous experimental data for HSA binding to build a quantitative model. Since our objective was to build many different models of ADME relevance with a consistent frame of informative descriptors we employed the VolSurf descriptors (chapter 2.2 and Cruciani et al. 2000) already used for correlation of 3D structures to human intestinal absorption.

PROCEDURE

The set of 95 drugs and drug-like molecules from the study of Colmenarejo et al. (2001) was used. From experimental HPAC retention times, the log $K_{(HSA)}$ values were used for statistical analysis. All molecules were treated in their neutral form and converted into their 3D structures using CORINA (Sadowski et al. 1992). From GRID molecular interaction fields for water, dry and carbonyl oxygen probes, a set of 72 VolSurf descriptors (Cruciani et al. 2000) was computed and analyzed as described above. All descriptors were normalized using autoscaling and subjected to PLS analysis. In the process of model building, two structurally unique outliers were rejected (Ebselen, Captopril). From initial models with only 93 compounds, a set of 47 informative descriptors was selected using fractional factorial design (FFD) implemented in VolSurf (Baroni et al. 1993; Cruciani et al. 2000).

EVALUATION

This procedure led to a predictive 6 component PLS model for 47 VolSurf descriptors with an crossvalidated r^2(cv) value after leave-one-out crossvalidation

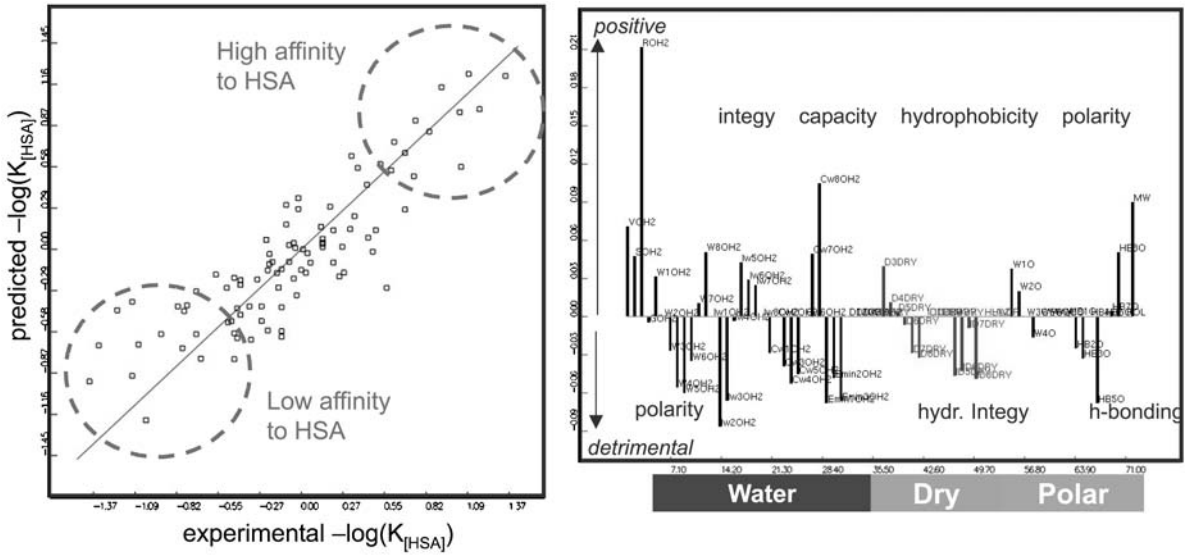

Fig. 6. Correlation of VolSurf descriptors with human serum albumin binding affinity for 93 drug-like molecules. *Left*: Predicted versus experimental $-\log(K_{(HSA)})$ from the final 6 component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of human serum albumin binding.

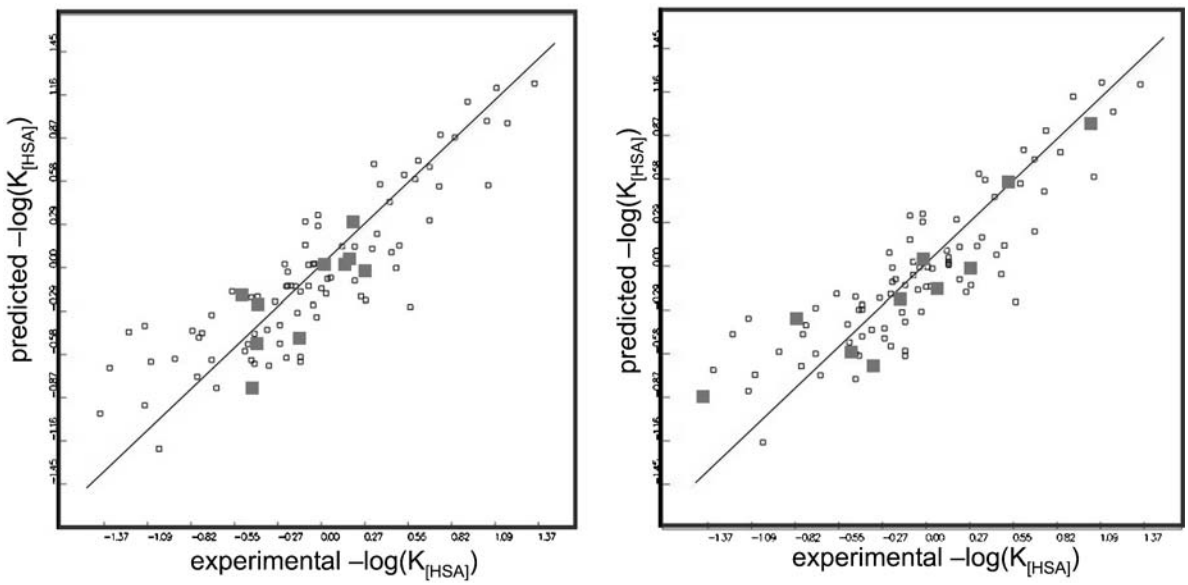

Fig. 7. Validation of VolSurf model for human serum albumin binding. Two submodels have predictive ability to external test sets. 10 compounds were removed by either experimental design on PCA scores (left) or following the literature (right).

of 0.671 and a conventional r^2 value of 0.755. Statistical validation using leave-two-out and leave-multiple-groups-out crossvalidation procedures underscores the significance of the final model. The graph of experimental versus calculated – $\log K_{(HSA)}$ values is shown in Figure 6 on the left. This overall model quality corresponds to the model reported by Colmenarejo et al. (2001).

The predictivity of this model to external compounds was further evaluated by splitting the dataset into a training set of 83 compounds and a test set of 10 molecules. One approach was to use the test set from Colmenarejo et al. (2001 model B), while another test set was generated using statistical design after a principal component analysis (PCA) on VolSurf descriptors (model A). The design was done by selecting

the most descriptive compounds from the PCA scores (Hudson et al. 1996). Scaling and variable selections were done independently for these smaller training sets, resulting in significant PLS models. For model A, a 6 component PLS model with a crossvalidated r^2(cv) value of 0.668 and a conventional r^2 value of 0.763 indicates a statistically significant model. The standard error of prediction (SDEP) of 0.233 for 10 external compounds counts for a model with high predictivity (see Figure 7 left). For model B, a crossvalidated r^2(cv) value of 0.646 and a conventional r^2 value of 0.745 with a SDEP of 0.274 were observed (see Figure 7), leading to the same conclusion. For both models, the graph of experimental versus calculated $-\log K_{(HSA)}$ values is shown in Figure 7. Both test sets are indicated by filled squares. In both cases the agreement between experiment and prediction was very good.

The analysis of PLS coefficients from the final model allows to assess the importance of individual descriptors contributing to the model. The corresponding PLS coefficient plot for the final 6 component model is shown in Figure 6 on the right. Factors like volume (VOH2), shape and rugosity (ROH2), integy moment from water interactions (Iw*OH2), hydrophobic contributions at lower energy levels (D3DRY), hydrophobicity (D3DRY), and molecular weight (MW) are positively correlated with HSA binding of drug molecules. In contrast, polarity (W*OH2), integy moment from the dry probe (Iw*DRY), and hydrogen bonding terms (HB5O) are negatively correlated to albumin binding. Any increase in those molecular properties is likely to decrease binding of drug-like molecules to HSA.

The interpretation for human serum albumin is in accord with literature findings and X-ray information on warfarin binding to the first drug-binding site in HSA (Petitpas et al. 2001). The influence of hydrophobicity in combination with geometry factors like shape, volume, and size is often discussed (Colmenarejo et al. 2001; 2003). It also is in accord with the findings of Kratochwil et al. (2002) demonstrating that lipophilicity influences albumin binding, but certainly not to a very significant extend. Obviously, factors like pharmacophoric similarity which might translate into different shape related VolSurf descriptors are more important. Any increase in polarity reduces HSA binding which can be understood by the mentioned physicochemical requirements of the albumin drug binding sites. The striking effect of shape descriptors on the other hand corresponds to the topological requirements of the binding sites.

CRITICAL ASSESSMENT OF THE METHOD

The presented general in silico model for HSA binding is built on a dataset from a solid in vitro assay conducted in a single laboratory. Hence, the quality of data certainly is not a limitation for this model. However, this assay is intrinsically not able to differentiate between drugs binding to different binding sites. This experimental uncertainty affects the overall quality of the model and adding another mechanistic dimension to the data. Recently, Hajduk et al. (2003) reported a detailed chemometrical analysis of 889 diverse molecules binding to the binding site in albumin subdomain IIIA only. This analysis was based on data from heteronuclear NMR correlation and fluorescence spectroscopy which enabled the measurement of dissociation constants to only one binding site. However, approaches like these are not routinely used in experimental profiling for this property.

Before such a model can be applied to another binding site or a different pharmaceutical company, careful validation and harmonization studies have to be conducted in order to ensure that the experimental data used for building the model correspond to a certain extend to those data used to evaluate it. Typical assays in this area even do not differentiate between drugs binding to albumin or other serum proteins like α-acid glycoprotein. Furthermore, there is a dynamic equilibrium between albumin-bound and free drug molecules. While binding is focused on one aspect in the transport of drug molecules, the kinetic aspect of k_{on} and k_{off} rates is equally important to understand the complex equilibrium between bound drug, free drug and drug bound often in a tighter fashion to its target protein. Those aspects have not been incorporated into such a model.

The present model is significant as it allows to guide library design and hit exploration on the basis of solid in vitro data without knowledge of close analogs. As drugs with high affinity for albumin exhibit markedly reduced efficacy in vivo this model offers a method to reduce albumin binding of potential candidates while maintaining high affinity at the therapeutic target. However, the real significant of such a model can only be assessed in the context of the experimental setup for drug discovery within a particular organization.

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II.C.2.3 Correlating 3D Structure to Intestinal Permeability for Thrombin Inhibitors

PURPOSE AND RATIONALE

During lead optimization, the use of chemotype specific models offers advantages over general models including the coverage of the relevant chemistry space for a discovery project and the availability of experimental data for a series of analogs usually from a single laboratory. Even if these data are not strict single mechanism data, there is often evidence that molecules on a particular chemotype share a particular mechanism of action.

Many pharmaceutical companies are actively working on the development of orally active inhibitors of the serine protease thrombin as one important enzyme in the blood coagulation cascade. In many cases, however, the systemic exposure to thrombin inhibitors following oral administration is limited due to low intestinal permeability rather than low solubility or metabolic instability. Most synthetic inhibitors require a highly basic functional group like benzamidine or guanidine for favorable interactions to the S1 pocket of thrombin, thus gaining a significant portion of its binding affinity. However, these moieties often cause low intestinal permeability.

To obtain more insight into the problem of permeability for benzamidine derivatives, Sugano et al. (2000) studied the membrane permeation of 51 benzamidine-based thrombin inhibitors in a rat everted sac permeability model. They reported significant membrane permeabilities in this in vitro model, which they attributed to passive paracellular transport, an absorption mechanism being different to transcellular permeability. Using Caco-2 cell experiments, they confirmed that opening the tight junctions increases permeability of some compounds which underscores that the paracellular pathway is dominating for this chemotype. Using physicochemical descriptors, the authors obtained significant models to understand structural determinants for paracellular intestinal permeability. This homogeneous experimental dataset for permeability of a congeneric series was used by us to build a quantitative local model employing VolSurf descriptors (above and Cruciani et al. 2000). Such a chemotype specific model in combination with similar models for related properties like solubility and others could be valuable during multidimensional compound optimization in the lead optimization phase. We use this model as example to illustrate the generation and use of related local models in optimization.

PROCEDURE

The set of 51 benzamidine-based thrombin inhibitors was taken from the study of Sugano et al. (2000). Experimental rat everted sac permeabilities were expressed as log(ESA) values.[2] The experimental permeability in this assay is expressed as ratio of outer (mucosal side) concentration of the drug and inner (serosal side) concentration after 1 h incubation of the everted sac of rat small intestine. All molecules were treated in their neutral form and converted into their 3D structures using CORINA (Sadowski et al. 1992). From GRID molecular interaction fields for water, dry, and carbonyl oxygen probes, a set of 72 VolSurf descriptors (Cruciani et al. 2000) was computed and analysed as described above.

[2] According to the authors, everted sac permeabilities were abbreviated by ESA.

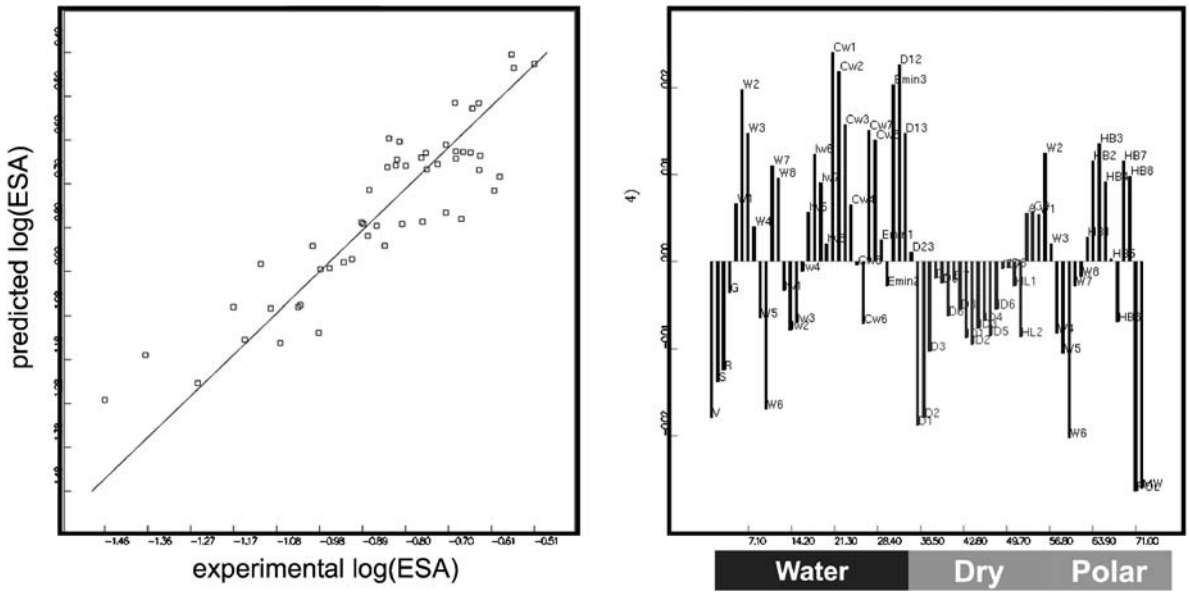

Fig. 8. Correlation of VolSurf descriptors with intestinal paracellular passive absorption for 51 benzamidine-based thrombin inhibitors. *Left*: Predicted versus experimental permeability log(ESA) from the 4 component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of paracellular permeability.

All descriptors were normalized using autoscaling and subjected to PLS analysis. No further variable selection was performed (Cruciani et al. 2000).

EVALUATION

This procedure led to a predictive 4 component PLS model for 72 VolSurf descriptors and 51 thrombin inhibitors. A crossvalidated r^2(cv) value of 0.599 after leave-one-out crossvalidation and a conventional r^2 value of 0.812 were obtained. Statistical validation using leave-two-out and leave-multiple-groups-out crossvalidation procedures underscores the significance of the final model. The graph of experimental versus calculated log(ESA) permeability values is shown in Figure 8 on the left. The overall model quality corresponds to the model reported by Sugano et al. (2000).

Based on the study of Sugano et al. (2000) and our predictive VolSurf model for this series, it can be concluded that factors like size and shape previously reported to affect paracellular permeability are indeed important to explain the local structure-permeability relationship of this chemotype. Usually, permeability via paracellular aqueous pore diffusion depends on the size of the solute and its diffusion coefficient in water. Another important factor is lipophilicity. Between intestinal absorption and both volume and lipophilicity, a negative correlation was reported for this series of thrombin inhibitors. In addition, hydrogen bonding properties and dipolarity are factors that determine the permeability of these benzamidine analogs. These findings are in good agreement to the interpretation of the VolSurf model from inspection of the PLS coefficient plot for the 4 component model (Figure 8 right). This plot is the result from the multivariate statistical analysis showing that factors like hydrophilicity from water interactions (W*), capacity factors (Cw*), and hydrogen bonding properties (HB*) are positively correlated with log(ESA). Increasing these descriptors will positively affect the passive paracellular permeability in this series. In contrast, factors like volume (V), surface area (S), shape (R), lipophilicity (D*), polarizability (POL), and molecular weight (MW) are negatively correlated with permeability.

CRITICAL ASSESSMENT OF THE METHOD

This example shows that a quantitative structure-permeability relationship could be established in a local series of thrombin inhibitors predominantly following a particular route of intestinal permeability via the paracellular pathway. The presented VolSurf model does not only agree to dominant factors discussed in the literature but also builds a statistical basis to quantify individual influences by informative descriptors of physicochemical relevance. Although permeability in general is related to different underlying mechanisms, there is evidence that in this local series the permeability follows only a single route. The detailed structure-permeability relationship on the other hand will guide drug discovery project teams

to introduce structural variations into the presented thrombin inhibitor scaffold and quantitatively assess the potential for paracellular permeability before synthesis. However, it is an intrinsic limitation of such a chemotype specific model that predictions for structurally less related structures are not possible. Furthermore, it cannot be ruled out that a particular derivate does not follow any longer this primary route of permeability which is assumed to form the basis for the present investigation. Beside the molecular modeling aspects of this model, the clinical relevance of paracellular transport, as shown by this example, remains to be elucidated.

Hence, local statistical models provide a qualitative ranking of candidates and, thus, are valuable for optimization of pharmaceutically relevant compounds, especially if combined with additional models to understand affinity, selectivity, or any particular pharmacokinetic behavior.

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II.C.2.4 Correlating 3D Structure to Rabbit Systemic Exposure for MMP-8 Inhibitors

PURPOSE AND RATIONALE

In search for potent and systemically available inhibitors of the matrix metalloproteinase MMP-8 (Matter et al. 1999; Matter et al. 2002) following oral administration, a local ADME model was derived to support lead optimization. For an internal series of inhibitors on the tetrahydroisoquinoline scaffold, hydroxamic acids for zinc ion binding in 3-position are essential for MMP affinity in first generation inhibitors. However, those compounds are characterized by insufficient pharmacokinetic properties and low systemic exposure following oral administration. Driven by X-ray and 3D-QSAR studies (CoMFA), alternative Zn^{2+} binding groups like carboxylates were investigated. In this series, the expected loss in affinity was compensated by optimally filling the MMP-8 S1' pocket close to the catalytic zinc ion. The design and structure-activity relationship (SAR) for this series is in agreement with protein binding site requirements and monitored multiple properties including selectivity against the undesirable MMP-1.

We decided to build a chemotype specific VolSurf model (chapter 2.2 and Cruciani et al. 2000) for 49 MMP-8 inhibitors to better understand molecular and physicochemical factors controlling the systemic exposure following oral administration in a rabbit animal model. This study provides an example where in vivo data have been employed fulfilling certain requirements to build a model for some pharmacokinetic aspects. However, one has to be aware that the obtained experimental data after oral dosing to rabbits do certainly not fulfill the requirement of representing only a single mechanism. Hence, in the initial phase of model building, compounds with metabolic instability from in vitro S9 assays and very low solubility were rejected from the dataset in order to primarily focus on the absorption aspect of the systemic exposure in rabbits following oral administration. This model illustrate the use of local models from in vivo data after filtering by in vitro properties in lead optimization.

PROCEDURE

The systemic exposure of a series of 49 MMP-8 inhibitors was determined following oral administration to rabbits under consistent conditions for inhibitor dosing and formulation. These inhibitors represent different scaffolds and zinc ion-binding functionalities. From the pharmacokinetic profiles of these animal studies the total area under the curve $AUC_{(oral)}$ was converted into $-\log(AUC_{(oral)})$ values and used to build the statistical model. All molecules were treated in their neutral form and converted into their 3D structures using CORINA (Sadowski et al. 1992). From GRID molecular interaction fields for water, dry, and carbonyl oxygen probes, a set of 72 VolSurf descriptors (Cruciani et al. 2000) was computed and analyzed as described above. All descriptors were normalized using autoscaling and subjected to PLS analysis. From initial models, a set of 46 informative descriptors was selected using fractional factorial design (FFD) implemented in VolSurf (Baroni et al. 1993; Cruciani et al. 2000).

EVALUATION

For these 49 MMP-8 inhibitors the systemic exposure in rabbits following oral administration

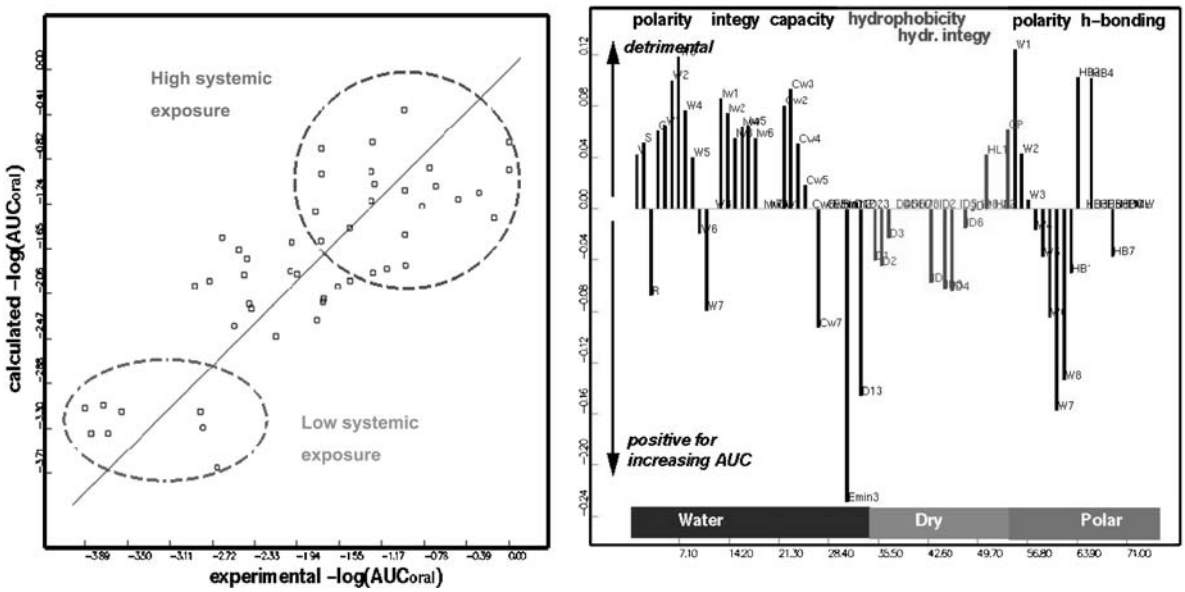

Fig. 9. Correlation of VolSurf descriptors with systemic exposure after oral administration in rabbits for 49 structurally diverse MMP-8 inhibitors. *Left*: Predicted versus experimental systemic exposure –log($AUC_{(oral)}$) from the 4 component PLS model. *Right*: PLS loadings showing the importance of VolSurf descriptors to the prediction of the systemic exposure in rabbits.

was successfully correlated to VolSurf descriptors. A semi-quantitative 4 component PLS model using 46 descriptors was obtained with a crossvalidated r^2(cv) value of 0.424 after leave-one-out crossvalidation and a conventional r^2 value of 0.646. Statistical validation using leave-two-out and leave-multiple-groups-out crossvalidation underscores the significance of the model. The validation suggests to apply the final model in a semi-quantitative way by classification of novel compounds into those with low, medium, or high systemic exposure. The graph of experimental versus calculated -log($AUC_{(oral)}$) permeability values is shown in Figure 9 on the left.

Further validation of this model was done by splitting the dataset into a training set of 39 and a test set of 10 compounds using statistical design after a principal component analysis (PCA) on VolSurf descriptors following the *most descriptive compound (MDC)* algorithm (Hudson et al. 1996). A 3 component PLS model with a crossvalidated r^2(cv) value of 0.457 and a conventional r^2 value of 0.673 indicates a statistically significant model. The standard error of prediction for 10 external compounds indicates medium predictivity but reasonable classification into low, medium, and high systemic exposure.

The analysis of PLS coefficients from the final model allows to assess the importance of individual descriptors contributing to the systemic exposure in rabbits following oral administration in this series. The corresponding PLS coefficient plot is shown in Figure 9 on the right. Please note that due to the chosen logarithmic scale for AUC values, favorable descriptors are now located in the lower area of this plot. Factors like shape (R), strength of interaction energy with water (Emin3), hydrophobicity (D*), integy moment from hydrophobic interactions (ID*), and interactions to the carbonyl O probe (W*) are favorable for the systemic exposure. Increasing these descriptors might lead to increased AUC after oral dosing. In contrast, polarity from the water probe interactions (W*), integy moment from the water probe (Iw*), capacity factor (Cw*), and hydrogen bonding terms (HB*) are negatively correlated to the systemic exposure.

This interpretation, although derived for a local series of MMP inhibitors, is in qualitative agreement to models for permeability and intestinal absorption. The effects of polarity versus hydrophobicity and the importance of hydrogen bonding interactions are consistently highlighted in comparison to general absorption model but with different importance of individual descriptors which have been tailored by PLS to the problem under study.

CRITICAL ASSESSMENT OF THE METHOD

While the chemical interpretation of this model qualitatively agrees to general models for permeability and absorption those conclusions were derived only for a small, limited series of analogs on representative scaffolds. Furthermore, all published models in this

field either describe human intestinal absorption or in vitro permeability assay data while there are significant differences between rabbits, rats and humans in terms of permeability and metabolism. Consequently, it cannot be ruled out that this model is very focused to the rabbit situation, because important animal studies to profile MMP-8 are conducted in rabbits. Although this model is not based on single mechanism data, the focus on a particular series of related scaffolds might help to partially solve this problem. The quality of the model also suggests that more than one mechanism actually is responsible for the observed pharmacokinetic profile, for which the AUC is only one appropriate way to summarize this behavior. Thus a combination of such a final model from in vivo studies combined with some appropriate filters from informative in vitro assays might help to resolve this problem.

The resulting semi-quantitative model was used in conjunction with structure-based docking and scoring, 3D-QSAR based affinity and selectivity predictions and in silico ADME models to estimate membrane permeability, solubility, and other key properties for the optimization process in this series. Hence, in this as well as in other series, multiple models can be collectively applied for ranking and prioritizing synthesis candidates and focused virtual libraries during advanced stages of multidimensional compound optimization.

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II.C.3 Conclusion and Outlook

In recent years, the pharmaceutical industry has come under increasing pressure to reduce the time as well as the expenditure for the discovery and development of novel drugs. As a consequence, sophisticated in vitro assays have been introduced aiming at the simulation and prediction of the in vivo situation. However, beside the generation of huge sets of data, all these assays still need the synthesis of the compounds. To overcome the issue of handling the generated information as well as to support medicinal chemists in selecting the most promising compounds for synthesis, in-silico approaches revealed to be indispensable tools. In-silico tools can be used to extract relevant information from large datasets, thereby assisting in the improvement of the understanding of factors controlling affinity, selectivity, as well as ADME and physicochemical compound properties. Furthermore, in-silico approaches are extremely valuable tools for the design of new compounds with improved properties during a multidimensional optimization phase. Hence, these tools represent a key technology in the transformation from time-consuming trial-and-error strategies to faster prediction approaches.

Facing the growing number of in-silico approaches, the list of tools discussed in this chapter can only represent a selection serving to illustrate their various applicabilities. The tools described in more detail have proven their suitability to support the success of drug discovery project teams. The ADME models discussed in this chapter are dominated by physicochemical molecular properties. Models for other ADME properties like cytochrome P450 inhibition or transporter binding have not been discussed because they are dominated by complementarity in protein-ligand interactions. Although also important, the discussion of these models was beyond the scope of this chapter.

In different phases of a drug discovery project, different in-silico models are applied namely general models in earlier phases and chemotype specific models later when more experimental information about analogs is available for a research team. Hence, the effective generation and storage of meaningful data in databases to address specific ADME and pharmacokinetic properties are essential for timely success of a project. That does not only imply the use of advanced in vitro assay technology towards single mechanism ADME data but also a data infrastructure for re-using valuable data and knowledge in subsequent design phases to effectively support lead optimization.

The problems of today's in-silico models for ADME properties are mainly associated with data availability, consistency, and quality to solve particular problems. Accurate and consistent date is an indispensable prerequisite for predictive models. In particular the use of multi-mechanism data often results in failures

in model building and application. Using complex data might only be possible, if congeneric series are monitored as related compounds might act by similar mechanisms.

Finally, it is important to note that neither a single approach is likely to solve all problems for a chemotype nor is an approach equally well suited for every problem. The challenge lies in the selection of the most suited model for a given problem and in the effective integration of experimental and computational tools. Once the suited in-silico tools are established, they can make a major contribution to the successful lead optimization process resulting in a novel drug.

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Chapter II.D
Absorption – in vitro Tests – Cell Based

Katharina Mertsch

INTRODUCTION

In the pharmaceutical industry we observed a change in paradigm during the 90s: project teams focused their search for potent compounds not exclusively on potency and specificity – they added pharmaceutical, biopharmaceutical, pharmacokinetic, metabolic, and toxicological characteristics for the most interesting compounds as well. The motivation to characterize compounds for a set of pharmacokinetic and toxicological parameters was driven not only by economic reasons. The number of hits from combinatorial and other chemical libraries raised the necessity to increase quality of nominated drug candidates to reduce the attrition rate in preclinical and clinical stages of project development. Nearly all pharmaceutical companies introduced an expanded assessment for drugability and developability of compounds at the stage of lead selection and lead optimization (Borchardt et al. 1998).

The integration of pharmaceutical, pharmacokinetic and toxicological criteria into discovery and development of compounds at the stage of compound lead nomination resulted in a higher percentage of successful clinical drug candidates. Most important criteria for developability and drugability of a compound include pharmacokinetic parameters like permeability, efflux potential, metabolic stability, metabolic profiling, protein binding, metabolic finger printing, inhibition and induction of major metabolising enzymes and safety aspects like Ames, HERG interaction potential, Irwin screening, Micronucleus tests, IC_{50} determination with important cells and others.

The present chapter will focus on one part of early ADME (Adsorption, Distribution, Metabolism, Excretion) strategy: the absorption and efflux screening.

Since many projects focus on the most accepted route of administration – the oral one – assessment of oral bioavailability potential for a compound is a very important part in early ADME screening.

In vivo different factors play a crucial role during absorption processes: disintegration of the dosage form, dissolution of the compound in different parts of the gastrointestinal tract (GI), metabolism in the GI tract and in the liver (so-called first pass), permeation across the intestinal mucosa, clearance. There is no integrative in vitro model being able to cover all aspects if in vivo absorption. Therefore an approach to divide the aspects into different test systems has been used: HTS methods for solubility testing, drug partitioning into membranes between aqueous and lipid phases, metabolic stability, permeability, CYP-inhibition and induction, efflux, protein binding and of course in silico methods and Lipinskis rule of 5 are used prior first animal experiments in pharmacokinetics. Due to the in vivo complexicity an in vitro prediction of absorption is only possible taking all factors of in vitro experiments together into account.

Absorption in the Gastrointestinal Tract

Absorption of compounds in the gastrointestinal tract occurs mainly in the small intestine with 3 different regions (duodenum, jejunum and ileum) and in the large intestine (colon). In the small intestine the uptake surface is increased by folds (3-times), villi (30-times) and microvilli (600-times). In the colon folds and villi are absent (Daugherty and Mrsny 1999).

The major physiological role of the GI, namely to provide a protection against pathogens and allow at the same time the permeation of nutrients and

vitamins, is supported by the brush border structure of microvilli and glycocalix. They form an enzymatic and physiological barrier. Mucus secreted by goblet cells and subepithelial glands interacts with compounds and can bind compounds (Larhed et al. 1998). During the passage through the GI a compound passes regions with different pH: from slight acidic to slight basic impacting solubility and stability. The protection against pathogens in the GI is provided by lymphoid cells producing antibodies and forming a part of the immune system called the gut-associated lymphoid tissue (GALT, Mestecky et al. 1987). In the small intestine lymphoid cells form structures called Peyers patches.

Peyers patches are covered by epithelial cells which can differentiate into M cells. M cells play an important role in the immunologic surveillance of the gut and are involved in specific functions like transport of particles, antigens, macromolecules. M cells seem to be involve in the absorption of intact proteins (Walker and Sanderson 1992). Peyers patches have been studied for uptake of macromolecules. Their proximal vicinity to immunocompetent cells is another hurdle for industrial use as a preferred uptake route (Daugherty and Mrsny 1999; Neutra 1998). M cells have been used to study uptake of lectins (for overview, see Daugherty and Mrsny 1999). However both cell types – Peyers patches and M cells – clearly are limited in their use as a preferred uptake route due to a small surface area they are covering and the limited capacity of absorbed molecules.

Epithelial cells covering the Peyers patches form the barrier in the intestine.

Mechanisms of Control and Barrier Function of Epithelial Cells

The main functions of the GI – digestion and absorption of nutrients, vitamins and cofactors as well as movements of ions and water – need a precise mechanisms of biochemical and physiological control to maintain barrier functions. The cells in the intestine are characterized by high enzymatic activity (lumen and wall), low permeability and typical resistance (between cells tight junctions are formed characterizing the very thight barriers in the organism), efflux pathways back into the gut lumen and first-pass metabolism. The barrier function of the gut is a crucial prerequisite for a normal function of intestine. Impairments lead to diarrhoea and other serious consequences.

Epithelial cells in the intestine form tight monolayers with tight junctions between polarized cells. Tight junctions of epithelial intestinal cells are studied extensively and contain among others ZO-1, occludin, claudin-1 and claudin-2. Tight junctions and complexes of adherence junctions in the membrane are closely connected to cytoskeleton. Studies with CACO-2 cells showed that localization of ZO-1 in the zonula occludens of tight junctions depends on Ca^{++} and Mg^{++} concentrations in the medium (Anderson et al. 1989). CACO-2 cells cultured in serum-free medium did not differ from cells cultured in serum-containing medium concerning brush border enzymatic activity but the transepithelial electrical resistance (TEER) was significantly lower in cells grown without serum reflecting an impairment of tight junctions (Hashimoto and Shimizu 1993; Jumarie and Malo 1991). Cells grown without serum show also a decreased transport of glucose, alanine and Gly-Gly (Hashimoto and Shimizu 1993). This might be related to incomplete differentiation and different expression of receptors and transporters without serum.

Biochemical part of the barrier contains alkaline phosphatase, γ-glutamytranspeptidase, dipeptidylpeptidase IV, aminopeptidase N, endopeptidase 24-11 and disaccharidases saccharidase, isomaltase, lactase. Among metabolising enzymes Phase I enzymes CYP 1A1 and 3A4 and Phase II enzymes glutathion-S-transferase, sulfotransferase and glucuronidase are present.

Uptake and efflux transporters complete the equipment of brush border membrane cells in performing controlled permeation of compounds.

Routes of Permeation across Intestinal Cells

Compounds can cross intestinal cells by different routes (Fig 1):

1. transcellular passive diffusion (drug absorbed from GI tract mainly in unionised form)
2. paracellular diffusion (size and charge dependent)
3. transcellular diffusion by endocytosis
4. transcellular diffusion using lipid particles
5. paracellular passive diffusion modulating tight junctions
6. carrier-mediated diffusion and active transport.

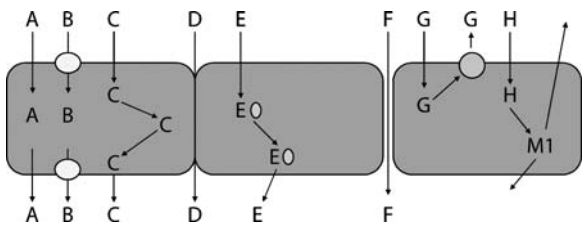

Fig. 1. Drug permeation across intestinal cells

A – transcellular passive diffusion
B – carrier mediated transport
C – transcellular diffusion by endocytosis
D – paracellular passive diffusion
E – transcellular diffusion by lipid particles
F – paracellular passive diffusion via modulation of T.J.
G – efflux
H – metabolism.

The surface area of brush border membranes is 1000 fold larger than paracellular surface area (Pappenheimer and Reiss 1987). Therefore, the probability for transcellular permeability is much higher than for paracellular permeability. Indeed, lipophilic drugs with rapid and complete absorption have a high probability for passive transcellular route. Hydrophilic drugs tend to pass cellular membranes via water filled pores in the paracellular pathway (for review, see Lee et al. 1991). However, there is also a part of hydrophilic molecules passing membranes by transcellular route (Nellans 1991). The paracellular pathway is used by some positively charged compounds whereas transcellular pathway is preferred with unionised compounds.

Regulation of tight junctions seems to be an attractive way of increasing bioavailability. There have been attempts to induce a transient damage of the intestinal mucosa and to enhance drug uptake. Surfactants, detergents, unsaturated cyclic ureas, fatty acids, bile acids, chelating agents were used with more or less serious consequences of transient entry of pathogens and lack of control concerning repair mechanism in the gut. None of the agents used so far resulted in an approval by the FDA for drug absorption support due to the risk of local epithelial trauma (Daugherty and Mrsny 1999).

Other interesting approaches to enhance drug absorption used cyclodextrins. They act as solubilizers of drugs or alter the physical structure of administered proteins.

Transcytosis through epithelial cells is of limited capacity.

Several processes limit the permeability: efflux and metabolism in the intestinal cells as well as high lipophilicity.

Receptor-mediated permeation offers an attractive pathway and has been studied for many drugs and compounds under evaluation.

The old view that the majority of compounds enters the organism by passive diffusion is no longer valid taking into account the complexity of intestinal membranes and number of transporters discovered during the last 10 years.

In vitro Models to Predict Membrane Permeability

As mentioned above due to the increased number of hits in several projects it is not feasible to test all compounds in in vivo experiments. Even cassette dosing does not solve the issue of limited in vivo capacities. In situ models like rat perfusion or inverted out gut segments have also only a limited capacity of throughput. They are used to solve special issues in projects.

Many different models have been introduced to assess permeability of a compound. Human intestinal cells (Hidalgo 1990; Artursson and Karlsson 1991), intestinal mucosal tissue or intestinal segments (Smith 1996; Fisher and Parsons 1949), in situ models with perfused intestinal mucosa (Lennernäs 1998; Griffiths et al. 1996), cellular models (T-84, HT-29, TC7 and others, Zweibaum et al. 1985; Meunier et al. 1995).

The majority of permeability screening is based on cellular assays mimicking intestinal adsorptive cells: CACO-2 cells (human colon carcinoma), and the CACO-2 clone TC7, HT-29, T84, IL-6 or MDCK cells (Madine Darby Canine Kidney). In this widely used and accepted cellular assays compounds are classified into 3 classes of permeability: high, medium, low. Moreover, the absorption potential of compounds within a chemical series can be compared concerning their apparent permeability coefficients (Artursson 1991; Hillgren et al. 1995; Artursson and Borchardt 1997).

CACO-2 are characterized by easy handling and at the same time resemble morphological and biochemical characteristics of intestinal cells.

Among 20 cell lines tested, CACO-2 cells were the only onces to differentiate spontaneously to intestinal enterocytes (Chantret et al. 1988). They resemble many characteristics of small intestinal epithelial cells (Hidalgo and Borchardt 1989) and are still the most widely accepted model in industry and academia (Artursson and Karlsson 1991; Artursson et al. 1996; Audus et al. 1990; Gan and Thakker 1997; Artursson and Borchardt 1997 and many others). Reduced demands for compound volumes in cellular assays allow for screening (Stevenson et al. 1995; Kuhfeld et al. 1994). CACO-2 cells predict permeability with reasonable accuracy and are used in many different test designs.

Their growth is characterized by the sequence of proliferation, confluency, differentiation. Morphologically they reveal typical brush border cells with columnar shape and microvilli, polarization, cubblestone morphology with flower-like clusters (Pinto et al. 1983; Hidalgo and Borchardt 1989), presence of

tight junctional and adherence junctional complexes (Daugherty and Mrsny 1999), biochemical features of intestinal cells and typical transporters expressed at permeability controlling membranes. Among biochemical markers of intestinal cells lumenally expressed sucrase isomaltase, alkaline phosphatase and aminopeptidase N increase in activity after proliferation phase with reaching confluency (Pinto et al. 1983; Zweibaum et al. 1983). Distribution of proteins to polarized membranes in intestine is a typical marker of differentiaton. In CACO-2 cells sorting to apical membranes involves trans-Golgi network (for sucrase isomaltase) or additional routes (aminopeptidase N and dipeptidypeptidase IV; Matter et al. 1990).

Other polarization factors characterizing CACO-2 cells are growth factor receptors (Hidalgo et al. 1989), bile acid transporters (Hidalgo and Borchardt 1990; Wilson et al. 1990), glucose transporters (Blais et al. 1987; Mahraoui et al. 1994), lipoproteins (Traber et al. 1987), neutral amino acid transporters (Hidalgo and Borchardt 1990). Additional enzymes and transporters like dipeptide transporter (Dantzig and Bergin 1990), PEPT1 (Saito and Inui 1993; Thwaites et al. 1994), vitamin B12 carrier (Dix et al. 1990), nucleoside transporters (Ward and Tse 1999), P450 enzymes (Boulenc et al. 1992), sulfotransferase (Baranczyk-Kuzma 1991), UDP-glucuronyltransferase (Peters and Reolofs 1992) complete the biochemical and enzymatic barrier of CACO-2 cells.

Cytosolic enzymatic activities (chymotrypsin-like, trypsin-like, cucumsin-like) were detected in CACO-2 cells at higher activities than in colonic and rectal mucosae (Bai 1995). This enzymes contribute to first pass effect in CACO-2 cells. At confluency in CACO-2 cells glutathion-S-transferase isoenzymes were detected with high activity. Interestingly, in non-confluent cells the placental π-form, characteristic for colonic cells was found whereas in confluent differentiated monolayers the μ-form was active being characteristic for intestinal cells (Peters and Reolofs 1989). Cytochrom P450 enzyme 1A1 was present and highly inducible in CACO-2 cells whereas CYP 1A2 was not active (Boulenc et al. 1992; Rosenberg and Leff 1993). Phenol sulfotransferase was expressed in mature cells (Baranchzyk-Kuzma et al. 1991). The major detoxification enzyme CYP 3A is expressed to a different degree in CACO-2 cells and clones: only clone MTX of HT-29 cells and TC-7 of CACO-2 cells exhibited significant activity of CYP 3A (Boulenc et al. 1992; Carriere et al. 1994). CACO-2 cells express antioxidant enzymes like superoxide dismutase, glutathion peroxidase, glutathion reductase and catalase (Grisham et al. 1990; Baker and Baker 1992). Induction and expression of UDP-glucuronosyltransferase (UGT1A1) and glutathion-transferase GST A1 were studied in CACO-2 cells (Svchlikova et al. 2004).

Efflux pumps from the ABC transporter family are expressed: multidrug resistance protein MDR1 (= Pgp), ABCG2 (= BCRP), MRP1 and MRP2 (Cordon-Cardo et al. 1990; Hunter et al. 1993 and others).

In summary, CACO-2 cells express many enzymes characteristic for intestinal cells involved in drug metabolism. The major drug metabolising enzyme, CYP 3A, is active only in selected clones pointing to polyclonal origin of CACO-2 and the necessity to characterize CACO-2 cells extensively for growth and experimental conditions used for given experiments.

While in the 90s the CACO-2 cell model has been widely characterized and used for different purposes Artursson and Borchardt (1997) raised in an review several questions to modify existing technologies. They discussed several questions which have been solved in the meantime in industry: small amounts of compounds for permeability experiments and the need for miniaturization of permeability tests, developing routine methods for testing compounds with low solubility, new cell growth and permeability test equipment, introduction of generic LC-MS analytical methods, aquisition and storage of data in a time frame consistent with high throughput pharmacological screening. Today, we use less than 40 μl of a 10 mM solution of the test compound; the permeability tests consider solubility of the compound in automated solubilization steps leading to prepared compound plates allover the world at different company sites. Many companies use 24-well plates or even 96-well plates for permeability experiments. Pipetting robots (TECAN and others) have been designed to perform all experiments automatically. Cell feeding robots have been introduced. Data storage and handling can be performed at different sites of a company at the same time. Screening of libraries was used to get an overview on a large amount of compounds but in the meantime strategies have changed and the trend is rather to support teams by secondary assays and sometimes sophisticated procedures differing from the high throughput method.

The experimental procedures which will be presented now consider only manual use. Protocols using robotic systems are not described due to great differences in the robotic systems. Nevertheless, many details refer to the use in an industrial environment.

II.D.1
Starting and Maintaining CACO-2 Cells

PURPOSE AND RATIONALE

CACO-2 cells from ATCC at passages 20–50 are used. CACO-2 cells are stored in cryovials in liquid nitrogen. Original cryovials from ATCC are handled as described in the ATCC procedure (see also Chen et al. 2002). This ensures constant cell source for many years once cells have been splitted, grown under the same conditions and than frozen in liquid nitrogen.

PROCEDURE

Cryotubes containing 2 Mio cells/ml are taken from liquid nitrogen tank and placed in an 37 °C water bath for approximately 5 min. After thawing up the suspension is transfered to a 50 ml centrifuge tube containing complete cultivation medium at 37 °C. As cell seeding/feeding medium DMEM/GlutaMAX I is used (Gibco, high glucose content, HEPES 25 mM) with following supplements:

1. 1 % NEAA (nonessential amino acids)
2. 10 % FBS (fetal bovine serum)
3. 40 μg/ml gentamycin (antibiotic additive).

The suspension is mixed carefully and than centrifuged at 150 g for 5 min. The cell pellet is resuspended after discarding freezing medium in cultivation medium and placed in an 175 cm^2 cultivation flask containing 80 ml complete medium. Cells are cultivated at 37 °C, 95 % humidity and 10 % CO_2.

Medium change is performed every 3 days. After 10 days cells are splitted.

Cell splitting starts with washing the attached cells with 20 ml PBS for 2–4 min. After removal of PBS trypsin/EDTA is added (20 ml) for 2 min. After removal of trypsin/EDTA 2 ml of trypsin/EDTA are added and incubated for 5 min at 37 °C with gently shaking the flasks. Trypsinization is stopped when cells start to detach by suspending the cells in 20 ml complete medium while trypsinization procedure stops. After a centrifugation step (to remove trypsin) at 150 g for 5 min cells are resuspended in complete medium and splitted to 3 flasks of 175 cm^2 (2.4–2.6 × 10^6 cells per flask).

After 7 days of cultivation with medium changes every 3 days (confluency 80–90 %) cells are trypsinated and prepared for use in 24-well plates.

EVALUATION

To ensure a constant and comparable viability cells are counted and cell viability is determined with trypan blue exclusion method. Results are compared from one week to the next.

MODIFICATIONS OF THE METHOD

Several modifications of the method are described in the literature (Artursson and Karlsson 1991; Hidalgo and Borchardt 1989 and many others). Modifications include cell culture medium, time of cultivation and frequence of medium change, variations of trypsinization methods and others. In an industrial environment cell cultivation methods are maintained over many years constant to reduce variability and ensure constant results in quality assessment protocols. Additionally to quality control parameters like TEER and permeability markers expression levels of major enzymes and transporters are checked.

II.D.2
Growth of CACO-2 Cells on 24-well Plates

PURPOSE AND RATIONALE

Transport studies are performed at 24-well or 96-well formats. Cells grown in 175 cm^2 flasks are moved to filter inserts and after 21 days of feeding and cultivation used in transport studies. The following method applies for use of 24-well filters of BD Falcon TM HTS 24-Multiwell Insert System. Alternatively, Costar 24-well filter systems could be used (non-coated, Transwell system). In many pharmaceutical companies cell permeability tests and feeding are performed with automatization equipment.

PROCEDURE

Trypsinization is performed as described above. Cells in the suspension are counted and prepared in a suspension containing 4.2–6.7 × 10^4 cells/cm^2. To each apical well of the 24-well plate 400 μl of the cell suspension are added apically. The Feeder Tray contains 40 ml of complete medium (feeding cells from the basolateral side).

Every 3 days medium is changed. Cells are cultivated in an incubator at 37 °C, 95 % humidity and 10 % CO_2.

After 21 days of cultivation and control measurements of monolayers cell density the permability experiment can be performed.

EVALUATION

Control measurements of the monolayer include measurements of transepithelial resistance with an Endohm Meter (World Precision Instruments, New Haven) and permeability measurements of mannitol and polyethylenglycol 4000 (marker for low

permeability) and of metoprolol (marker for high permeability) for example. Permeability experiments can start if the resistance measured is > 250 Ohm/cm^2. Permeability coefficient of mannitol, a standard permeating by passive diffusion, should be in a defined range before permeability experiments start. Several laboratories start permeability tests if permeability coefficient for mannitol is below 1 × 10E-06 cm/sec.

CRITICAL ASSESSMENT OF THE METHOD

The decision for selection of filter support membranes, coating, pore size, medium and medium additives depends on the design of the study. In an industrial environment manual coating is not performed due to labor intensity; precoated filters are cost intensive. It is known that in vivo cells are growing on an extracellular matrix and that in vitro mimicking this conditions by coating with collagen I, II, III or IV improves cell attachment, spreading, migration and time to reach confluency (Hidalgo et al. 1989; Basson et al. 1992). On the other hand decision for experimental procedures is also driven by factors like costs, labor intensity and time.

Different filter supports have been tested: nitrocellulose (NC), polycarbonate (PC), aluminium oxide (AO), polyethylenterephtalate (PET). NC filters have shown reduced non-specific binding compared to AO filters but seem to interact with marker PEG and steroids (Nicklin et al. 1992). AO filters displayed only half of permeability of PC for taurocholic acid. Reproducibility of binding and transport experiments was improved with PC filters (Hidalgo et al. 1989). Pore size is an important factor which needs to be considered and tested: CACO-2 cells migrate through pores > 1 μm (Tucker et al. 1992; Hilgers et al. 1990). PET filters are translucent and allow microscopic observation as well as staining procedures.

Cells grown on PC showed lower TEER and about three fold higher permeabilities for a series of thrombin inhibitors (Walter et al. 1995).

CACO-2 cells display a heterogenicity from batch to batch and as a function of time and culture conditions due to different selection pressure (Vachon et al. 1992; Wilson et al. 1990; Jumarie and Malo 1991; Karlsson et al. 1994). Therefore the cells have to be characterized carefully for those parameters important in a study design. Not only TEER and permeability of marker compounds are important characteristics of the system (for review, see Delie and Rubas 1997) but also expression of enzymes, transporters and proteins as a function of time and experimental conditions. Delie and Rubas (1997) point in their review to importance of passage number of CACO-2: several authors described changes in morphology, TEER, proliferation and permeability characteristics with increased passage number of cells (Walter and Kissel 1995). In an industrial environment where a test system is used over many years and necessarily data need to be compared within and between projects over a long time a test system needs to be robust and well controlled with quality standards and cell cultivation standards (passage number etc).

MODIFICATIONS OF THE METHOD

Modifications of the method include manual or automated cell feeding in 24-well plates, media modifications, possible coating of filter surfaces, pore size of filter membranes. Other modifications include quality assurance criteria (TEER, permeability values).

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EXAMPLE

Examples of CACO-2 cultivation for use in permeability tests are given in Artursson and Karlsson (1991), Artursson (1991), Hilgers et al. (1990), Stevenson et al. (1995), Walter et al. (1995), Wilson et al. (1990).

II.D.3 Permeability Assay Conditions

PURPOSE AND RATIONALE

Permeability of compounds across a cell layer is measured in order to determine the absorption potential of a compound or a chemical series and select compounds for in vivo studies. Apparent permeability coefficients can be used to compare compounds within a series for ranking. Between different laboratories comparison of compounds should be done only on the basis of classification (high, medium, low) since permeability coefficients can differ between the labs (Artursson et al. 2001; see Critical Assessment of the Method).

PROCEDURE

Permeability Experiment

The design of the 24-well plates containing filter supports is shown in Figure 2.

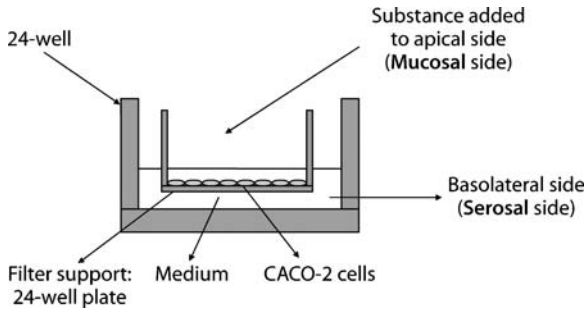

Fig. 2. Filter insert system for permeability studies

As experimental buffer for washing and permeability assay HBSS, pH 7.4 is proposed.

Compound dilutions should be performed from 10 mM compound stock solutions in DMSO at the day of experiment. Compounds should be taken from micro well plates prepared by the different robot systems; dilution is prepared with buffer or intermediate steps (containing DMSO) to avoid precipitation of compounds with low solubility. Final DMSO concentration should not exceed 0.5 %. Alternatively, compound dilutions are prepared manually to result in maximal final concentration of DMSO of 0.5 %.

Compound concentration should be chosen on the basis of solubility data. Compound concentrations of 50 μM should not be exceeded in routine tests. Concentrations lower than 6.25 μM should be avoided due to possible analytical limitations.

As an example, 4 different categories of compound concentrations could be used due to differences in projects and compound characteristics: 50 μM, 25 μM, 12.5 μM, 6.25 μM.

As assay time 1h is proposed.

In an industrial environment very often one time point determinations are performed in triplicate using incubator conditions (37 °C, 1 h) with shaking or non-shaking conditions.

Samples are collected in triplicate from both the basolateral and apical compartments after 1 h.

Triplicate samples are also taken from the dosing solution.

Analytical Methods

All analytics should be performed on LC/MS systems: in industrial labs very often ion traps or quadrupole systems are used. Single ion monitoring in either positive or negative mode (compound dependent) is used in HTS conditions. Local LC conditions (injection volume, mobile phase gradients) should be applied to accommodate variations in LC equipment. The selected LC column stationary phase can be used routinely in different labs (for example, C-18; phase selection is compound dependent).

Mobile phase consists of varying amounts of ammonium acetate (5–10 mM) or formic acid and acetonitrile.

All samples should be kept in a stack cooler during injection routine. According to limited solubility of compounds in projects compounds could be screened at different concentrations (as described above). Analytical samples should be run than at either 6.25, 12.5, 25 or 50 μM depending on solubility results of plate/project.

Dosing and apical samples will be diluted routinely to a final concentration of not more than 2.5 μM (depending on linearity of LC-MS measurment range and MW of compound).

One injection per sample should be done (for the procedure described, routinely ten samples will be acquired per compound: buffer blank, 3-basolateral 1 hr, 3-apical 1 hr and 3-dosing solution).

EVALUATION

Apparent permeability coefficients (Papp in cm/sec) are calculated according to the following equation

$$\text{Papp(cm/sec)} = (dQ \times V)/(dt \times S \times C_0)$$

where S is the surface area of the filter in cm^2, C_0 is the initial concentration of the compound on the donor side, dQ/dt is the amount of compound transported during the 60 min experiment and V is the volume of the receiver chamber.

In industrial labs standardized worksheets are used and results automatically appear in data bases (globally or at a certain site of the company). Results are than given for example as: mean Papp (single point, cm/sec) with standard deviation (n = 3), Absorption rating (Low, Medium, High), mean % transport/h, mean % mass recovery, mean % remaining donor.

Mean % mass recovery is calculated from the sum of the basolateral and apical quantities of compound at 1h as a % of the original dosing solution at 0 h.

Mean % remaining donor is calculated as quantity of compound remaining in the apical compartments at 1h as a % of the original dosing solution at 0 h.

Each recovery should be individually checked to see if it falls within a predefined threshold. The partial recovery is calculated as the sum of the percent transport and the percent remaining donor (Peak Area Donor 1 h + Receiver/Donor 0 h). As quality assurance criteria for example partial recovery $> 65\,\%$ and partial recovery $< 125\,\%$ could be accepted and then the result value is included in the calculation of the average Transport/h or the average Papp/single point.

If the recovery for 2 or more wells fall outside of 65 %–125 % range, then no result can be reported and experiment is usually repeated.

The assay is evaluated in all laboratories by correlating in vivo absorption data of standard compounds with in vitro permeability values (see Artursson and Karlsson 1991). Since CACO-2 cells differ due to cultivation procedures and possible selection of populations/clones of cells this correlation curve should be established in every laboratory. It is not possible to use a curve from literature for prediction of in vivo permeability from inhouse in vitro data. Every laboratory should use its own curve. A comparison of Papp values from different labs for compound ranking is not possible (Artursson et al. 2001). Therefore, many industrial labs use a classification of compounds into 3 classes of permeability: high, medium, low. This classification is very well comparable between different laboratories. Test compounds analysed in different labs should be classified into the same class. On the basis of correlation curve between in vivo absorption and in vitro permeability data a sigmoidal curve can be calculated and 2 important thresholds are usually set: at in vitro Papp values corresponding to 20 % in vivo permeability and to 80 % (Artursson et al. 2001; Bailey et al. 1996 and others). Other labs use 90 % threshold values according to a FDA proposal. Compounds with in vitro Papp below the first threshold value (at 20 % in vivo permeability from standard curve) are classified as low permeable; compounds with Papp values above the higher threshold are classified as high permeable and compounds with permeability values between the 2 thresholds are classified as medium. An example of different permeability values in laboratories is given in the table below.

Due to a high throughput in industrial laboratories very often so called mean Papp single point is calculated from 3 filters with standard deviation meaning that only one time point (60 minutes) is used for calculations. Alternatively or in addi-

Table 1 In vitro Papp (cm/sec) values corresponding to 20 % and 80 % in vivo permeability data from sigmoidal correlation curve with standard compounds.

% Human Absorption	Rating	Laboratory 1	Laboratory 2	Laboratory 3
20 %	Low	< 3.2×E−07	< 0.3×E−06	< 8×E−07
20–80 %	Moderate	3.2×E−07 to 1.25×E−06	0.3×E−06 to 3.9×E−06	8×E−07 to 6×E−06
80 %	High	> 1.25×E−06	> 3.9×E−06	> 6×E−06

tion, mean % transport/h can be calculated from 3 filters.

If 2 or more filters do not meet acceptance criteria compound assay should be repeated (exception: compounds with unusual features causing for example a discrepancy in mass balance due to high plastic binding).

Standard markers should be included in all experiments. Usually marker compounds for different permeability classes are used like metoprolol for high permeability and radioactive mannitol for low permeability. Quality assurance criteria define accepted upper permeability values for mannitol (in the case of mannitol many laboratoratories use 1.0 × E-06 cm/sec). Permeability values higher than upper limit should lead to rejection of the test.

Integrity control of the monolayer is based on measurements of both TEER (transepithelial resistance measurements) and permeability for a standard marker (radioactive mannitol or Lucifer yellow as non-radioactive alternative).

TEER is usually measured at the beginning (0 h) and end of the assay (1h). Monolayers with TEER values below an acceptance value should rejected (for example, TEER < 250 Ohm/cm^2 at 0 h could be used as criterion to exclude cell monolayers from experiment). TEER acceptance criteria should also be defined (for example +/– 30 % change in TEER at 1h from TEER value at time zero means that the experiment can not be accepted).

Again, quality criteria should be defined in every lab independently: while Papp values (upper permeability criteria) and TEER limits can not be transferred from one lab to another one, recovery data (% of start solution) are comparable.

Additional comments are usually included like leaky monolayer, solubility below 6.25 μM, variability in data.

Recently in some industrial labs blind probe test have been introduced: 5 selected standards are analysed in permeability tests at different sites and results are compared (rating, recovery). The qualification and classification of the compounds to the criteria low permeability (< 20 %), moderate (20 – 80 % permeability) or high (> 80 % permeability) should not differ among different labs. This blind probe tests can be used as quality assurance if repeated several times a year.

CRITICAL ASSESSMENT OF THE METHOD

In vivo as well as in vitro permeability is reduced due to the aqueous boundary layer or unstirred water layer (UWL). Shaking can reduce the UWL using different shaking frequencies (Hidalgo et al. 1991; Karlsson and Artursson 1992). However, the experimental conditions need to be tested carefully to avoid damage of monolayers. In side-by side diffusion systems the UWL was 52 μm (Karlsson and Artursson 1992). In vivo thickness of UWL was found 40 μm (Strocchi and Levitt 1991).

TEER, normalized to surface, was 188–221 Ohm/cm^2 in CACO-2 cells and 78–125 Ohm/cm^2 in colon (Rubas et al. 1996). The presence of villi and crypts in vivo with a higher surface and a different cellular composition (goblet cells, M cells, higher permeability in crypts) compared to a cell monolayer might lead to higher permeability. Tanaka et al. (1995) compared permeability of FITC-Dextran (MW 4000) in CACO-2 cells and rat jejunum and colon: the permeability was 10 fold decreased in CACO-2 compared to jejunum and 5 fold lower than in colon. TEER in CACO-2 was 470 Ohm × cm^2 in this study, 40 Ohm × cm^2 in rat jejunum and 80 Ohm × cm^2 in rat colon. Permeability of standard markers like PEG was compared between CACO-2 cells and colon (Artursson et al. 1993). The conclusion for cellular studies is to select cells with acceptable TEER and permeability values and to perform quality assurance tests on a regular basis.

Several authors compared permeability of CACO-2 with in situ perfused intestine or everted intestinal rings (Lennernäs et al. 1996; Rubas et al. 1993; Jezyk et al. 1992). Their conclusion is that CACO-2 cells with TEER below 300 Ohm/cm^2 give permeabilities for hydrophilic drugs comparable to intestinal

tissue. The trend for hydrophilic compounds concerning permeability was CACO-2 > colon > small intestine.

Further limitations of cellular layers in comparison to in vivo conditions are absence of mucus, different expression of enzymes (lack of CYP 3 in CACO-2) and transporters. There have been attempts to introduce mucus secreting cells (HT29) to the CACO-2 permeability system (Wikman and Artursson 1993; Wikman-Larhed and Artursson 1995; Behrens et al. 2001; Pontier et al. 2001). Cells with a higher permeability than CACO-2 for example, IEC-18 cells (Ma et al. 1992; Duizer et al. 1995) or immortalized cells 2/4/A1 (Milovic et al. 1996) have been studied. This attempts were only partly successful: in coculture systems cells did not mix as in vivo, goblet cells produced not enough mucus, permabilities for standards increased more than expected.

However, this systems need further evaluation. For an industrial environment coculture systems need to be robust and reliable being at the same time easy to handle. On the other hand, convincing advantages have to be shown to change the monolayer system to a coculture model or to change the cell system used. In many companies CACO-2 cells are used since many years and an immense data base is available.

Another aspect to consider is the effect of food, increasing or decreasing uptake characteristics of a compound. Diet, physiological status, diseases influence permeability as well.

Prognosis of a compounds permeability should be made stressing limitations of the model. There is no bioavailability prognosis from in vitro data – a cellular assay can provide only permeability potential through a biological membrane. The membrane, in most cases CACO-2 cells, is very similar to what we observe in vivo in the small intestine and resembles many characteristics to in vivo enterocytes. CACO-2 cells can be used for prediction of different pathways across intestinal cells. Best correlation occurs for passive transcellular route of diffusion. Passive paracellular pathway is less permeable in CACO-2 and correlations are rather qualitative than quantitative for that pathway. CACO-2 cells are an accepted model for identification of compounds with permeability problems, for ranking of compounds and selection of best compounds within a series. Carrier-mediated transport can be studied as well using careful characterization of transporters in the cell batch or clone as a prerequisite for transporter studies.

There are advantages of cell culture models in permeability testing which should be exploited adequately. Differentiated human cells with similarities to human intestinal epithelia can be used for screening procedures, tests for absorption enhancers and sensitive cytotoxicity screening, uptake and secretion studies as well as functional bioassays to study proteins, transporters or enzymes generating reproducible data. Mechanistic studies are increasingly introduced to clarify issues and enable studies for structure-activity relationship for a selected issue.

MODIFICATIONS OF THE METHOD

Modifications include all details described in the method above (time of permeability experiment, concentration ranges, shaking, quality criteria and acceptance parameters).

Modifications to the analytical procedure presented are of course necessary if different equipment is used or if a compound class can not be analyzed with the standard procedure proposed.

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EXAMPLE

Examples of CACO-2 permeability tests are given in Artursson and Karlsson (1991), Artursson (1991), Hilgers et al. (1990), Stevenson et al. (1995), Walter et al. (1995), Wilson et al. (1990). Additionally, CACO-2 have been used in testing excipients (Saha et al. 2000) and applying simulated intestinal fluid (Ingels et al. 2004).

II.D.4 Efflux Experiments Using CACO-2 Cells

PURPOSE AND RATIONALE

Multidrug resistance (MDR) is an important and common cause of drug resistance in cells. MDR is mediated by the increased expression of energy-dependent drug efflux pumps from the ABC-transporter family (Borst et al. 1999). Efflux transporters like P-glycoprotein (P-gp, or MDR1), encoded by the MDR1 gene, Multidrug resistance associated proteins (MRP1 and 2), lung resistance protein (LRP) and breast cancer resistance protein (BCRP = ABCG2) are efflux transporters present in the gastrointestinal tract (Ambudkar et al. 1999; Borst et al. 2000; Jonker et al. 2000; Doyle et al. 1998).

MDR1 is a 170 kDa transmembrane protein member of the ATP binding casette (ABC) transporter family. It is localized at the apical secretory surface of various tissues (e.g. liver, kidney, gastrointestinal tract, blood brain barrier) where it mediates the active transmembrane transport of a variety of lipophilic substrates, which tend to be large, aromatic and amphiphilic. MDR1 can extrude/exclude a wide range of structurally diverse drugs (Ambudkar et al. 1999). MDR1 limits the oral absorption of a number of drugs by transporting them back from the intestinal cells into the gut lumen. Recent studies have revealed the overlapping substrate specificity of CYP3A4 and MDR1 and many substrates of CYP3A4 are also substrates or inhibitors of MDR1. The functional expression of MDR1 has also been shown in Caco-2 cells.

MDR1 and members of the MRP family have substantial substrate overlaps (Borst et al. 2000).

MRP1 and MRP2 have substrate specifity overlaps for amphiphilic anions, or gluthathione- glucuronate, or sulfate-conjugates (König et al. 1999). The transporter cMOAT (canalicular multispecific organic anion transporter; i.e., cMRP or MRP2) responsible for the active excretion of amphiphatic anionic conjugates formed by phase II conjugation into bile, is also found in the intestine.

For MRP2 following substrates have been identified and characterized so far: Glutathion disulfide, leukotrienes (C4, D4, E4, N-acetyl E4), glutathion conjugates (DNP, BSP, metals Sb, Bi, As, Cd, Cu, Ag, Zn), glucuronide conjugates (bilirubin, T3, P-nitrophenol, grepafloxacin), bile acid conjugates (glucuronides, sulfates), organic anions (folates, methotrexate, ampicillin, ceftriaxone, cefadoxine, grepafloxine, pravastatin, temocaprilate).

BCRP is an ABC half-transporter and is expressed at very high levels in the intestine, liver, kidney and blood-brain barrier. However, the identified list of substrates and/or inhibitors is limited yet: Mitoxantrone, topotecan, doxorubicin, flavopiridol, SN-38, Lysotracker green, BBR 3390, BODIPY-Prazosin, Rhodamin 123. As inhibitors GF 120918 and fumitremorgin C were identified (Doyle et al. 1998; Litman et al. 2000).

In the small intestine expression data for MDR1, ABCG2, MRP's and LRP have been detected (Kool

et al. 1997; Doyle et al. 1998; Fromm et al. 2000; Maliepaard et al. 2001). However, systematic studies are still necessary for the intestine as the major barrier for oral absorption.

Efflux studies are very often performed in cellular systems which are tight enough to perform flux and efflux studies (CACO-2, MDCK). Taipalensuu and colleagues were the first to study expression and quantitative relationship in detail for CACO-2 cells in comparison to jejunal levels (2001). Jejunal transcript levels of the different ABC transporters is spanning a range of three log units with rank order: BCRP~MRP2 > MDR1 ~MRP3~MRP6~MRP5~MRP1 > MRP4 > MDR3 (Taipalensuu et al. 2001). Transcript levels of 9 of the ABC transporters correlate well between CACO-2 cells and jejunum, only BCRP exhibits a 100fold higher expression in the in vivo system (Taipalensuu et al. 2001).

Many laboratories use CACO-2 cells as a standard method for assessment of efflux. Since the standard CACO-2 cell assay is very well established, easy to use, reproducible and reliable the corresponding efflux assay can give valuable and helpful data for project support in a screening approach. A prerequisite for the interpretation of efflux data is a characterization of efflux transporters present in the system used and a set of standard efflux markers checked regularly (like digoxin for MDR1).

PROCEDURE

As experimental buffer for washing, permeability and efflux assay HBSS, pH 7.4 is proposed.

Compound dilutions should be performed from compound stock solutions in DMSO, 10 mM at the day of experiment. Compounds should be taken from micro well plates prepared by the different robot systems; dilution is prepared with buffer or intermediate steps (containing DMSO) to avoid precipitation of compounds with low solubility. Final DMSO concentration should not exceed 0.5 %. Alternatively, compound dilutions are prepared manually to result in maximal final concentration of DMSO of 0.5 %.

Compound concentration should be chosen on the basis of solubility data. Compound concentrations of 50 μM should not be exceeded in routine tests. Concentrations lower than 6.25 μM should be avoided due to possible analytical limitations.

As an example, 4 different categories of compound concentrations could be used due to differences in projects and compound characteristics: 50 μM, 25 μM, 12.5 μM, 6.25 μM.

In contrast to permeability tests, compounds are added not only to apical compartment (to determine flux A–B) but in an additional experiment to basolateral compartment (efflux B–A). As assay time 2 h is proposed.

An additional experiment is performed usually to determine active efflux. For this experiment flux and efflux assays are performed at 4 °C in comparison to 37 °C. Since efflux is an active process requiring ATP an experiment at 4 °C will show a reduced efflux rate if an active process is involved.

As for the standard CACO-2 assay, very often one time point determinations are performed in triplicate using incubator conditions (37 °C, 2 h).

Samples are collected in triplicate from both the basolateral and apical compartments after 2h. Triplicate samples are also taken from the dosing solution. Analytics are performed as described in 2.3.

EVALUATION

Apparent permeability coefficients (Papp in cm/sec) are calculated according to the following equation

$$\text{Papp(cm/sec)} = (dQ \times V)/(dt \times S \times C_0)$$

where S is the surface area of the filter in cm^2, C_0 is the initial concentration of the compound on the donor side, dQ/dt is the amount of compound transported during the 60 min experiment and V is the volume of the receiver chamber.

Ratio of B–A/A–B is calculated and compared to the ratio of standard compounds.

As standards digoxin, etoposide, Cyclosporin A, Colchicine (all for MDR 1), Bromosulfophthalein, Furosemide (for MRP2) and Metothrexate, topotecan (for ABCG2) can be used. Efflux ratio of the compound is compared to ratio of standards.

CRITICAL ASSESSMENT OF THE METHOD

Cellular efflux assays have a lower throughput as binding studies or competition studies. The Calcein assay used very often to identify MDR1 substrates can offer interactions only with one binding site of MDR1. Consequently the test fails in identification of those substrates or inhibitors interacting with the second binding site, with both or with a so called interaction site (Litman et al. 2001). Two very important studies have been performed by Schwab et al. (2003) and Polli et al. (2001) comparing several different MDR1 assays for identification potential towards known substrates/inhibitors of MDR1. Both studies conclude that HTS assays such as calcein

or rhodamine 123 assay are not sufficient to detect MDR1 interacting compounds. ATPase assay can provide additional information but fails also for some known MDR1 interacting compounds. In the ATPase assay colchicine, digoxin, etoposide (Schwab et al. 2003) and Cyclosporin A, GF120918, colchicine, etoposide, doxorubicin, vincristine (Polli et al. 2001) were not identified. Inhibition (or competition) assays such as calcein assay failed to identify colchicine, digoxin, etoposide (Schwab et al. 2003) and colchicine, etoposide, vincristine, taxol and others (Polli et al. 2001). Rhodamine 123 assay again did not identify colchicine, digoxin, etoposide, ranitidine and others (Schwab et al. 2003). The rank order for compounds in MDR1 efflux (based on a comparison of efflux ratio B–A/A–B in both studies) is different between both test systems used (MDR1-MDCK cells in Pollis study and LLC-MDR1 and LLC-mdr1a cdells in Schwabs study). Compounds with high intrinsic permeability (midazolam, nifedipine) could overcome efflux and were not identified as MDR1 interacting compounds (Schwab et al. 2003). Verapamil was not effluxed due to high membrane partitioning, too (Polli et al. 2001). Both studies conclude that it is not sufficient to rely on one HTS assay. The authors propose to perform several assays in a cascade strategy: first HTS assays using fluorescent read-outs (calcein, rhodamine), followed by ATPase assay and/or cellular transport assays. However, following this strategy etoposide, colchicine, digoxin would have been not identified as interacting MDR1 substrates. Therefore, at the stage of early drug candidate identification efflux assays using well characterized cell systems are very useful. Given that the rank order of effluxed compounds was different (taking ratio as comparison) it is necessary to stay in one efflux system for comparative studies and to check in overexpressing cell systems such as MDCK and LLC-PK1 protein sequence and molecular weight of transfected efflux transporters. Other efflux transporters should be checked as well because due to transfection they could be over- or underexpressed or be expressed in a different glycosylation form. Please pay attention to the Critical assessment part in Efflux inhibition studies (II.D.5).

MODIFICATIONS OF THE METHOD

Modifications include all details described in the method above (time of permeability experiment, concentration ranges, shaking, use of standards, quality criteria and acceptance parameters). Since in CACO-2 cells efflux for standard compounds varies due to heterogenicity and batch variations several teams used selected CACO-2 cells. Cells set under selection pressure with vincristine (Eneroth et al. 2001) or digoxin (Takara et al. 2002). Other teams selected subclones of CACO-2 cells with higher expression rates of MDR1 (Horie et al. 2003).

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EXAMPLE

Examples of efflux studies using CACO-2 and other cells expressing efflux transporters are given in the studies of Schwab et al. (2003), Polli et al. (2001), Pachot et al. (2003) and others.

II.D.5 Efflux Inhibition Experiments Using CACO-2 Cells

PURPOSE AND RATIONALE

Once a compound is identified in an efflux assays it is sometimes of importance to discriminate between efflux, inhibition potential and to determine the efflux transporter responsible for efflux of the compound.

We are still lacking good inhibitors interacting efficiently with only one efflux transporter. The majority of inhibitors interacts with several efflux transporters (to a different degree) and are not selective.

Nevertheless, inhibition assays are used very often as a first step before performing more specific efflux assays.

The list of substrates and inhibitors for MDR1 is extensive due to the fact that we are aware of different binding sites (two binding sites minimum) and interacting sites (capacity of competition or inhibition at an additional site of MDR1, see Litman 2001).

However, in two recently published studies from Roche (Schwab et al. 2003) and Glaxo (Polli et al. 2001) our limitations in studying MDR1 became obvious: several compounds known as substrates or inhibitors of MDR1 were not clearly identified, others were identified only by one or two of the test methods (either ATPase assay or calcein-efflux or rhodamine efflux or cellular efflux studies).

The greatest precision and best identification is still achieved with cellular efflux assays. However, cellular efflux assays do not have high throughput capacity and can therefore be applied only for a limited number of compounds.

PROCEDURE

The conditions for inhibition assays using CACO-2 cells are as described above for efflux assays.

In contrast to permeability tests, compounds are added not only to apical compartment (to determine flux A–B) but in an additional experiment to basolateral compartment (efflux B–A). As assay time 2 h is proposed. At the same time, an inhibitor is added and results of permeability and efflux assays compared to results obtained without inhibitor. Additionally, a selection of inhibitors covering different efflux transporters is used. After a preincubation period used in many studies (15 minutes for example) test compounds are added and flux/efflux studies performed.

As for the standard CACO-2 assay, very often one time point determinations are performed in triplicate using incubator conditions (37 °C, 2 h).

Samples are collected in triplicate from both the basolateral and apical compartments after 2 h. Triplicate samples are also taken from the dosing solution. Analytics are performed as described in 2.3.

EVALUATION

Apparent permeability coefficients (Papp in cm/sec) are calculated according to the following equation

$$P_{app}(\text{cm/sec}) = (dQ \times V)/(dt \times S \times C_0)$$

where S is the surface area of the filter in cm^2, C_0 is the initial concentration of the compound on the donor side, dQ/dt is the amount of compound transported during the 60 min experiment and V is the volume of the receiver chamber.

Ratio of B–A/A–B is calculated and compared to the ratio of standard compounds. In addition B–A/A–B ratio determined in the inhibition experiment is compared to efflux ratios determined without inhibition. Given that efflux of a compound can not be inhibited by an inhibitor one should conclude that more than one efflux transporter are involved in the efflux. Adding two or more inhibitors at the same time provides maximal inhibition.

As standard inhibitors verapamil is used very frequently, Cyclosporin A, Colchicine (all for MDR 1) GF120918 is used University labs; Bromosulfophthalein, Furosemide (for MRP2) and Metothrexate, topotecan (for ABCG2) can be used. Inhibitors used in University labs for ABCG2 include also GF120918 and fumitremorgin C.

Table 2 Frequently used efflux standard substrates and inhibitors.

Efflux transporter	Compound	Substrate (S)/inhibitor (I)
MDR1	Digoxin	S
	Etoposide	S (interacts with MRP1)
	Cyclosporin A	S/I
	GF 120918	I (interacts with BCRP)
	Verapamil	S/I
	Daunorubicin	S/I
	Ritonavir	S/I
	Vinblastine	S/I (interaction with MRP)
	Colchicine	S/I
	RU 487	S/I
MRP2	Probenecid	I (interacts with MRP1)

CRITICAL ASSESSMENT OF THE METHOD

In section II.B.1.4 dealing with efflux studies it was discussed that rank order of effluxed compounds was different in different test systems (taking ratio as comparison). The conclusion was to stay in one efflux system for comparative studies and to check in over-expressing cell systems such as MDCK and LLC-PK1 protein sequence and molecular weight of transfected efflux transporters. Other efflux transporters should be checked as well because due to transfection they could be over- or underexpressed or be expressed in a different glycosylation form. It was noted that animal cells transfected with a human gene express a protein migrating to a lower molecular weight (Evers et al. 1996). Pig kidney epithelial cells LLC-PK1, when overexpressed with MDR1, expressed a protein with a MW at 120 kD. Tang et al. (2002) detected in MDCK-MRP2 overexpressing cells, in MDCK-WT and CACO-2 cells 2 bands (cross-reaction of the MRP2 antibody): at 150 and 190 kD. In MDCK-MRP2 cells only the band at 150 kD was overexpressed. The authors discuss possible differences in glycosylation as a reason for this phenomenon. In the same study it was found that Michaelis-Menten constants Km and Vmax differed between CACO-2 and MDCK-MRP2 cells for vinblastine. Similar observations were reported by Soldner et al. (2000) for losartan and by Lentz et al. (2000) for vinblastine in MDR1 studies. Affinities for the inhibitors Cyclosporin A, MK 571, vincristine and etoposide were lower in CACO-2 cells compared to MDCK-MRP2 (Tang et al. 2002). On the other hand, reserpine had no affinity in MDCK cells at all and daunorubicin showed lower affinity in MDCK-MRP2 compared to CACO-2. Summarizing all data, authors discuss as a possible reason for those differences between CACO-2 and MDCK cells a different lipid composition of the membrane which could lead to a different orientation of MRP2 and MDR1. Additionally, drug partitioning into membranes may be different resulting in differences in substrate/inhibitory specificity and binding kinetics (Romsicki and Sharom 1999; Ferte et al. 2000; Tanaka et al. 1997; Tang et al. 2002).

Moreover, since in the Schwab study differences in the human MDR1 and mouse mdr1 were detected concerning substrate recognition (ritonavir, saquinavir were negative in mdr1a cells, verapamil, terfenadine, quinidine were less active in mdr1a than in MDR1) and differences occurred between porcine brain endothelial efflux and LLC-PK1-MDR1 (vinblastine) in the future efflux systems containing animal transporters have to be considered, too. In addition we have to keep in mind that in rodents two efflux transporters correspond to human MDR1: mdr1a and mdr1b which have both different substrate specificity (for Cyclosporin A, terfenadine, verapamil, vinblastine). And, complicating the story even more, some compounds have been identified in mdr1a as strong substrates for mdr1a but were not good substrates for human MDR1. Keeping in mind that in pharmacokinetics studies are performed in rats and mouse before going into dogs or mini pigs PK parameters could be different between the species due to different substrate specificity of transporters.

However, for MDR1 we have a panel of studies available and we are aware of complications and considerations. There are several models addressing binding sites and the modulatory site. For many other transporters we have to expand knowledge before we can perform routine studies with in vitro designs for all transporters.

MODIFICATIONS OF THE METHOD

Modifications for efflux inhibition studies are as frequent as efflux studies and include all parts of the method (permeability time, inhibitors, determination of Papp at single time point versus several time points).

Tang et al. (2002) used [3H]-vinblastine as a substrate for MRP2 studies (Evers et al. 1998). Inhibitors were Cyclosporin A (25 μM) for MRP2 and MDR1 (Tanaka et al. 2000; Nies et al. 1998; Smith et al. 1998), GF120918 (2 μM) for MDR1 (Hyafil et al. 1993; Utsunomiya et al. 2000) and MK571 (50 μM) for MRP2 (Walle et al. 1999).

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EXAMPLE

Examples of efflux studies using efflux inhibitors are given in Tang et al. (2002), Tanaka et al. (1997) and others.

II.D.6 Transporter Uptake Studies Using CACO-2 Cells

PURPOSE AND RATIONALE

During the last 10 years many uptake transporters as well as efflux transporters have been discovered in the GI and especially in the small intestine. They allow uptake of ions, amino acids, peptides, nucleic acids, sugars, organic acids, vitamins, cofactors and nucleosides. On the other hand, efflux transporters ensure protection of the organism from unwanted pathogen or compound delivery. Subsequently, cellular systems have been used to study uptake in mechanistic studies in more detail. Additionally inhibition studies provide hints on potential drug–drug interactions.

CACO-2 cells resemble many features of entherocytic intestinal cells. In gene chip analyses 38 % of 11 559 genes were detected in 16 day-old CACO-2 cells. 170 of those genes were transporters and channels, 443 were transporters, channels and metabolic enzymes (Sun et al. 2002). In human duodenal probes 44 % of the total of 12 559 gene sequences were detected. Permeability values between CACO-2 and duodenum which were compared resulted in good correlation for passively absorbed drugs whereas correlation coefficients for actively absorbed drugs were lower in CACO-2 than in duodenum.

Nevertheless, several uptake transporters have been studied successfully in CACO-2 cells as possible drug delivery systems. CACO-2 cells are a suitable model for uptake studies given that they are characterized for the studied transporter (Anderle et al. 2003). Whereas permeability values for actively transported compounds may differ substantially from in vivo situation a ranking of compounds for higher or lower permeability seems to be possible.

PROCEDURE

As experimental buffer for washing, permeability and efflux assay HBSS, pH 7.4 is proposed.

Compound dilutions should be performed from compound stock solutions in DMSO, 10 mM at the day of experiment. Compounds should be taken from micro well plates prepared by the different robot systems; dilution is prepared with buffer or intermediate steps (containing DMSO) to avoid precipitation of compounds with low solubility. Final DMSO concentration should not exceed 0.5 %. Alternatively, compound dilutions are prepared manually to result in maximal final concentration of DMSO of 0.5 %.

Compound concentration should be chosen on the basis of solubility data. Uptake experiments should be performed using Petri dishes or filter supports. In the case of filter supports a permeability experiment has to be performed at the same time to control basolateral amount of compound.

Uptake studies for peptides have been performed by adding the compound to the apical side of the filter (pH 6.5 for optimal function of oligopeptide transporter). The pH at the basolateral side was 7.4. After a preincubation period (10 min, 37 °C) cells should be washed and incubated for 15 min (or for a shorter time period) with the compound under evalation. After incubation period medium is removed, cells are washed 3 times with ice-cold pH 7.4 buffer to stop further uptake and to remove unbound compound (for details see Tamura et al. 1996). Cells should be scraped and dissolved in ice-cold buffer. If a radioactive compound is studied, cells and filters are dissolved in a Ready-Safe scintillation cocktail and radioactivity is determined in a liquid scintillation counter. Alternatively, for non-radioactive compounds LC-MS or HPLC detection is recommended.

An alternative is a time-dependent study where cells are scraped and uptake is stopped at different times over 15 minutes. In both cases (in time-dependent studies and one-time-point determinations) protein content is used to express uptake in nmol/mg protein.

In another study uptake of biotin was studied with confluent CACO-2 cells cultured on 12-well plates (Balamurugan et al. 2003). Labeled and unlabeled biotin was added to cell monolayers and reaction terminated after 3 minutes by adding ice-cold buffer (2 ml). Cells were rinsed twice in ice-cold buffer, digested with 1 ml of 1 N NaOH, neutralized with HCl and than counted for radioactivity. Protein content of cells was determined with a Bio-Rad kit (Richmond, US).

A comparable uptake assay to study uptake of peptides and compounds via peptide transporter was described by D'Souza et al. (2003).

EVALUATION

The uptake is expressed in nmol/mg protein and compared to standard drugs. In addition, time-dependent uptake experiments provide uptake kinetic parameters.

CRITICAL ASSESSMENT OF THE METHOD

An uptake assay using CACO-2 monolayers grown on filter dishes needs to be combined with permeability studies to assess the amount of compound permeating during the short time of uptake. Studies with cells grown on Petri dishes or multi-well plates have the disadvantage that they may be performed with cells not fully differentiated. Uptake and efflux transporter expression increases with differentiation. Moreover, some transporters require pH gradients and the 3-dimensional filter geometry to function with optimal activity.

Transporter studies have been performed with CACO-2 cells for many different transporters of the Solute Carrier system (SLC). The use of transporters as drug targeting systems to increase bioavalability is discussed since several years. Many transporters from the SLC family are being evaluated for their drug targeting use. The following part of the critical assessment will deal with SLC transporters identified in the intestine and their possible role in respect to drug targeting.

Uptake Transporters in the Intestine

During the last 10 years many uptake transporters as well as efflux transporters have been discovered in the GI and especially in the small intestine. They allow uptake of ions, amino acids, peptides, nucleic acids, sugars, organic acids, vitamins, cofactors and nucleosides. On the other hand, efflux transporters ensure protection of the organism from unwanted pathogen or compound delivery. Figure 3 illustrates the most important transporters and enzymatic systems identified in enterocytes.

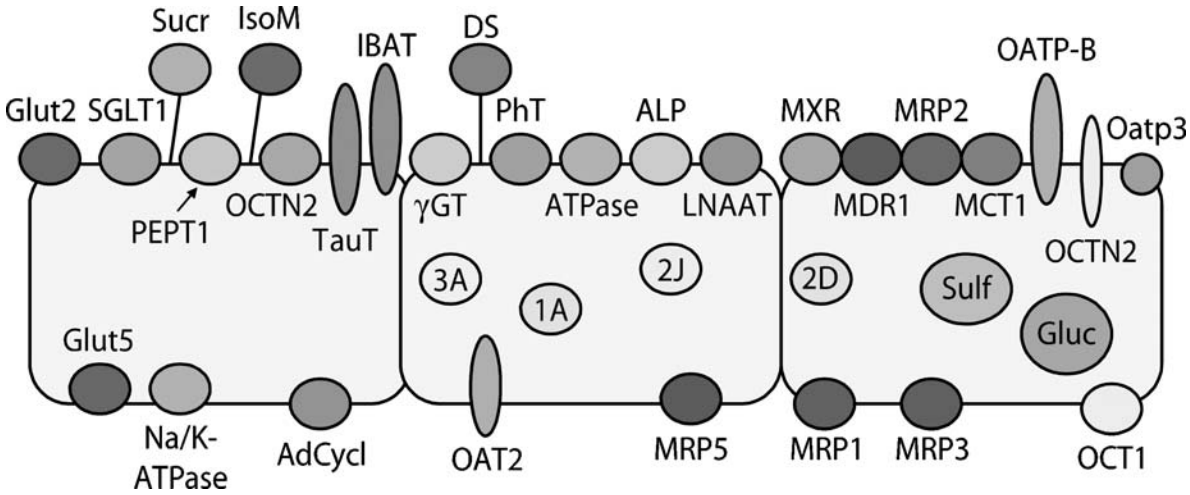

Fig. 3. The intestinal barrier: Sucr – sucrase; IsoM – isomaltase; DS – disaccharidase; TauT – taurocholic acid transporter; MCT1 – monocarboxylic acid transporter; PhT – phosphate transporter; LNAAT – amino acid transporter; 3A, 1A, 2D, 2J – Phase I metabolizing enzymes; Gluc – glucuronidase/Phase II, sulf – sulfatase/Phase II; PEPT1 – di/tripeptide transporter; OCT, OCTN2 – organic cation transporters; IBAT – intestinal bile acid transporter; AdCycl – adenylate–cyclase; MDR, MRP – ABC transporters; ALP – alkaline phosphatase; γGT – gamma–glutamyltranspeptidase; OAT – organic anion transporter; OATP – organic anion transporting polypeptide; Glut, SGLT – glucose transporters

Subsequently, several of the transporters have been used as drug delivery systems.

Amino Acid Transporters

Several uptake amino acid transporters have been identified at the apical side of brush border membrane enterocytes: heterodymeric amino acid transporters (HAT system) with apical transporters LAT1-4F2hc and b0.+AT and the systems PAT, B0, Beat/Taut, A and B0,+. At the basolateral side the HAT transporters y+LAT1-4F2hc, LAT2-4F2hc and ASC1-4F2hc are expressed together with other systems: A, y+, XAG- and Tat (for overview, see Steffansen et al. 2004). In Table 3 the transporters mentioned are listed with gene name and substrate specificity. However, the capacity of amino acid transporters seems to be limited for an effective oral delivery approach. On the other hand substrate specificity is limited as well. For review refer to Verrey (2003), Palacin and Kanai (2003), Verrey and Wagner (2003), Halestrap and Meredith (2003), Ganapathy et al. (2001).

Nucleoside Transporters

At the apical side of intestinal enterocytes concentrative nucleoside transporters CNT1, CNT2 and CNT3 are identified whereas at the basolateral membrane equilibrative nucleoside transporters ENT1 and ENT2 are localized. Table 4 gives an overview on nucleosides, nucleobases and drugs being transported by nucleoside transporters (for detailed review see Steffansen et al. 2004; Ritzel et al. 2001 and 2002).

The capacity for drug transport by nucleoside transporters is different: the CNT's are discussed as potential drug uptake candidates since they have micromolar affinities. Several antiviral and anti-cancer drugs are transported by CNT's like 3-azido-3-deoxythymidine (AZT) and gemcitabine by CNT1, 2,3-dideoxyinosine by CNT2 and 5-fluorouridine, zebularine, gemcitabine, cladribine and fludarabine by CNT3 (Ritzel et al. 2001 and 2002).

Sugar Transporters

Hexose transporters are divided into two families: the sodium-dependent glucose transporters (SGLT, transport of hexoses against a concentration gradient) and the Glut family (transport of hexoses down a concentration gradient). At the apical membrane of enterocytes SGLT1, Glut2 and Glut5 and SGLT6 have been identified. Basolaterally hexoses are transported by Glut5 and SGLT6. The localization of SGLT3 and SGLT4 is unknown. SGLT1 transports D-glucose, D-galactose, alpha-methyl-D-glycopyranoside, inositol, proline, pantothenate, iodide, urea, myoinositol, glucose derivatives. SGLT6 is capable of transporting D-glucose. SGLT3 transports D-glucose and Myo-inositol. Glut5 transports D-fructose whereas Glut2 identifies D-glucose, D-fructose and streptozotocin. For reviews please refer to Wright and Turk (2003), Uldry and Thorens (2003) and Stuart and Trayhurn (2003). Examples of drugs being transported by hexose transporters are rare so far.

Table 3 Amino acid transporters, substrates and localization. A – apical side of the membrane; B – basolateral side of the membrane.

Transporter	Gene name	Specificity	Substrates	Cellular localization
LAT1-4F2hc	SLC7A5/SLC3A2	Large NAA	Gabapentin, mephalan, baclophen, L-Dopa	A/B
LAT2-4Fhc	SLC7A8/SLC3A2	NAA	Cys	B
B0.+AT	SLC7A9/SLC3A1	CAA	Gabapentin	A
ATB0	U75284	NAA	Pregabalin	A
CAT1	SLC7A1	CAA		B
Y+LAT1-4F2hc	SLC7A7/SLC3A2	CAA		B
Y+LAT2-4F2hc	SLC7A6/SLC3A2	CAA NAA		B
A	SLC5A4	NAA	Glu, imino acids	A/B
ATB0.+	SLC6A14	NAA, CAA	Pregabalin	A
TAUT	SLC6A6	Tau	Tau, Ala	A
TAT1	SLC16A10	AAA	L-Dopa	B
EAAC1	SLC1A5	Asp/Glu	Asp-3	B
ASC1-4F2hc	SLC7A10/SLC3A2	Ala, Ser, Cys, Gly, Thr	Aminobutyric acid, βalanine, D-Ser	B
PAT1	SLC36A1	NAA	D-Ser, D-cycloserine, GABA	A

Table 4 Nucleoside transporters, their substrates and localization. A – apical side of the membrane; B – basolateral side of the membrane.

Transporter	Gene name	Specificity	Substrates	Cellular localization
CNT1	SLC28A1	Purine nucleosides	Zaltidabine, cytarabine, cladribine, gemcitabine, 5 deoxy-5-fluorouridine	A
CNT2	SLC28A2	Pyridine nucleosides	Adenosine, cladribine, didanoside	A
CNT3	SLC28A3	Purine and pyridine nucleosides	5-fluorouridine, floxuridine, zebularine, gemcitabine, zalcitabine	A
ENT1	SLC29A1	Purine, pyrimidine nucleosides	Cladribine, cytarabine, fludarabine, gemcitabine, zalcitabine, didanoside	B
ENT2	SLC29A2	Purine, pyrimidine nucleosides	Gemcitabine, didanoside	B

Peptide Transporters

In the intestine following peptide transporters have been identified: PEPT1, peptide/histidine transporter PHT1, PHT2 and the peptide transporter PT1 (Herrera-Ruiz et al. 2001). PT1 belongs to the cadherin family; all other peptide transporters belong to the Solute Carrier Family SLC15A.

For PEPT1 an impressive list of compounds (drugs and prodrugs) has been presented during the last years all of which in retrospective have

been identified as PEPT1 substrates (for example beta-Lactamantibiotics, cephaslosporins, ACE and rennin-inhibitors, trombin-inhibitors, bestatin, prodrugs of acyclovir and gancyclovir (Friedman and Amidon 1989; Walter et al. 1995; Bretschneider et al. 1999; Brandsch et al. 2004 and others). A very comprehensive overview is given in Nielsen et al. (2002). Several reviews deal with PEPT1 as a potential drug delivery target and show thrapeutic applications and prodrug approaches (Daniel and Kottra 2003; Nielsen et al. 2002; Steffansen et al. 2003 and 2004).

Monocarboxylate Transporters (MCT)

Eight different Monocarboxylate transporters have been identified playing an important role in the cellular metabolism by transporting endogenous substrates like monocarboxylates, pyruvate, L-lactate, propionate, butyrate (Halestrap and Price 1999). The best studied transporter, MCT1, is localized basolaterally (according to Tamai et al. 1995). Other authors detected MCT1 in apical intestinal membranes (Ritzhaupt et al. 1998). Tamai studied the uptake and kinetics of several organic acids like benzoic acid, nicotinic acid, pravastatin and salicylic acid (Tamai et al. 1995). Some beta-lactam antibiotics like cefdinir and phenethicillin and carindacillin have been proposed as MCT1 substrates (Tsuji et al. 1993; Itoh et al. 1998; Li et al. 1999).

Fatty Acid Transporters

In the intestine fatty acid translocase (FAT), fatty acid transporting protein (FATP4) and fatty acid binding protein (FABPpm) have been identified (for review, see Stahl 2003). Natural substrates are long chain fatty acids like myristate, oleate, palmitoate.

Organic Anion Transporters and Transporting Polypeptides

In the intestine only few members of the large group of organic anion transporters have been found: OAT2 (SLC22A7) and OATP-B (SLC21A9) responsible for the transport of many different compounds (Burckhardt and Wolff 2000; Tamai et al. 2000; Kobayashi et al. 2003). Drugs like fexofenadine, methotrexate, pravastatin and ouabain are substrates of OATP's (Cvetkovic et al. 1999; Abe et al. 2001; Bossuyt et al. 1996; Dresser et al. 2002). Their role and substrate specificity are currently evaluated in detail.

Organic Cation Transporters

The family of organic cation transporters is represented at the apical membrane of intestinal cells by OCTN2, whereas the basolateral membrane is equipped with OCT1 (Sekine et al. 1998). In the liver OCT1 plays a crucial role in uptake of positively charged drugs and compounds preparing hepatic metabolism and elimination. In the intestine this transporter is localized basolaterally, making a drug targeting questionable: compounds once entering the cells would be identified by OCT1 and excreted via sinusoidal membrane directly for uptake into hepatocytes (for review, see Koepsell and Endou 2003; Koepsell et al. 2003).

OCTN2 was identified as an Na+/carnitine cotransporter and is also located in the apical membrane of proximal tubular cells. Substrates of OCTN2 are TEA, verapamil, pyrilamine, choline and quinidine (Koepsell et al. 2003).

Phosphate Transporters

It is believed that the major part of inorganic phosphor absorption in the intestine occurs via Na^+-dependent phosphate cotransporter NaPi-Iib (SLC34A2, Xu et al. 1999). Small drugs like phosphocarbonic acid and foscarnet are transported by this transporter (Swaan et al. 1995; Tsuji and Tamai 1996). However, the substrate range is rather limited including only inorganic phosphate compounds.

Bile Acid Transporters

Bile acids are reabsorbed from the intestine by the bile acid transporter ASBT (SLC 10A2). Substrates are cholate, taurocholate, glycoursocholate, glycodeoxycholate (Craddock et al. 1998; Wong et al. 1996). Conjugation of oligopeptides to bile salts increased their bioavailability substantially (Kramer et al. 1994). However, it is important to ensure that possible ASBT mediated drug uptake should not interfere with bile salt absorption. It is known that patients with decreased bile salt uptake have higher risk for colorectal carcinomas may be due to increased bile salt concentration in the GI (Wang et al. 2001).

Vitamin Transporters

Several vitamin transporters are expressed in the intestine: SVCT1 responsible for ascorbinic acid uptake (Wang 2000), THTR-2 (SLC 19A3) showing high affinity for thiamine (Eudy et al. 2000; Nguyen et al. 1997; Said et al. 1996). Biotin is transported by the SMVT (Balamurugan et al. 2003). The vitamin transporters are discussed for possible drug targeting although their uptake capacities are low.

Transports with Unknown Localization

Several transporters have been identified in intestinal tissue without defining the localization: SGLT3

and SGLT4 for glucose, PHT2 (PTR3) for peptide/histidine, MCT8 for monocarboxylates/thyroid hormone, FABpm for fatty acids (oleate, myristate, palmitate, stearate, arachidonate, linoleate), RFC1 for folate, THTR2 for thiamine and SMVT for biotin (Steffansen et al. 2004).

MODIFICATIONS OF THE METHOD

Modifications are used to study different uptake transporters. Inhibition studies provide information about transporters involved in uptake. An alternative are cells overexpressing uptake transporters or oocyte studies with one expressed uptake transporter.

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EXAMPLE

Examples of transporter uptake studies utilizing CACO-2 cells can be found in D'Souza et al. (2003), Bretschneider et al. (1999), Tamai et al. (1995), Walter et al. (1995) and Balamurugan et al. (2003).

Chapter II.E
Absorption – in-vitro Tests – Non-Cell Based

Markus Kohlmann

INTRODUCTION

The predominant absorption pathway for the majority of drugs after oral dosage is the passive transcellular diffusion pathway. On the transcellular pathway compounds have to cross a number of biological membrane barriers. Biological membranes consist of lipid bilayers featuring a polar headgroup region at the interface to the aqueous phase and a non-polar region in the centre of the bilayer. In order to cross a biological lipid bilayer membrane, drugs have to interact with the hydrophilic headgroup region of the membrane and enter it subsequently from the bulk water environment. This is followed by diffusion through the hydrophobic acyl chain region of the bilayer. Consequently the drugs have to exit the hydrophilic headgroup region again into the bulk water environment at the inner side of the membrane. The overall process is driven by the concentration gradient of the compound across the membrane.

The permeability of a drug is determined by the physicochemical properties that influence all of the steps involved in the transcellular diffusion. Lipophilicity is a main parameter influencing permeation. It is important that lipophilicity is a mixed parameter expressing hydrophobic and membrane affinity properties as well as hydrogen bonding and ionisation properties of the drug depending on the lipophilicity scale and lipophilicity measurement method used. Lipophilicity is no inherent physicochemical property of a compound but its value depends on the method used to determine it. Lipophilicity is traditionally expressed as the partition coefficient between water and n-octanol called logP (see chapter about physicochemical parameters). Lipophilicity, expressed as octanol-water partition coefficients, correlates to drug absorption in a bell shaped curve. However, a number of alternative assays for estimating lipophilicity expressed as partition coefficients have been proposed (other organic solvents as isotropic partitioning media, RP HPLC, IAM HPLC), which all differs in their lipophilicity scale. Real lipid bilayer membranes (liposomes) have also been used for partition experiments. The intention of these assays is to improve throughput of logP measurements or to find a better correlation of lipophilicity values to transcellular absorption of drugs than using the octanol-water partitioning system.

Several in vitro assays using filter immobilized artificial membranes exist for the estimation of permeability (Parallel artificial membrane permeation assay, PAMPA). In these assays the permeation of a compound is followed directly by estimating the amount of compound on either side of the membrane barrier. The results of these experiments are expressed as permeability values rather than lipophilicity values.

A number of review articles and books described recently the methods used in pharmaceutical research to early on estimate the absorption potential of a compound by means of a variety of techniques.

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II.E.1 HPLC Methods for Lipophilicity Determination

PURPOSE AND RATIONALE

Lipophilicity is a main parameter influencing a drug's intestinal absorption and is generally expressed as the partition coefficient logP between water and n-octanol (Hansch et al. 1964). The partition coefficient logP is used if the partition behaviour of the neutral form of an analyte is considered; the distribution coefficient logD is used if a mixture of chemical forms of the same analyte is under investigation. LogD values take the ionisation state of a compound into account. This is achieved by measuring the distribution behaviour of a given compound at a fixed pH. A wide range of correlation between lipophilicity expressed as logD(P) octanol-water ($logP_{OW}$) values and biological properties of compounds have been described over the years including biological absorption and membrane partitioning (Kerns 2001).

The partition coefficient logP or the distribution coefficient logD between water and n-octanol are determined traditionally by the shake flask method (see chapter about physicochemical parameters). The shake flask method is a rather labour intensive method and not suited for high throughput analysis needed in pharmaceutical research.

The use of hydrophobic stationary phases and aqueous mobile phases in chromatography on so-called reversed phase (RP) materials is widely established. Reversed phase materials typically consist of alkyl chains (mainly C18 in length) immobilised on silica material. The chromatographic retention of a solute on such a system directly depends on its partition between the aqueous mobile and the hydrophobic solid phase (Berthod et al. 2004). Therefore lipophilicity (expressed as partition coefficient logP between stationary and mobile phase, can be estimated from the retention behaviour of the solute of interest on reversed phase material as reviewed by Nasal et al. (2003).

$$\log P = \log k - \log\left(\frac{V_s}{V_m}\right) \qquad (1)$$

V_m and V_s are the volumes of the stationary and mobile phase respectively; k is the retention factor that is derived from the retention time (t_R) taking the dead time (t_0) into account.

$$k = \frac{t_R - t_0}{t_0} \qquad (2)$$

The advantage of using a chromatographic system is much higher throughput compared to shake flask assays. The retention time of an analyte is concentration independent and therefore no quantification has to be done to establish lipophilicity values. Chromatographic measurements are easily automated including peak picking and data evaluation. Another advantage is that disturbing impurities are separated from the compound of interest during the chromatographic experiment and will not interfere with the estimation of lipophilicity. However, the volume of the stationary phase cannot be estimated easily, so that the partition coefficients have to be calibrated using a set of compounds with known partition coefficients in order to achieve comparable results.

PROCEDURE

The lipophilicity of compounds can be measured with any commercially available HPLC equipment; most commonly UV absorption is used as detection system for the chromatography. The retention factor (retention time) of a compound is the analytical parameter needed to measure lipophilicity. To measure the retention factor, the retention time of the compound under investigation has to be determined reproducibly together with the dead time. The dead time is determined using the retention time of a not retained substance on reversed phase columns like buffer salts.

Isocratic methods are widely used to determine lipophilicity. In principle, injecting a solute onto a reversed phase system using a purely aqueous mobile phase would directly determine the partition coefficient. However, to cover a broad range of lipophilicity, a variety of mobile phase mixtures have to be used as most of the drug substances does not elute from reversed phase columns using purely aqueous mobile phase. Organic co-solvents in the mobile phase have a strong influence on the measured partition between lipophilic stationary phase and mobile phase and have to be considered. Generally a number of different experiments with increasing percentage of organic solvent (not exceeding about 50–70 % organic solvent) have to be carried out and the determined retention factors are plotted against the organic solvent ratio used. The retention factor k_w extrapolated to 0 % organic solvent is taken from this plot. Valkó et al. (1997) and Du et al. (1998) showed that instead of using a number of different isocratic experiments

at different organic solvent ratio, one fast gradient experiment on short reversed phase columns could be applied. If a fast gradient is used, any retention time defines a specific mixture ratio of organic and aqueous solvent. Therefore the retention index k_w could be determined directly from one experiment just by measuring the retention time. Short reversed phase columns are recommended in order to keep the organic solvent ratio low at the point of elution from the column.

As mobile phase, different buffers at different pH values (mainly 6.8 or 7.4) with organic modifiers are used. Results obtained with methanol as co-solvent were shown by Baczek et al. (2000) to correlate better to octanol-water partitioning data than results using acetonitrile as organic co-solvent. The H-bonding capabilities of the alcohol seem to be the reason for that.

EVALUATION

The variety of stationary phases available commercially and the lack of standardisation between different laboratories makes it difficult to compare the retention factors k directly as comparison of lipophilicity. Therefore different lipophilicity scales obtained from chromatographic data (isocratic or gradient elution) have been introduced. A calibration of the individual chromatographic system with compounds of known lipophilicity is used in all cases.

The determination of log k_w as the retention factor at 0 % organic modifier estimated from isocratic chromatographic analysis was described earlier. Log k_w is one of the widest used chromatographic lipophilicity parameters and is usually correlated to $LogP_{OW}$ values estimated using the traditional shake flask method. OECD guidelines (1989) suggest a calibration of k_w values with experimental $LogP_{OW}$ values and the expression of reversed phase chromatographically estimated partition coefficients directly as logP values.

The lipophilicity index φ_0 was defined by Valkó et al. (1993). φ_0 is the volume percent of organic modifier in the mobile phase by which the retention time is twice the dead time, which means the retention factor k is equal to 1. It was reported that using the φ_0 scale, the inter laboratory comparability was improved compared to using log k_w. The correlation with traditionally determined log P_{OW} values was shown to better (Valkó 1993) using the φ_0 index. The analytical advantage of the φ_0 value is that it can be estimated from bracketing experimental results and not from extrapolation to 0 % organic modifier.

The CHI index was introduced by Valkó et al. (1997) in order to match the lipophilicity index φ_0 of isocratic experiments with results from gradient elution. The lipophilicity results from gradient elution experiments are calibrated using standard compounds with known φ_0 value estimated using isocratic elution.

The results of chromatographic lipophilicity measurements using gradient elution are often directly expressed as logD values. In that case estimated retention factors are calibrated with known $logD_{OW}$ values of a standard set of compounds used as external or internal standards. However, it has to be pointed out, that the correlation between estimated logD values from reversed phase chromatography and octanol-water distribution coefficients $logD_{OW}$ is low for a diverse set of compounds. The reason is the different organic partition media used in the experiments.

Several review articles recently summarised the use of reversed phase chromatography to measure partition coefficients (Valkó 2004; Berthod et al. 2004; Nasal et al. 2003).

CRITICAL ASSESSMENT OF THE METHOD

The lipophilicity measurements using reversed phase HPLC are fast and reliably and the equipment for the analysis is available in almost every laboratory dealing with drug discovery. Using reversed phase chromatography the high throughput needed to cope with the high numbers of compounds delivered from combinatorial or parallel synthesis can easily be achieved. As LCMS analysis on fast generic gradients is used nowadays as the standard tool for purity estimation in drug research the very same method offers a very robust way to estimate lipophilicity of high numbers of compounds on the run.

However, there are obvious differences between the classically determined octanol-water partition data and the reversed phase chromatographic partition data due to the different nature of the partitioning solvents. The correlation between shake flask octanol water partition coefficients and chromatographic retention factor k_w (0 % organic solvent) was described to be low (Valkó 2004) when structurally diverse compound sets were analysed. Using the φ_0 or the CHI lipophilicity index an improvement in agreement to $logP_{OW}$ was reported (Valkó 1993, 1997).

However, related to drug absorption, both classical octanol-water partition coefficients and chromatographic partition coefficients using the different chromatographic scales have shown to be predictive. If the exact octanol-water partitioning behaviour of a solute has to be analysed the reversed phase chromatographic experiments seem to be no substitute for classical shake flask experiments. For comparison

measurements and ranking of lipophilic properties of a high number of compounds, the reversed phase chromatography technique using fast gradient elution seems to be the method of choice.

MODIFICATIONS OF THE METHOD

In order to improve the correlation between isocratic log k_w values and $logP_{OW}$ values for diverse sets of compounds, Lombardo et al. (2000, 2001) used an octanol saturated mobile phase and a Supelcosil LC-ABZ (polar amide column) as stationary phase. Isocratic separation is used in their work. The obtained log k_w data are calibrated with known logD/logP values of a training set and the obtained lipophilicity data from unknown samples are reported as ElogP (ElogD) values. These authors pointed out that ElogD values correlate very well with traditionally obtained $logD_{OW}$ values in case of neutral and basic compounds. Donovan et al. (2002) used new polymer based reversed phase columns with no residual free silanol groups which might influence the retention behaviour of bases for their fast gradient lipophilicity measurements. They calibrated the system with internal standards of known logP values and expressed lipophilicity as logP. They found a very good correlation of their chromatographic determined logP values with $logP_{OW}$ values from shake flask measurements.

Fast gradients and short reversed phase columns are the usual features of generic gradient LCMS analysis in modern drug discovery. Kerns et al. (2003) combined the experiment to check for purity and identity of synthetic products with the estimation of logD using reversed phase HPLC by using a fast methanol gradient on polaris C_{18} material. Camurri et al. (2001) used the LCMS technology in order to further improve the throughput of chromatographic lipophilicity measurements. Mixtures of compounds were injected onto the chromatographic system and each peak was identified by mass spectrometry. They could measure the lipophilicity of up to 32 different compounds at the same time using this approach.

Apart from the hydrophobic interactions provided by the alkyl part of the molecule, octanol has also hydrogen-bond acceptor and donor functions like lipid membranes have. This property of n-octanol made the octanol-water distribution coefficient that widely used. However, n-octanol or reversed phase materials cannot mimic the interfacial character of the bilayer structure. The ionic interactions between membrane phospholipids and solute are also not represented in the properties of octanol or reversed phase materials. To overcome this issue, alternative stationary phases have been developed to determine lipophilicity values more related to intestinal absorption than to classical $logP_{OW}$ values. These stationary phases are made of silica material with covalent bound phospholipids as introduced by Pidgeon at al. (1998). These immobilized artificial membranes (IAM) stationary phases having diacetylphosphatidylcholine (PC) with or without glycerol backbone bound to silica are now commercially available [Regis technology, Morton Groove, IL]. Kararli et al. (1995) showed that PC is relevant to intestinal absorption as PC together with PE (phosphatidylethanolamine) comprise a major portion of the intestinal brush border membranes.

The lipophilicity measured using IAM columns generally is expressed as the retention factor k_{IAM} or k_{IAMw} of a solute. Valkó et al. (2000) also applied gradient elution to IAM columns and a CHI_{IAM} index was defined similar to the CHI values as described for reversed phase chromatography. Log k_{IAMw} values of neutral solutes often correlates with other lipophilicity scales but charged solutes generally result in a different lipophilicity scale. Log k_{IAM} values have been correlated successfully with blood-brain barrier distribution, small intestine absorption and CaCo2 cell permeation as summarised by Stewart et al. (1998). The respective correlations were shown to be superior to the correlation with octanol-water partition coefficient or HPLC determined lipophilicity in another review by Yang et al. (1996). However, this is not generally the case, and Taillardat-Bertschinger et al. (2003) recently stated that the best lipophilicity descriptor used to predict membrane permeation differs according to the compounds under investigation.

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II.E.2
Lipophilicity Determination Using Liposomes

PURPOSE AND RATIONALE

Lipophilicity expressed as logP_{OW} correlates with membrane affinity and other biological properties as summarised in Kern's (2001) review on physicochemical profiling. However, the interfacial (anisotropic) character of bilayer membranes and the ionisable phospholipid head groups of biological membranes influence the partition properties of drugs. These effects are not reflected using octanol-partitioning experiments. To analyse these influences the partition of solutes between water and charged bilayer membranes should be measured.

For this purpose liposomes are used as lipid phase. Unilamellar liposomes are artificial lipid bilayer vesicles. They can be considered as real model bilayer membranes as they ideally consist of a circular bilayer membrane. The hydrophobic acyl chains are assembled in the hydrophobic core of the liposome whereas the hydrophilic head groups point to the water in the inside and outside of the vesicle. Liposomes can be produced from a variety of lipids and from mixtures of lipids. This possibility allows studying the influence of membrane constituents on the partition of solutes. Krämer et al. (1997) studied the influence of the presence of free fatty acids in membranes on the partition behaviour of propranolol. The influence on α-Tocopherol in membranes on the partition behaviour of desipramine has been reported recently (Marenchino et al. 2004) using a liposome model.

Balon et al. (1999a) reported improved correlation between liposome partition and human intestinal absorption in comparison to the correlation of intestinal absorption to logD_{OW}. Tammela et al. (2004) showed a good agreement between CaCo2 permeation results and liposome data.

The general use of liposomes and the evaluation of the obtained data have been recently reviewed by Plember van Balen et al. (2004) and Krämer (2001).

PROCEDURE

For liposome partition experiments freshly prepared liposomes of defined size are incubated with an aqueous solution of the analyte. The partition coefficient is calculated from the ratio of compound present in aqueous environment and lipid environment in equilibrium.

In order to prepare liposomes, the lipid preparation is dried at low temperature under an inert gas atmosphere (protect the lipid from oxidation). The lipid film is swollen with water or buffered aqueous solution and several freeze-thaw cycles are carried out to get optimal rehydration of the lipid. The rehydrated lipid preparation is filtered using membrane filters with defined pore size. After repeated filtration steps (extrusion) an unilamellar liposome preparation with a defined size distribution is obtained. Large unilamellar vesicles (LUV) are produced in this way. LUV's are about 100 nm in size; the thickness of the lipid bilayer is about 4 nm. Even smaller liposomes can be derived from sonication (sonication probe or ultrasonication bath). Separation of the prepared liposomes

according to size using sepharose chromatography or ultra centrifugation ensures a homogenous size distribution of the liposomes (Krämer 2001). Balon et al. (1999b) used these sonicated small unilamellar vesicles (S-SUV) for partition experiments.

Several methods are used to determine the partition coefficient of solutes between water and liposomes.

Equilibrium dialysis is used in a number of examples to analyse the ratio of lipid-bound to free analyte. Krämer et al. (1998) described the use of equilibrium dialysis by separating the liposome suspension and the water phase by a semi-permeable membrane. The analyte is dissolved in the water compartment of the system and diffuses into the liposome compartment. If equilibrium is reached, the remaining concentration of the analyte in the water compartment is determined by means of a quantification method (mainly HPLC or LCMS, fluorescence techniques) and the partition coefficient is calculated. Krämer et al. (1997) used a radio tracer substance as analyte to quantify the compound in both compartments using liquid scintillation counting.

The partitioning behaviour of an ionisable compound can also be followed directly using a two-phase potentiometric titration using commercial instrumentation (Sirius analytical Instruments) as shown by Avdeef et al. (1998).

EVALUATION

The partition experiments are generally evaluated in terms of a partition coefficient that is calculated from the concentration ratio of analyte bound to liposomes or dissolved in the aqueous phase. For calculating the partition coefficient the amount of lipid in the system have to be known.

Using equilibration dialysis the distribution coefficient logD is calculated using the following equation (Plemper van Balen et al. 2004)

$$\log D = \log\left(\frac{V_{LB}\,(C_{LB} - C_B)}{V_{Lipo} \cdot C_B} + 1\right) \qquad (3)$$

C_{LB} and C_B represent the analyte concentration in the liposome-containing compartment and the aqueous compartment respectively. V_{LB} is the volume of the liposome-containing compartment and V_{Lipo} defines the total volume of lipid phase (total amount of lipid used).

The distribution coefficient logD expresses the distribution of a solute if a mixture of ionisation states of the analyte molecule is present at a defined pH value. The partition coefficient logP is strictly expressed for a specific ionisation state of a solute. LogP values can be obtained from liposome partition experiments if experiments are carried out under a number of defined pH conditions.

Using the potentiometric titration approach (the same approach is also used to determine $\log P_{OW}$ values and described in the paragraph about physicochemical properties), first the substance in aqueous solution is titrated against standard acid or base in order to obtain the pKa value. In presence of liposomes, the experiment is repeated and a shift in the observed pKa value (pKa^{app}) might be noted as analyte has disappeared from the aqueous phase into the liposome phase. From the shift in observed pKa^{app} value the partition coefficient of protonated analyte and neutral analyte can be calculated (Pember van Balen et al. 2004).

$$pKa^{app} = pKa - \log\left(\frac{1 + r \cdot P^{B}}{1 + r \cdot P^{BH+}}\right) \qquad (4)$$

The ratio of organic to aqueous phase is expressed as r, P^B is the partition coefficient of the unionised, P^{BH+} is the partition coefficient of the protonated analyte. Using this approach depending on the nature of the analyte, several titrations (for monoprotic compounds at least two experiments) using different ratios of lipid to water phase have to be carried out in order to determine the partition coefficients of the solute. The result is a partition coefficient–pH profile for the compound and its distinct ionisation states.

CRITICAL ASSESSMENT OF THE METHOD

Using liposomes for membrane affinity studies has the great advantage that liposomes are a nearly one-to-one model of biological bilayer membranes. Liposomes can be generated from a variability of lipids and mixtures of lipids in order to study the influence of the membrane constituents on the partition behaviour of drug candidates.

Mainly the electrostatic interactions of liposomes are closer to those of biological membranes than in the case of n-octanol or reversed phase solid phases. The partition coefficients for neutral molecules mainly correlate well with n-octanol partitioning data or chromatographic lipophilicity scales including IAM whereas for charged or zwitterionic molecules strong differences between partitioning in liposomes and octanol were described. Using liposomes the difference between charged and uncharged molecules concerning their partitioning coefficient is much lower as compared to the $\log P_{OW}$ case. Therefor the use of liposomes might help in understanding the role charged species play in the overall membrane permeation

process of drugs. Balon et al. (1999a) showed that the correlation of liposome partition data with human intestinal absorption is superior to n-octanol partition experiments in some cases whereas Österberg (2001) showed equivalent correlation of different lipophilicity scales including IAM and liposome partitioning compared to human fraction absorbed. Plember van Balen (2004) pointed out that the mechanism for permeation might be different for different ionised species of the same molecule or members of the same chemical series, which makes correlation of data complicated.

However, the throughput of liposome partition assays is limited as preparation and validation of liposomes are very time consuming. That seems to be the main reason why liposome partitioning is not widely used in the pharmaceutical industry to date. On the other hand, the results obtained using liposome partitioning show that liposomes are unique tools to study the bilayer membrane affinity (MA) of drugs and their correlation to intestinal membrane absorption.

MODIFICATIONS OF THE METHOD

To overcome the throughput problem in liposome partition experiments, a number of methods have been introduced. Beigi (1998) immobilised liposomes onto sepharose material by gel bead swelling and used it as solid phase in chromatography experiments (Immobilized Liposome Chromatography, ILC). A bell shaped correlation between the retention behaviour on the liposome columns and human fraction absorbed was reported by these authors. However, the immobilisation of liposomes using gel bead swelling is not very stable over an extended time range and column-to-column reproducibility seems to be an issue. Liu (2002) coupled avidin covalently to sepharose material and used biotin tagged phospholipids to immobilise liposomes on this stationary phase. They reported improved column stability compared to the gel bead swelling approach. Good correlation to human fraction absorbed was shown using these results. Österberg (2001) compared liposome partition with ILC and other lipophilicity scales. An excellent correlation between liposome partitioning data and ILC results was shown. Even extracted biological membranes have been used in ILC experiments by Engvall and Lundahl (2004), however the stability of the columns was shown to be limited.

The use of solid-supported lipid membranes (SSLM) to measure membrane affinity was recently reported by Loidl-Stahlhofen (2001a, 2001b). To produce solid-supported lipid membranes a single phospholipid bilayer membrane is non covalently attached to a solid support. In contrast to covalent immobilisation of liposomes on solid materials for ILC or the impregnation of lipids on filter materials (PAMPA), the solid supported lipid membrane is reported to retain the physiological fluidity and its unilamellarity. SSLM material for membrane affinity measurements is commercially available as TRANSIL material by NIMBUS Biotechnology (Leipzig, Germany). The compounds under investigation are incubated in buffered solution with TRANSIL material of known lipid amount (V_{lipid}) provided by the manufacturer in a 96 or 384 well format. After incubation the concentration in the aqueous phase (N_{water}/V_{water}) and in the solution before incubation (N_{total}) is determined by HPLC and membrane affinity is calculated using the following equation (Loidl-Stahlhofen et al. 2001a):

$$MA = \frac{c_{lipid}}{c_{water}} = \frac{V_{water}}{V_{lipid}} \times \frac{N_{total} - N_{water}}{N_{water}} \qquad (5)$$

A good correlation between $logMA_{SSLM}$ and logMA estimated from equilibrium dialyses using liposomes in solution was reported. The TRANSIL approach is a unique use of liposome like real bilayer membranes as high throughput method for the estimation of membrane affinity.

Liposomes have also been attached to sensor chip surfaces used in surface plasmon resonance (SPR) instruments. SPR biosensors measure the quantity of a compound bound to an immobilised partner in real time without the need for fluorescent or radioactive labelling. SPR is an optical phenomenon that occurs when total internal reflection of light at metal surfaces is examined. Changes in SPR occur due to changes in the refractive index at the metal surface (up to about 300 nm). Binding of compounds to a metal-immobilised partner will change the refractive index near the metal surface. Therefore binding of compounds to metal surface immobilized partners can be determined as changes in the refractive index in SPR. Liposomes are attached to the dextran matrix that covers the gold surface of a SPR chip. In SPR experiments the compound of interest is injected in buffered solution to a liposome and reference surface at low flow rates. The result of a SPR experiment is called sensogram, which shows the association and dissociation of an interaction partner to the immobilised system in real time. Baird (2002) used blanc and liposome chip surfaces at the same time and difference sensograms between the control surface and a liposome surface were analysed. The plateau binding response expressed as response units (RU) for the individual analytes is the measure for membrane affinity in these

experiments. Membrane affinity expressed in RU has been successfully correlated to fraction absorbed in humans by Danelian et al (2000).

SPR also offers the unique possibility to study the kinetics of the membrane solute interaction if a dilution series of the compound under investigation is injected into the system. Abdiche (2004) clustered compounds with the same apparent partition coefficient K_D according to their binding kinetics and differentiated mainly between fast off and slow off rate drugs.

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II.E.3 Parallel Artificial Membrane Permeability Assay (PAMPA)

PURPOSE UND RATIONALE

The use of partition coefficients between water and lipophilic media is of wide use in pharmaceutical research. As discussed in the last chapters, different lipophilicity scales are used to describe the lipophilicity of a compound and relate it to its absorption behaviour in vivo. Differences between the $logP_{OW}$ and partitioning between phospholipids and water (mainly determined using liposomes) for diverse compounds have been described leading to the development of the immobilized artificial membrane chromatography system. However, also the predictivity of the IAM system is limited and only a small number of membrane systems are available.

Arturrson et al. (1991) described the permeation across biological cell layers (CaCo2 system and others, see chapter about in vitro permeation assay, cell based) in order to predict the human intestinal absorption of a solute. However, the throughput of CaCo2 assays is limited due to the long growing cycles of the cells and because the assay itself is very laboratory intense. Therefore it is not easy applicable to the high throughput needed in drug research in a combinatorial chemistry and high throughput-screening environment. The need for a fast, easy and cheap method to asses permeability in vitro led to the development of the parallel artificial membrane permeability assay (PAMPA) that uses the filter immobilised membrane

concept to analyse membrane permeation. The development of PAMPA was possible because of the former work by Thompson et al. (1982) and others about filter supported lipid bilayers (black lipid membranes BLM) that showed that stable lipid layers can be formed on filter supports and that the lipid layer is able to close the filter pores enabling measuring the permeation across this lipid layer barrier. PAMPA is a permeability assay whereas the assays described until now in this chapter were purely partition assays. Permeation across a biological membrane is the sum of several partition events between the aqueous environment and the lipophilic membrane plus the diffusion event within the biological membrane and the passage through the unstirred water layer at both sides of the membrane. Using octanol as barrier, partition between octanol and water and permeation through octanol immobilised on filters were correlated by Camenisch (1997) and a sigmoidal relationship was encountered similar to the correlation between logD and CaCo2 permeability indicating the importance of directly measuring permeation of solutes through artificial membranes instead of solely correlating partitioning data. Kansy (1998) first proposed a high throughput permeation assay called Parallel Artificial Membrane Permeation Assay (PAMPA) using an artificial phospholipid membrane brought onto a supporting hydrophobic filter. They presented a promising hyperbolic correlation between human absorption and PAMPA permeability however with a steep initial slope. The correlation allowed classifying compounds in classes of low, intermediate, and high permeability. Their focus of the PAMPA method is to offer a "physicochemical high throughput screening" method for large library sets in order to allow a multidimensional compound optimisation considering both physicochemical and biological activity data. Now PAMPA is used in a variety of labs worldwide and a commercial instrument and software is available (pION Inc.). In the year 2002 a specialised symposium was held on the use of PAMPA in pharmaceutical research (http://www.PAMPA2002.com) indicating the rapid progress of the technique.

PROCEDURE

PAMPA is generally carried out in the 96 well format using a PAMPA sandwich construction. The sandwich consists of a standard 96 well plate and a filter plate put on top (Microtiter plate filter plates that are commercially available). The bottom standard 96 well plate is filled with buffer so that the liquid surface will be in contact with the filter material of the filter plate put later on top. The filter material was impregnated with lipid solution (lecithin etc.) in an organic solvent (dodecane etc.) and put carefully on top of the donor plate in order to avoid any air bubbles between the liquid surface and the filter plate. Then the filter plate is filled with buffer and the permeation experiment is started by the addition of the analyte to the donor plate on top of the sandwich. As the focus of PAMPA is on high throughput, the concentration of the analytes in the acceptor wells after incubation is determined by fast UV measurements at various wavelengths using a UV plate reader. The analyte concentration in the acceptor well after permeation is compared to the analyte concentration in the donor solution before incubation to estimate permeation of the analyte under investigation.

PAMPA is often used at various pH values in order to measure permeability pH profiles as the permeability of ionisable compounds depends heavily on the pH of the buffer. As the pH range of the intestinal tract varies between pH 6 and pH 8 this is the range of pH values that mostly is used. Kerns (2004) recommended to measure from pH 4 to pH 7,4 in order to predict both bases and acids correctly. Ruell (2003) used permeation pH profiles from pH 4 to pH 9 together with the pKa values of the compounds under investigation to establish the optimum pH value for a single pH PAMPA measurement.

EVALUATION

The permeation in PAMPA experiments can be followed directly as the percentage of compound permeated into the acceptor compartment after a given time (%T) as described in the original work by Kansy et al. (1998) which reflects an equilibrium analysis at a given time point.

$$\%T = 100 \times \frac{C_A^{end}}{C_D^{start}} \tag{6}$$

C_A^{end} is the concentration of the solute under investigation in the acceptor well after incubation and C_D^{start} is the concentration of the analyte in the donor well at the beginning of the experiment.

Permeation in PAMPA experiments is also expressed as a flux rate P_{app} with the unit [cm/sec] under the assumption that transport equilibrium is not reached and back-transport can be neglected in course of the experiment. P_{app} values represent kinetic information and describe the flux of compounds over the membrane.

$$P_{app} = \frac{V_A}{A \times (C_D - C_A)} \times \frac{dC_A}{dt} \tag{7}$$

V_A is the volume in the acceptor well, A is the filter area, C_D is the concentration of analyte in the donor compartment at the beginning of the experiment, C_A is the concentration of analyte in the acceptor compartment at the beginning of the experiment and (dC_A/dt) is the increase of drug concentration during the course of the experiment.

P_{app} values are often calculated from the ratio of analyte signal in the acceptor compartment at a single time point divided by the analyte signal in equilibrium without membrane barrier. Also % T and P_{app} values are related to each other following the equation taken from Zhu's (2002) work:

$$P_{app} = \frac{V_D \times V_A}{(V_D + V_A) \times A \times t} \times \ln\left[\frac{100 \times V_A}{100 \times V_D - \%T\,(V_D + V_A)}\right] \quad (8)$$

Both P_{app} and %T values have been correlated to fraction absorbed in human and hyperbolic curves were encountered. This correlation is calibrated using a set of compounds with known human absorption properties and unknown solutes are classified according to their position on the hyperbolic correlation curve into groups of high/low/medium absorbed compounds.

Avdeef (2001) described the influence of membrane retention of solutes observed in PAMPA experiments on the data evaluation. Membrane retention is analysed using a mass balance between compound left in donor compartment after incubation and compound determined in acceptor compartment after incubation. For calculating P_{app} under consideration of membrane bound fraction Avdeef (2001) used a equation different from equation (2) where the total amount of solute is substituted by the non-membrane bound fraction of solute in the equation.

CRITICAL ASSESSMENT OF THE METHOD

The PAMPA technology started to evolve with Kansy's (1998) work published in 1998 and since then the number of published work and the use of the technique in industry and academia grew tremendously. The development of a commercially available instrument/software shows the impact the development of PAMPA had on the drug research. Nowadays PAMPA is the method of choice for high throughput in vitro cell free permeability assays in pharmaceutical industry. The high throughput possibilities offered by PAMPA are superior to the other in vitro permeability techniques (including logD measurements using RP HPLC) at least if UV absorbance is used as analytical tool.

Also the fact that only transcellular permeation is regarded in PAMPA experiments is valuable as it allows comparing PAMPA results to cellular permeation experiments that feature all possible permeation mechanism including paracellular or active transport and active efflux. Kerns (2004) recommended this comparison to get an insight into the permeation mechanism applied by a compound under investigation. The possibility to obtain permeability pH profiles is also really helpful to identify the relevant permeability value of a compound and cannot be determined by cellular assays due to the limited pH range usable with living cells.

PAMPA is relatively insensitive to DMSO percentage or other solubility mediating formulations in donor or acceptor compartment which gives the possibility to study the permeability of compound from different formulations which is not easily possible using living CaCo2 cells. Liu (2003) used that approach in conjunction with LCMS analyses to study the permeability of poorly liquid soluble compounds.

On the other hand, PAMPA is a purely artificial method and PAMPA membranes do not reassemble real lipid bilayer structures as barriers for permeation but much thicker barriers. The thickness and material of the supporting PVDF filters also influences artificially the permeation of compounds depending on the lipophilicity of the compounds more than the thin polycarbonate filter does in CACo2 experiments. Also the best choice of membrane constituents for PAMPA experiments is still under investigation and it seems that it will depend a lot on the goal of the PAMPA experiment which membrane is used (e.g. blood brain barrier permeation or intestinal absorption). One has to take into account that PAMPA today is a summary term on a lot of different methods applied in different laboratories using different membrane constituents, sink conditions, permeation times etc., which makes inter laboratory comparison difficult.

Therefore the knowledge of the lipophilicity expressed as $\log P_{OW}$ will still be of high interest in drug research. Pampa will not substitute CaCo2 or other cell types for the study of permeation but it is able to deliver predictive data about transcellular permeation of a high number of analytes. More time can then be used for the cell-based assays to investigate in depth the mechanism of permeation or efflux of compounds of interest.

MODIFICATIONS OF THE METHOD

The PAMPA permeability depends heavily on the artificial membrane used in the experiment. Today

the lipid choice varies between the labs. Kansy et al. (1998) used a solution of 1–20 % lecithin in dodecane or hexadecane for their work. Sugano (2001) presented improvements on the used membrane composition in terms of better correlation of the permeability data to the % absorption data in human and higher overall permeability. Theses authors used a so-called biomimetic lipid made from phosphatidylcholin (0,8 %), phosphatidylethanolamin (0,8 %), phosphatidylserine (0,2 %), phosphatidylinositol (0,2 %) and cholesterol (1 %) dissolved in 1,7 octadien. Li (2003) used porcine brain lipids as artificial membrane to investigate blood-brain permeation and showed correlation between in vivo assays for brain permeation and PAMPA assay. The commercial available membrane system used in pION systems (double sink PAMPA^{TM}) consist of 20 % phospholipid in dodecane featuring 16 % negative charge on the surface as used eg. by Bermejo (2004). Also the filter support was proven to have influence of the permeability determined by PAMPA experiments. Zhu (2002) used a hydrophilic PVDF membrane as support and could obtain permeability results yet after 2 hours of incubation in contrast to 15 hours using the hydrophobic filter materials used by other authors. Wohnsland and Faller (2001) used polycarbonate material instead of PVDF because of the reduced thickness of the polycarbonate material and more uniform surface structure as compared to the PVDF filters. Whereas generally PAMPA is used with artificial membranes composed of mixtures of phospholipids with organic solvents, Wohnsland and Faller used a permeation screen using hexadecane on a polycarbonate filter support. The use of pure alkane as lipid barrier for permeation allows measuring permeation through and partitioning between the alkane phase and water in the same experiment. The permeation data using hexadecane were correlated to % absorption in human and a good correlation was reported showing the predictive character of this assay.

Ruell (2003), Wohnsland (2001) and others discussed the influence of the unstirred water layer on both sides of the filter plate on the results from PAMPA assays. Avdeef (2004) introduced recently individual stirring in each well of the 96 well plate using stirring disks rotating parallel to the membrane surface for PAMPA experiments. PAMPA experiments as short as 15 minutes due to the intense stirring were reported.

In intestinal absorption the concentration gradient over the lipid membrane is the driving force for permeation of compounds. The concentration of compound at the inner side of the membrane is hold very small due to removal of compounds from the membrane through the blood flow or binding of the compound rapidly to plasma protein (Youdim 2003). These sink conditions are mimicked in some PAMPA protocols by trapping the analytes in the acceptor compartment using a pH gradient between donor and acceptor well. Zhu (2002) presented a method where acids and bases have to be analysed under different sink conditions aiming in trapping an ionised species in the acceptor compartment. Double-sink^{TM} conditions are offered commercially by pION. This method combines pH gradients between donor and acceptor compartments depending on the acid/base properties of the analytes with the use of a surfactant in the acceptor compartment mimicking serum albumin and trapping mainly neutral compounds. Using sink conditions, Zhu (2002) found that underestimation of permeability in PAMPA compared to CaCo2 assays is reduced in comparison to iso pH experiments.

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Chapter II.F
Distribution – in vitro Tests – Protein Binding

Jens Riedel

INTRODUCTION

The protein binding plays an important role in the pharmacokinetics and pharmacodynamics of a drug. Plasma binding can also be viewed as a circulating site of storage, releasing the drug to tissue with higher affinity. Only the unbound drug is thought to be able to diffuse across membranes and to interact with an effector site to produce a therapeutic response.

The extent of protein binding in the plasma or tissues controls the volume of distribution and affects both hepatic and renal clearance. The volume of distribution (V) depends on the fraction unbound in plasma (f_u), the fraction unbound in tissue (f_{uT}), the volume of tissue (V_T), and the volume of plasma (V_P) by the equation:

$$V = [f_u / f_{uT}]V_T + V_P.$$

For all drugs with a volume of distribution value $>$ 30 L, changes in f_u therefore translate directly into changes in V (Rowland and Tozer 1995). According to their incidence on drug distribution two types of drug plasma binding can be distinguished. It can be restrictive, when the quantitative distribution of the drug is limited, respectively vice versa non-restrictive, when the distribution is not limited. A restrictive binding is characterized by blood concentrations higher than tissue concentrations. Large volumes of distribution values indicate a non-restrictive plasma binding. Especially if the binding capacity of some tissues are greater than that of the circulating protein and the greatest part of the applied dose penetrates into tissues (Urien et al. 2001).

The influences of plasma binding on drug elimination may be best understood by consideration of clearance. All organ clearance models incorporate a protein-binding term. For example the conversion of the intrinsic clearance (CL_{int}) to the hepatic clearance (CL_{hep}) involves the use of equations describing the well-stirred and parallel tube models of hepatic clearance (Pang and Rowland 1977; Wilkinson and Shand 1975).

$$CL_{hep} = \frac{Q * f_u * CL_{int}}{Q + f_u * CL_{int}} \text{ (Well-stirred model)} \quad (1)$$

$$CL_{hep} = Q * \left(1 - e\frac{-CL_{int} * f_u}{Q}\right) \quad (2)$$

(Parallel tube model)

Q: hepatic blood flow

In consideration that (hepatic) clearance is a product of (hepatic) blood flow and (hepatic) extraction ratio of the drug ($CL_{hep} = Q_{hep} * E_{hep}$) two types of elimination can be distinguished. For very highly extracted drugs E_{hep} approaches the factor 1. As the product $CL_{int} * f_u$ for these drugs is greater than Q, CL_{hep} approaches Q. Under this perfusion rate-limited condition changes in fu have only small influence on the hepatic clearance. Conversely the impact of the unbound fraction on the clearance is higher for drug with a small hepatic extraction ratio (Rowland and Tozer 1995).

Only the unbound fraction in plasma is filtered in the kidney. Therefore, the rate of filtration is the product of glomerular filtration rate (GFR) and unbound plasma concentration. For a drug which is only filtered and all filtered drug is excreted into the urine, the renal clearance (CL_R) is the rate of filtration product:

$$CL_R = f_u * GFR. \quad (3)$$

The binding to plasma or subcellular liver fraction can be taken into account for the prediction of human pharmacokinetic parameters either from preclinical and/or in vitro metabolism data (Obach et al. 1997; Mahmood 2000). Obach (1999) showed by comparison the in vivo investigated clearance values and clearance values projected from in vitro intrinsic clearance data of 29 drugs that the inclusion of blood and liver microsomes binding values gave the best agreement.

The unbound fraction of the drug can be included for the calculation of the safety margin, based on the exposure of the drug from toxicological results in animal and the expected exposure in the first human study.

The binding of a drug, respectively its amount of unbound fraction can be change by co-medication of another drug. The bound fraction of the primary drug is displaced by the secondary drug, which shows a higher affinity to the protein. The increase of free fraction can cause an increase of pharmacological effects, side effects or and toxicological effects. The quantitative and clinical importance of the drug-drug interaction from plasma protein binding depends on the total amount of the initial drug in the body that is bound to plasma before displacement, the extent of displacement, the extent of binding to tissue structures, and the apparent volume of distribution. However, the importance of displacement has been controversial discussed by several authors. Rolan (1994), Sansom and Evans (1995) and Benet and Hoener (2002) have presented theoretic arguments about the limited cases when drug interaction has been regarded as significant. Rolan has shown with several examples that drug interactions, which have been attributed to plasma protein binding displacement, can be explained by other mechanism (e.g. inhibition of metabolism). He suggests an algorithm to determine the clinical significance of potential PPB displacement interactions. An interaction is likely and a clinical study should be performed to quantify the effects, if the drug of interest has a plasma protein binding above 90 %, a narrow therapeutic index, the hepatic extraction ratio is high, and the drug is given intravenously.

The plasma protein profiles in humans are influenced by age, gender, and disease (Verbeeck et al. 1984; Grainger-Rousseau et al. 1989). Elderly tend to have decreased plasma protein concentrations. Disease may cause hypoalbuminemia as found in patients with burns, cancer, cardiac failure, cystic fibrosis, enteropathy, inflammations, liver impairment, malabsorption, nephrotic syndrome, renal failure, sepsis, and trauma. In acute renal failure the concentration of total plasma concentration, particularly of albumin, is significantly reduced (by approximately 25 %). Significant changes in plasma composition have been observed in pregnancy and in neonates (Notarianni 1990). Pregnancy results in decreased drug-protein binding to albumin. Foetal albumin present in newborn infants has less binding affinity for acidic drugs. Thus, free drug concentrations in neonates are higher that those observed in children or adults.

The PPB of racemic drugs is potentially stereoselective, as a consequence of chiral discriminative properties of the binding sites of the protein fractions. However, the limited available data on the binding of enantiomers indicate the differences are small (Pacifici and Viani 1992). The concentrations of total protein, albumin and α-acid glycoprotein in plasma differs slightly between human and animal species (Davies and Morris 1993). Interspecies differences in the stereoselectivity of protein binding have been reported for various drugs (Lima 1988; Lin et al. 1990, 1991).

Beside the measurement in whole plasma, the PPB can be characterized according to the participated proteins. It may involve, in the simplest case one, or even several proteins. The most frequent situation is that of a drug-albumin complex. Since albumin is the protein having the highest concentration in plasma. It is by far the most important drug carrier. The plasma level of albumin in a healthy adult is 35 to 50 g/L (Rowland and Tozer 1995), with a mean value of 41.8 g/L (Davies and Morris 1993). It is mainly involved in acidic drug binding and has three major drug binding sites. Non carboxylic acids (e.g. endol derivates) bind to site I. Also known as the warfarin site. Site II binds carboxylic acids through hydrophobic forces and is also selective for benzodiazepine and indole compounds (Fehske et al. 1981). Site III is specific for cardenolides and biliary acids (Kragh-Hansen 1985, 1990). Oral anticoagulants, oral hypoglycaemics and nonsteriodal anti-inflammatory drugs (NSAIDs) are examples of drugs that are extensively bound to albumin with a fraction unbound less than 10 % and sometimes less than 1 %.

The second main plasma protein fraction is α_1-acid glycoprotein. The normal plasma levels are 0.4 to 1.0 g/L (Rowland and Tozer 1995). As the protein contains an anionic charge, it attracts basic molecules (Muller 1989; Kremer et al. 1988). Drugs with high affinity to α_1-acid glycoprotein include synthetic opioids (e.g. fentanyl, methadone), antiarrhythmic agents (e.g. lidocaine, disopyramide) and tricyclic antidepressants (Jack 1992). Plasma concentrations of α_1-acid glycoprotein increases in the presence of stress, inflammation, malignancy, myocardial infarction and various haematology disorders.

Other blood components, such as erythrocytes, lipoproteins and α-, β- and γ-globulins and tissue proteins are also capable of drug-protein interactions.

The addition of anticoagulants to the plasma can alter the PPB and should be evaluated prior. Hence, the binding is determined in plasma (e.g. of human) collected using potassium EDTA, sodium citrate,

and lithium heparin anticoagulants and compared against serum binding. The effect of an anticoagulant is considered acceptable, if the ratio of the free fraction in plasma to serum is within 0.8 and 1.25.

The affinity of drugs to proteins can be pH dependent. Hence, it is necessary to control the pH of the plasma prior. The pH value can be adjusted to an appropriate standard value (pH 7.4) by introducing carbon oxide or nitrogen gas. Also the stability of the drug in the matrix (e.g. plasma) should be verified prior to the assay. For instable compounds the degradation (e.g. esterase) can be avoided by addition of appropriate inhibitor (e.g. sodium fluoride).

General Considerations

From the great variety of methods for the determination of protein binding three separation methods, equilibrium dialysis (ED), ultrafiltration (UF), and ultracentrifugation (UC) and a non-conventional method with the binding to immobilized proteins has been chosen. The first methods are undoubtedly the most widely used because of their simplicity and general applicability to many different systems. Other methods e.g. size exclusion chromatography, capillary electrophoresis, or spectroscopic methods have been not described. Oravcová et al. (1996) gives a comprehensive review and comparison for these applications.

The methods, described in this chapter are for the determination of adherent protein binding, which is reversible. Irreversible covalent binding (e.g. caused by reactive intermediates) has not been considered.

Analytics

The applied analytical method depends on the binding assay used, and on the phase of drug development when the protein binding is performed respectively, on the availability of radiolabeled substance. Generally the highest purity of the compound is preferred, to avoid interferences with contaminants or degradation products.

In the stage of drug discovery, LC/MS-MS analytics is the method of choice to quantify the unbound drug concentration. The sensitivity can be increased by the use of radiolabeled substance. But, the radiochemical purity, isotope decay, if not ^{14}C-label is used; as well a sufficient specific activity must be taken into consideration (Wright et al. 1996). The concentrations of radioactivity in bound and unbound fraction are measured by liquid scintillation counting. The use of radiolabeled material allows easily examination of the potential of adsorption. However, the identity of the drug in unbound fraction should additionally be verified by HPLC-radiometry detection. In the early phase of drug discovery high throughput screening assays with 96 well plates, fluorescence- or UV- readers, are employed.

Evaluation of Binding Data

In characterising the protein binding several parameters of a drug can be experimentally determined – the unbound plasma concentration (c_u), the bound plasma concentration (c_b), the fraction bound (f_b), and the fraction unbound (f_u). The determination can be performed at one defined concentration (e.g. 10 μM) or within a range including the pharmacological and toxicological relevant concentrations.

Additionally, the equilibrium association constant (K_A), equilibrium dissociation constant (K_D) and the number of protein binding per sites per class of binding site (n) are results of interest (Wright et al. 1996).

The drug protein association can be schematically represented as:

$$c_u + P \overset{K_A}{\longleftrightarrow} c_b$$

P: protein concentration

Whereas K_A is defined as $[c_b]/[c_u] * [P]$, and $K_A = 1/K_D$.

The equilibrium association constant K_A, respectively the corresponding dissociation constant (K_D) provides information on the affinity or the strength of the drug-protein association. A highly protein bound drug will typically have a K_A value ranging from 10^5 to 10^7 L/mol, and a drug with low to moderate protein binding will usually have a K_A value ranging from 10^2 to 10^4 L/mol (Wright et al. 1996).

Derivation of the second order equation for protein interactions yields an equation that describes c_b as a function of c_u:

$$c_b = \frac{n * P * K_A}{1 + K_A * c_u}. \tag{4}$$

This equation describes the bound drug concentrations as a function of unbound drug concentrations. A binding system with a single class of non-saturable binding sites can be described by

$$c_b = n * P * K_A * c_u. \tag{5}$$

If the binding system consists of 2 classes of binding site it is characterised by equation 6:

$$c_b = \frac{n_1 * P_1 * K_{A1}}{1 + K_{A1} * c_u} + \frac{n_2 * P_2 * K_{A2}}{1 + K_{A2} * c_u} \tag{6}$$

Graphical Methods

The graphical methods for the estimation of the protein binding parameters are limited to protein systems, which have only 1 class of binding sites. If the protein concentration P is known the product $n*K_A$ can be calculated by using equation 4. The slope of the regression line is equal to $n*K_A*P$.

For saturable binding, the following graphical methods can be used after rearrangement and linearization of equation 5:

The Double-Reciprocal Plot

$$\frac{1}{v} = \frac{1}{n * K_A * c_u} + \frac{1}{n} \tag{7}$$

$$\text{Where } v = \frac{c_b}{P} \tag{8}$$

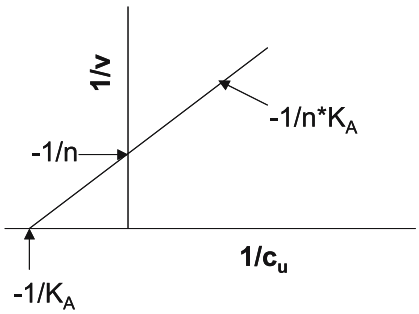

Fig. 1. Double–Reciprocal Plot. The y-intercept is –1/n and the x-intercept is $-1/K_A$.

The Scatchard Plot

$$\frac{v}{c_u} = n * K_A - v * K_A \tag{9}$$

$$\text{Where } v = \frac{c_b}{P} \tag{10}$$

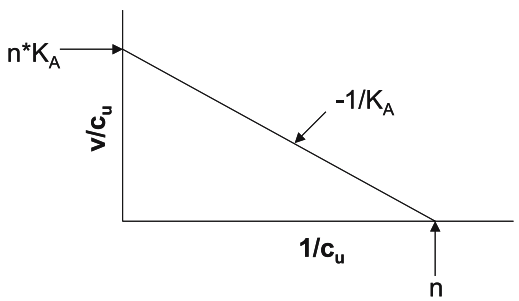

Fig. 2. Scatchard Plot. The y-intercept is $n*K_A$ and the x-intercept is n. The slope is $-1/K_A$ (Scatchard 1949).

The Rosenthal Plot

This modified Scatchard Plot is used if the protein concentration is unknown (Rosenthal 1967).

$$\frac{c_u}{c_b} = n * K_A * P - c_b * K_A \tag{11}$$

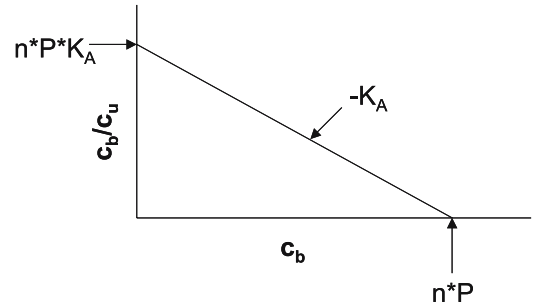

Fig. 3. Rosenthal Plot. The y-intercept is $n*P*K_A$, the x-intercept n*P and the slope $-K_A$.

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II.F.1 Ultrafiltration

PURPOSE AND RATIONALE

The ultrafiltration (UF) with semipermeable, size-exclusion membranes produces a separation of the free drug from macromolecules by employing a pressure gradient, which forces small molecules through the membrane. UF can either be used in an individual filtration unit or in a 96 well plate format system.

Conventional Ultrafiltration (Individual Devices)

Equipment & Material

- Plasma pool of different species
- Stock solution of the drug prepared in an appropriate organic solvent (e.g. 10 mM, DMSO)
- Phosphate buffered saline pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl)
- Calibrated pH-Meter
- Nitrogen gas and carbon dioxide gas
- Glass wool for lab purpose
- 1.5 mL microcentrifuge tubes
- Thermostatized centrifuge with fixes angle rotor (angle of inclination 33°) (e.g. Hettich Rotixa RP 001260 Tuttlingen Germany)
- Disposable individual ultrafiltration unit (e.g. Centrifree Micropartition System No. 4104 Millipore Bedford MA USA). It consists of two compartments – the sample reservoir and filtrate cup. A hydrophilic ultrafiltration membrane with a defined molecular cut off (10 kDa) separates them.
- Single pipettes.

PROCEDURE

1. The frozen plasma is slowly thawed in cold water, or over night in the refrigerator and filtered over glass wool. Protein binding of some drug is pH-dependent. Therefore the pH value of the freshly thawed plasma has to be adjusted to pH 7.3–7.4 by CO_2 respectively, N_2. In the lab, carbon dioxide gas may be released by vaporizing small quantities of dry ice in an Erlenmeyer flask with plastic tube and small pipette for the introduction of the gas. Due to the strong pH lowering effect of the gas, the CO_2 should be introduced carefully and in small quantities. If the pH value falls below pH 7.3–7.4, it can be re-adjusted by degassing with nitrogen.
2. Aliquots of the stock solution diluted to 10 μM are added to 1 mL of pH-adjusted plasma, protein fraction or buffer. The amount of organic content should not exceed more than 2 % of the initial spiked plasma. Higher content could adversely interfere with protein binding.
3. The spiked sample (1 mL) is immediately placed in the ultrafiltration unit. Closing the sample reservoir with a provided cap before the centrifugation can prevent changes of the pH during the determination.

4. The sample is filtered through the membrane by centrifugation in a fixed angle rotor at 1000–2000 g. The flow rate depends on the protein concentration in the sample, which can differs between the species, the starting volume, the relative centrifugal forces, the rotor type, and the temperature. For the determination of the protein binding, the volume of the obtained filtrate should be a fifth (approx. 200 μL) of the initial volume of sample. Hence, for each matrix the centrifuge time has to be determined beforehand.
5. In parallel the non-specific adsorption has to be investigated by using physiological PBS buffer or recommended, protein free serum filtrate.
6. The concentration of the free drug (c_u) in the ultrafiltrates or the concentration of bound fraction (c_b) in the retentate can be quantified by LC-MS/MS by means of a calibration curve. If radiolabeled substrate has been applied, liquid scintillation counting can be employed.

Complementary to the simple adsorption test, the mass balance of the separation could be investigated. This method is more accurate to determine the loss of free drug, which may occur due to variations in sample preparations methods, non-specific protein binding and to metabolism. The latter may also be tested in separate stability tests in plasma or applied matrix.

The mass balance is calculated by determining volumes by gravimetric and drug concentrations in both the retentate and ultrafiltrate.

Automated Ultrafiltration in a 96 Well Plate System

Equipment & Material

- Plasma pool of different species
- Stock solution of radiolabeled or non-radiolabeled drug prepared in an appropriate organic solvent (e.g. 10 mM, DMSO).
- Phosphate buffered saline (PBS), pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl)
- Calibrated pH-Meter with one bar measuring chain
- Pure nitrogen (N_2) and carbon dioxide (CO_2). In the lab, carbon dioxide gas may be released by vaporizing small quantities of dry ice in an Erlenmeyer flask with stopcock, plastic tube and small pipette for the introduction of the gas
- Incubator
- 96 well plate Mixer
- Centrifuge with swinging bucket rotor and sealed microtiter plate carriers
- Liquid Scintillation Counter (LSC) or LC/MS-MS-equipment
- MultiScreen Filter Assembly with Ultracel-PPB Membrane No. MAPPB1010 (Millipore Corp – Bedford MA USA)
- Single and multichannel pipetters
- Non-sterile troughs
- Microplate 96 deep-well (2 mL/well)
- 15 mL graduated centrifuge tubes
- 1.5 mL microcentrifuge tubes.

PROCEDURE

1. Adjusting the pH of the plasma
2. The frozen plasma is slowly thawed in cold water, or over night in the refrigerator and filtered over glass wool. Protein binding of some drug is pH-dependent. Therefore the pH value of the freshly thawed plasma has to be adjusted to pH 7.3–7.4 by CO_2 or N_2. Due to the strong pH lowering effect of the gas, the CO_2 should be introduced carefully and in small quantities. If the pH value falls below pH 7.3–7.4, it can be re-adjusted by degassing with N_2
3. Using a 1.5 mL microcentrifuge tube, prepare a 0.5 mM solution of each drug in PBS
4. Add 80 μL of the drug solution to 7.92 mL of plasma or plasma fraction in a 15 mL centrifuge tube for a final test solution concentration of 5 μM
5. Mix and incubate at 37 °C for 60 minutes
6. Transfer 300 μL/well of each plasma/drug mixture to one column of each of two Multi- Screen plates with Ultracel-PPB fitted with receiver plates. Cover the assemblies with sealed plate covers or the supplied extended centrifugal covers
7. Centrifuge at 2000 g (37 °C) for 45 minutes
8. Remove a 25 μL aliquot of the retentate volume (for purposes of determining the mass balance) and a 25 μL aliquot of the filtrate volume (for purposes of determining the concentration of free drug). It is possible that a concentrated protein layer forms on the membrane during filtration, so care should be taken to thoroughly mix the retentate prior to removing the aliquot
9. The filtrate remaining in the receiver plate is then analyzed for volume determination using either spectrophotometry by determining the dilution factor of a known aliquot of a chromophore added to the filtrate, or spectrophotometry by using the near infrared (NIR) absorption of water according to standard protocols, or gravimetric determination (Millipore Application Note AN1733EN00)
10. 25 μL of each sample or standard dilution is either analyzed by LC/MS-MS or LSC. The analytical

method depends on the use of radiolabeled or non-radiolabeled drug. For good quantitative analysis, 4 to 6 point standard curves are generated

11. Concentrations are determined for filtrates, retentates, controls and standard dilutions

EVALUATION

Final results are given as percentile fraction unbound (f_u) of percentile fraction bound (f_b) for a defined concentration.

$$f_u\,[\%] = \frac{c_u}{c_{initial}} * 100\% \tag{12}$$

$$f_b\,[\%] = f_u\,[\%] - 100\% \tag{13}$$

c_u: Concentration in ultrafiltrate [μM or μg/mL]

$c_{initial}$: Concentration added [μM or μg/mL]

The fraction of non-specific adsorption (f_{ads}) is calculated as:

$$f_{ads}\,[\%] = \frac{\text{conc. in filtrat of adsorption test}}{c_{initial}} * 100\% \tag{14}$$

Determination of Mass Balance (MB):

$$MB\,[\%] = \frac{c_u * V_u + c_{ret.} * V_{ret.}}{c_{initial} * V_{initial}} * 100\% \tag{15}$$

V_u: Volume of ultrafiltrate [mL]

V_{ret}: Volume of retentate [mL]

$V_{initial}$: Initial volume [mL]

$c_{ret.}$: Concentration in retentate [μM or μg/mL].

As well, the evaluation of equilibrium association constant (K_A) and the determination of the number of protein binding per sites per class of binding site (n) is possible, if different concentrations of the drug are applied (see chapter "Evaluation of Binding Data").

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Rapid, efficient and simple technique
- Commercially available kits
- Lack of sample dilution and volume shifts
- No requirement of a non-physiological buffer
- Applicable for plasma and plasma fraction as well for other different types of biological matrices, including tissue homogenates
- If automated 96 well plate ultrafiltration system is used, high throughput with sample handling by robotic system.

Disadvantages

- Potential of non-specific binding (NSB) to filter membrane or plastic devices. Low recovery from either protein-filtrate or buffer indicates adsorptive losses and/or membrane rejection
- Leakage of bound drug through membrane
- Deviation of the drug protein binding equilibrium because of the change in the protein concentration, especially in case of high binding drugs. The concentration unbound may remain constant even though the proteins became concentrated in the upper reservoir as the amount of plasma water increases in the ultrafiltrate (Judd and Pesce 1982; Withlam and Brown 1981)

Ultrafiltration has been used to determine the protein bound fraction of many drugs, such as methadone (Wilkins et al. 1997), phenylacetate and phenylbutyrate (Boudoulas et al. 1996), etoposide (Robieux et al. 1997), doxorubicin and vincristine (Mayer and St-Onge 1995), disopyramide (Echize et al. 1995), and ketamine and its active metabolites (Hijazi and Boulieu 2002). Schumacher et al. (2000) have shown the applicability for the determination of erythrocyte/plasma distribution. The method of UF has been applied in the measurement of free unaltered thyroxin or after displacement by salicylate as well after displacement by heparin in healthy people and in patients with non-thyroidal somatic illness (Faber et al. 1993). The protein binding of tritium labeled, antidiabetic repaglinide and its displacement by warfarin, furosemide, tolbutamide, diazepam, glibenclamide and nicardipine were determined by ultrafiltration (Plum et al. 2000).

The reliability and applicability of the UF has been done in comparison to the classical method of equilibrium dialysis for several drugs, like thiopental (Christensen 1979), disopyramide (Norris et al. 1982), valproic acid (Barre et al. 1985).

Although different filter material procedures were applied in these studies the comparison gave comparable values for the free fraction of the drug for total concentrations between the two methods. Beside its use in in vitro assay, this method is widely applied for routine free drug monitoring of ex vivo PPB in clinical laboratories.

The implementation of new materials for filter membrane and plastics (e.g. polypropylene with PTFE) for the ultrafiltration units has diminished the disad-

vantages of non-specific binding (NSB) and the break trough of protein, respectively protein bound drug. Eight radiolabeled drugs (taxol, digoxin, prednisone, testosterone, warfarin, propranolol, methotrexate, ibuprofen and mannitol) were analyzed for their drug recovery The applied Ultracel YM membrane with a nominal molecular weight limit of 10 K Da exhibits less than 2 % NSB and > 99.5 % retention of serum proteins (Millipore, Application Note AN1735EN00). Although the improvement in the devices, the determination of the non-specific binding of new drug by recovery experiment is recommended and taken into account by a correction factor, if necessary.

MODIFICATIONS OF THE METHOD

Pre-treatment of the filter membrane (regenerated cellulose, molecular cut off 10 KDa) with 5 % Tween 80 or benzalkonium chloride showed significantly less non-specific binding for compounds that had a tendency toward high membrane binding (Lee et al. 2003).

With rising temperature the drug binding decreases. The determination can be performed either at room temperature (approx. 22 °C) or under more physiologically condition at 37 °C. The chosen temperature should be maintained in the plasma sample, and during the separation by pre-warming the rotor or using a heated centrifuge.

The UF can either performed in a single ultrafilter unit or in a 96 well plate ultrafiltration system, latter in a semi-automatic high-throughput determination with sampling handling by a robotic system (Fung et al. 2003; Jordan et al. 2000).

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II.F.2 Equilibrium Dialysis

PURPOSE AND RATIONALE

The equilibrium dialysis (ED) is based on the establishment of an equilibrium state between a protein compartment and buffer compartment, separated by a membrane, which is permeable only for a low-molecular weight ligand. Although there is no "standard method" for binding measurement, ED is often regarded as

the "reference method" for the determination of drug–protein-binding profile (Oravcová et al. 1996).

Equilibrium Dialysis (Individual Device)

Equipment & Material

- Fresh or frozen heparinized plasma of different species (pool), collected from fasted animals and humans). If frozen plasma is used, the pH value has to be adjusted (see adjustment of pH at chapter ultrafiltration)
- Stock solution of the radiolabeled, respectively non-radiolabeled drug prepared in an appropriate organic solvent (e.g. 10 mM ethanol)
- Phosphate buffered saline pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl)
- Equilibrium dialysis apparatus, e.g. variable speed dialyser Dianorm-4 (Dianorm Munich Germany). The dialyser is equipped with 1 mL macro cells (4.5 cm^2 working surface area) and cellulose dialysis membrane (63 mm diameter, molecular cut off weight 10 KDa) The apparatus is placed in a water bath with a circulating system to obtain a constant temperature of 37 °C in the system.

PROCEDURE

Preconditioning of Membrane: prior to dialysis, the membranes are pre-conditioned by soaking in water for 15 min, followed by soaking in PBS for 30 min. The cells are filled at ambient temperature, and when fully loaded the Dianorm apparatus is placed in a water bath at 37 °C and started at 12 rpm.

Determination of the time to equilibrium (ET): The ET is determined by dialyzing freshly spiked plasma containing the high (10 μg/mL) and intermediate (0.05 μg/mL) test concentrations against PBS. At time points 1, 2, 3, 4, and 6 hours of dialysis, a small sample/50 μL) of plasma and PBS is removed from each cell. And the concentration analyzed by LSC or LC/MS-MS. The optimal ET is then determined.

Equilibrium Dialysis: The ED is performed for five test concentrations (0.01, 0.05, 0.1, 1 and 10 μg/mL) for each of the test plasma samples against PBS. At each concentration, four cells, each containing 1 ml of spiked plasma and PBS, are prepared for each plasma type. After the time to reach the equilibrium (e.g. 4 hr), which was determined in the preliminary test, the dialysis is stopped. Aliquots of 200 μL plasma sample or PBS buffer are taken from each cell. To determine the concentration bound in plasma (c_b) or the free or unbound fraction (c_u) in the buffer the samples are analyzed by LC/MS-MS of LSC. The analytical method depends on the use of unlabeled or radiolabeled substance. The initial concentrations (c_{inital}) of each spiked plasma (aliquots of 200 μL) can also be determined.

EVALUATION

Final results are given as percentile fraction unbound (f_u) or percentile fraction bound (f_b) for a defined concentration.

$$f_u\,[\%] = \frac{c_u}{c_{initial}} * 100\% \tag{16}$$

or if $c_{initial}$ is not determined:

$$f_u\,[\%] = \frac{c_u}{c_u + c_b} * 100\% \tag{17}$$

$$f_b\,[\%] = \frac{c_b}{c_{initial}} * 100\% \tag{18}$$

$$f_b\,[\%] = f_u\,[\%] - 100\% \tag{19}$$

c_u: concentration in buffer [μM or μg/mL]

$c_u + c_b$: concentration in plasma [μM or μg/mL]

$c_{initial}$: concentration added [μM or μg/mL]

If different concentrations are applied, the evaluation of equilibrium association constant (K_A) and the determination of the number of protein sites (n) are possible (see chapter "Evaluation of Binding Data").

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Easy to set up and to use
- Temperature controlled.

Disadvantages

- Long time to reach equilibrium, up to 24 hrs, potential degradation of test substance as a result of thermal or metabolic instability in plasma
- Volume shift (Tozer et al. 1983)
- Donnan effects (Hindering of the passage of free ligand) (Mapleson 1987)
- Non-specific adsorption to dialysis apparatus and membrane (Henricsson 1987; Henry and Mitchell 1981)
- Overestimation of the free fraction resulting from slight leakage of protein (or fragments of it) into the dialysate. The absence of protein in the dialysate should be verified by protein assay (Bowers et al. 1984)
- pH shift during ED due to continuous loss of CO_2, need of adjustment and control (Brørs and Jacobson 1985)

24-Multiwell Plate Equilibrium Dialysis

Equipment & Material

- Freshly or frozen heparinized plasma of different species (pool), collected from fasted animals and humans. If frozen plasma is used, the pH value has to be adjusted (see adjustment of pH in the chapter ultrafiltration)
- Stock solution of the radiolabeled, respectively non-radiolabeled drug prepared in an appropriate organic solvent (e.g. 10 mM ethanol)
- Phosphate buffered saline pH 7.4 (0.1 M phosphate buffer/0.09 M NaCl)
- Incubator
- Plate shaker (orbital or reciprocating)
- Disposable 24-well BD Gentest Serum Binding System with 24 well dialysis insert, containing individual Spectrapor 2 membranes and 24 well BD Falcon Microplate (BD Biosciences Bedford MA USA Cat. No 453700)
- Single and multichannel pipetters
- Liquid Scintillation Counter (LSC) or LC/MS-MS-equipment.

Procedure

1. Preconditioning of Membrane: The plate is soaked for 20 min in de-ionized water thereafter in 30 % absolute alcohol in de-ionized water. Then the plate is washed with water and soaked for 20 min in PBS used for the dialysis experiment.
2. Test well: Bottom chamber: serum protein spiked with test compound; Top chamber: PBS buffer only
3. Control well: Bottom chamber: test compound in PBS buffer; Top chamber: PBS buffer only
4. The bottom chamber is loaded with 742.5 μL of plasma/serum protein for the test well, respectively PBS buffer for the control wells. To both 7.5 μL of 100 × concentrated compound (solved in DMSO) is added. To both top chamber of control and test well 250 μL of PBS buffer is added
5. The plate is shaken for 20 hours on medium setting. The shaker is placed in humidified incubator to reduce evaporation.
6. Aliquots (100 μL) from the upper chamber of the control and test wells are removed.
7. The concentration of test compound in the upper chamber of the test well ($c_{\text{test well upper}}$) and the concentration of compound in the upper chamber of the control well ($c_{\text{control well upper}}$) are analyzed by LC/MS-MS or LSC.
8. Alternatively, plasma protein binding can be determined without using a control well by comparing the concentration of compound in the upper chamber ($c_{\text{control well upper}}$) to the total concentration (sum of $c_{\text{control well upper}} + c_{\text{control well lower}}$). This approach conserves test compound, but requires an analytical technique that can measure the concentration of compound in high protein samples (plasma).

EVALUATION

Final results are given as percentile fraction unbound (f_u) or percentile fraction bound (f_b) for a defined concentration.

$$f_u\,[\%] = \frac{c_{(\text{test well})}}{c_{\,(\text{control well})}} * 100\% \tag{20}$$

Or (for single well approach, if control well is not applied):

$$f_u\,[\%] = \frac{c_{(\text{test well upper chamber})}}{c_{(\text{test well upper chamber})} + c_{(\text{test well lower chamber})}} * 100\ \% \tag{21}$$

$$f_b\,[\%] = f_u\,[\%] - 100\% \tag{22}$$

Also evaluation of equilibrium association constant (K_A) and the number of protein binding per sites per class of binding site (n) is possible if different concentration are applied (see chapter "Evaluation of Binding Data").

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Commercial available disposable 24-well system
- Easy set and use
- High throughput compatible with robotic systems
- Temperature controlled.

Disadvantages

- Long time to reach equilibrium, up to 24 h and more
- Volume shift
- Donnan effects (Hindering of the passage of free ligand)
- Non-specific adsorption to 24 well dialyse
- Overestimation of the free fraction as result from slight leakage of protein (or fragments of it) into the dialysate, the absence of protein in the dialysate should be verified by protein assay
- pH labiality during ED due to continuous loss of CO_2
- Potential degradation of test substance as a result of thermal or metabolic instability in plasma.

MODIFICATIONS OF THE METHOD

An alternative ED method in 96 well plate format has been reported by Kariv et al. (2000). The authors used a disposable equilibrium dialyser with a 10 KDa ultrathin membrane, co-developed with Amika Corp. (Columbia MD USA). The binding of three well-studied drugs, propranolol, paroxetine and losartan with low, medium, high binding properties, respectively were tested to validate the method. The data of free fraction correlated with the published values determined by conventional ED.

The vertical design of 96 well dialyze block of Banker et al. (2003) allows the robotic system the access to the sample and dialysate site. The dialysis block with partially separated bars is reusable. The validity of the system was tested with ten different standard compounds, in comparison to standard ED, and literature data.

To equalize the osmotic pressure and therefore to attenuate the volume shift, dextran 2.5 % (w/v) was added to the dialysate (Lima et al. 1983). However, this approach is inconvenient for high throughput. That the test compounds show no affinity to dextran has to be proven in an additional experiment.

Plum et al. (1999) showed the applicability of a step-wise ED for the plasma binding determination of the two strong adhesive ^{125}I-labeled proteins insulin aspart and insulin detemir.

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II.F.3 Ultracentrifugation

PURPOSE AND RATIONALE

The ultracentrifugation (UC) method achieves separation of free and protein bound drugs by centrifugation of the sample in a tube without a membrane. It makes use of the fact that sedimentation of solutes depends upon their molecular weight. The first time Steinberg and Schachmann (1966) demonstrated theoretically and practically the potential of the method.

PROCEDURE

Equipment & Material

- Freshly or frozen heparinized plasma of different species (pool), collected from fasted animals and humans. If frozen plasma is used, the pH value has to be adjusted (see adjustment of pH at chapter ultrafiltration)
- Stock solution of the radiolabeled, respectively non-radiolabeled drug prepared in an appropriate organic solvent (e.g. 10 mM ethanol)
- Centrifuge tubes (e.g. Ultra Clear tubes, Beckmann Palo Alto CA)
- Ultracentrifuge (e.g. Airfuge Beckmann Palo Alto CA)
- Single pipettes
- Liquid Scintillation Counter (LSC) or LC/MS-MS-equipment

Procedure

1. Plasma samples are spiked with labeled or unlabeled drug to give specific concentrations (ranging from pharmacological to toxicological relevant concentrations). An aliquot (200 μL) is either transferred to LSC or LC/MS-MS to quantify the initial concentration ($c_{initial}$).
2. The centrifuge tubes are filled with identical volume (200 μL) of spiked plasma and loaded into the rotor of the ultracentrifuge. The cooled sample is centrifuged for 4 hr at an air pressure of 30 psi and 150 000 × g. The sample is cooled during and after the centrifugation to 4 °C to avoid thermodynamic interferences.

   The time needed to obtain a “protein free” top layer should be determined In a series of preliminary

experiments. Therefore, the centrifuge is loaded with a spiked plasma sample. The samples are spun at 150 000 × g and the Airfuge stopped at different time intervals. An aliquot of 20 µL top layer is removed and the concentration is measured. The time where the concentration is constant is chosen for the experiment.

3. From the supernatant consecutive aliquots of 20 µL are taken without disturbing the remaining plasma sample. The concentrations of the top layers are quantified in the same manner like $c_{initial}$ by means of a calibration curve.

EVALUATION

After the measurement of the concentration of the individual consecutive aliquots, the mean ($c_{supernatant}$) of the constant values are chosen. A significant higher amount in the first aliquot indicates a binding to lipoproteins.

Final results are given as percentile fraction unbound (f_u) or percentile fraction bound (f_b) for a defined concentration.

$$f_u\,[\%] = \frac{c_{(supernatant)}}{c_{(initial)}} * 100\% \qquad (23)$$

$$f_b\,[\%] = f_u\,[\%] - 100\% \qquad (24)$$

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Alternative method to ultrafiltration and equilibrium dialysis
- Elimination of the problems associated with free membrane effects like fewer non specific binding (NSB)
- No dilution of the sample, native status of plasma.

Disadvantages

- High cost of ultracentrifuge
- Time consuming (up to 18–24 hours centrifugation time)
- Limited number of sample can be spun simultaneously
- Intricate manual process, no automation applicable
- Interferences according sedimentation, thermal back diffusion, viscosity and floating of lipoprotein in the supernatant fluid
- Self-sedimentation of the test compound with molecular weight > 300 Da.

Ultracentrifugation (UC) is an alternative to both equilibrium dialysis and ultrafiltration and enables the separation of the free and protein fraction in a more physiological environment. The UC is a method of choice for compound with a high affinity to non-specific binding. It eliminates the problems associated with free membrane effects. Without the necessity to add a buffer system, a dilution observed at the equilibrium dialysis is avoided.

However several comparative studies showed quantitative discrepancies between the results obtained by UC compared to ultrafiltration, respectively equilibrium dialysis (Barre et al. 1985; Kurz et al. 1977). Verbeeck and Cardinal (1985) investigated the plasma protein binding of phenytoin, salicylic acid, propranolol, phetidine and chlorpromazine using UC. The binding data was comparable to those obtained by equilibrium dialysis, except for chlorpromazine. The latter has been explained by the author in relation to the binding of the compound to a very low-density lipoprotein fraction, which floats after the centrifugation step. A micro scale UC was evaluated by Nakai et al. (2003). It showed a good correlation of the protein binding of 10 compounds, with reported values determined by ultrafiltration and equilibrium dialysis.

MODIFICATIONS OF THE METHOD

Depending on the available ultracentrifuge different relative centrifugation forces have been used, ranging from 120 000 g up to 436 000 g (Nakai et al. 2003). Depending on the affinity of the test substrate for non-specific binding different material of ultracentrifuge tubes can be applied.

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II.F.4 Binding on Silica Beads Immobilized Protein Fraction

PURPOSE AND RATIONALE

This method is based on the affinity of the new chemical entity to a membrane, consisting of human serum albumin immobilized on Transil beads. The silica beads are coated with egg yolk phosphatidylcholine and are commercially available under the trademark Transil from Nimbus (Nimbus Leipzig Germany).

PROCEDURE

Equipment & Material

- Stock solution of drug prepared in an appropriate organic solvent, diluted to appropriate concentration (e.g. 10 μg/mL). The amount of the solubility modifier should be below 5 % in the final concentration
- Transil "Ready to go plates" for 10 compounds per plate; for high precision assay, order no.: SP200096 (Nimbus Biotechnology Leipzig Germany)
- UV-translucent plate (suitable for UV-measurement above 250 nm)
- Multichannel pipette
- UV plate reader and/or LC/MS-MS (needed for quantification of UV inactive compounds)
- Centrifuge with rotor for 96 well plate or vacuum filtration device.

Procedure

1. The plates are delivered in frozen state filled with a total volume of 270 μL of buffer for control well (column 1 and 7) and 270 μL of buffer/varying amounts of Transil for sample well. The plates are slowly defrosted immediately before measurement.
2. To each well (control and sample) 30 μL of compound solution has to be added. If UV spectroscopy method is used for quantification the wells H7–H12 have to use as reference sample to account for the absorbance of the UV-plate (not necessary for LC/MS-MS).
3. Re-suspend the liquid at least three to six time by swirling the beads around using a multichannel pipette to ensure complete mixing of the compound and the beads.
4. Separate the unbound fraction by short low speed centrifugation (15 min/10 000 g). Alternatively a vacuum filtration with collector plate can be applied.
5. Transfer a fixed volume (100 μL) of the filtrate into a UV-translucent plate.
6. UV-plate reader and/or LC/MS-MS determine the concentrations of the unbound fraction at varying HSA concentration.

EVALUATION

The data is transferred into an Excel workbook supplied by Nimbus to conveniently calculate the dissociation constant (K_D).

In the high affinity assay for each compound, five assay points are generated and evaluated via a Scatchard type analysis using a linearized binding equation.

Rearrangement of

$$c_b = \frac{n * P * K_A}{1 + K_A * c_u} \tag{25}$$

with $K_A = 1/K_D$ leads to the dissociation constant (K_D)

$$K_D = c_u * \frac{1 - \frac{c_b}{P}}{\frac{c_b}{P}} \tag{26}$$

with the assumption $c_{protein} \gg c_b$ simplifies equation 26 to

$$K_D = \frac{P * c_u}{c_{initial} - c_u}. \tag{27}$$

This condition is valid for compounds with low and medium affinity also compound concentrations larger than the protein concentration. Otherwise equation 26 has to be used, where the total compound concentration $c_{initial}$ has to be determined.

The bound fraction f_b is calculated by equation 28.

$$f_b = \frac{c_b}{c_{initial}} = 1 - \frac{1}{1 + P/K_D} \tag{28}$$

P: Concentration of the protein in the blood. For HSA it is usually 40 g/L, corresponding to 588 μM.

CRITICAL ASSESSMENT OF THE METHOD

Advantages

- Fast and easy, high throughput assay, applicable to a robotic systems in 96 or 386 well plate format
- Simple quantification by hints of UV-plate reader, MS measurement is only needed for UV-inactive compounds
- "Ready to go plates" are commercially available
- Determination of dissociation constant K_D and fraction bound
- Small amount of compound is needed
- Low influence of non specific binding for the determination of K_D.

Disadvantages

- Only applicable to determine the binding to human serum albumin
- For UV-inactive compounds LC/MS-MS is needed; which diminishes the throughput.

MODIFICATIONS OF THE METHOD

Schuhmacher et al. (2003) published a method, where Transil beads in suspension has been used for the determination of protein binding in diluted plasma and plasma/erythrocyte distribution for strongly bound compounds. For HTS of the method 96 well plates with removable glass inserts filled with Transil suspension were applied. The comparison with the classical methods ultrafiltration and equilibrium dialysis showed for eight compounds comparable precision and accuracy of the binding parameters.

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Chapter II.G
Perfused Organs

Andreas W. Herling

II.G.1
In Situ-perfused Isolated Intestinal Segments and Bile Secretion in Anaesthetized Rats

PURPOSE AND RATIONALE

The gut technique of in situ-perfused isolated intestinal segments in rats is used to determine the rates of intestinal absorption or intestinal secretion of a candidate compound, as well as in combination with a bile fistula to detect the hepatobiliary elimination of a candidate compound. By perfusion of intestinal segments (duodenum, proximal and distal part of the jejunum, and ileum) the site of intestinal absorption or secretion of a candidate compound can be determined.

PROCEDURE

In general, rats are fasted overnight and are anesthetized with an intraperitoneal injection of 60 mg/kg pentobarbitone and anesthesia is maintained by a s.c. infusion of pentobarbitone at a rate of 20 mg/kg/h for the desired length of the perfusion experiment. Animals are laparotomized by a median incision and the common bile duct is cannulated with a polyethylene (PE) 10 catheter. Duodenum, or jejunal segments, or the ileum are cannulated for single pass perfusion or recirculated perfusion. After flushing the lumen of the respective intestinal segment with 40 ml of medium, perfusion is performed in situ at a very low continuous flow rate of 0.5 ml/min, ensuring a minimal perfusion pressure. The candidate compound is added to the perfusion medium at an appropriate concentration to reach detectable compound levels in blood (plasma) and bile. Body temperature and perfusion medium are maintained at 37 °C. Bile aliquots are collected at 10 to 30 min intervals. Blood samples (100 µl) are collected from the jugular vein every 30 minutes in EDTA tubes. At the end of the perfusion experiment the livers are removed from all animals, total weight is determined and the livers are immediately frozen for determination of tissue level of the compound in liquid nitrogen and then stored at –20 °C until analysis. For analysis of tissue levels of the candidate compound the frozen livers are homogenized in water (1 g tissue in 1 ml water (or solvent to extract the candidate compound from the tissue)) using an ultra-turrax. A continuous bile flow throughout the experiment is the best measure of the functionally integrity of the organ (typically bile flow are shown in Fig. 1). Animals with an initial bile flow below 100 µl/30 minutes should be excluded from the final evaluation of the data.

EVALUATION

The concentrations of the compound are determined using appropriate analytical methods for the respective candidate compound in the perfusate at the end of the perfusion experiment, plasma, bile and hepatic tissue. From the data the site of intestinal absorption using different perfused intestinal segments in separate experiments as well as the degree of hepatobiliary elimination can be estimated.

Appropriate analytical methods with sufficient sensitivity are used for detection of the candidate compound in the perfusate, plasma, bile and liver tissue. From the total amount of the candidate compound in the perfusate of the respective intestinal segment at the end of the perfusion experiment in relation to the total added amount of compound at time 0, the total enteral absorption rate can be estimated. The decline in the concentration of the candidate compound in the perfusate is a measure of the enteral absorption, the uptake, metabolism and elimination of the compound by the liver. The concentration in the plasma is a measure of the bioavailability. The appearance of the candidate compound in the bile is a measure of the hepatobiliary elimination of the compound. The tissue level of the candidate compound is a measure of the uptake and

accumulation of the candidate compound in the liver. Ideally the analytical method for the detection of the candidate compound included also the detections of known metabolites of the candidate compound in plasma, bile and liver tissue.

In addition the total hepatobiliary elimination of the candidate compound (sum of all collecting intervals (concentration × secreted volume per collecting interval)) can be calculated.

MODIFICATIONS OF THE METHOD

Various modifications are reported with respect to the experimental setup (single pass or recirculated intestinal perfusion) as well as the site of blood collection, e.g. mesenteric vessels for estimation of the intestinal absorption rate (DeGraw RT, Anderson BD 2004). vs. peripheral veins for estimation of systemic availability of the candidate compound. This method is widely used for investigation of intestinal absorption of nutrients by using radioactive tracers (e.g. cholesterol, glucose) and their interference with the candidate compound (Arts et al. 2004). In addition the secretion of the candidate compound into the intestine can be studied by peripheral administration of the compound into a peripheral vein and subsequent determination of the appearance of the candidate compound in the intestinal perfusate (Merino et al. 2003; Berggren et al. 2004). Also variations are reported using chronically isolated intestinal loops in rats (Poelma et al. 1992).

CRITICAL ASSESSMENT OF THE METHOD

During anesthesia intestinal motility is dramatically reduced, which secondarily might influence the intestinal absorption of the candidate compound. Bile flow is relatively constant during the first 2 to 3 hours but declined during prolonged anesthesia (Fig. 1) due to the interruption of the enterohepatic circulation of bile acids.

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EXAMPLE

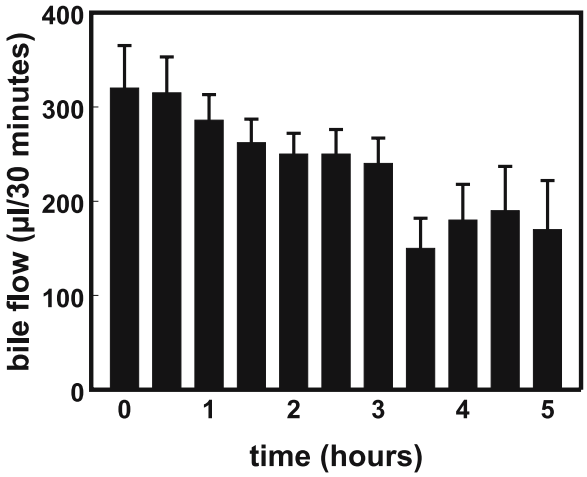

Fig. 1. Representative time-course of bile flow in anesthetized rats with separate drainage of the bile. Values are mean +=– SEM, n = 4.

II.G.2 Isolated Perfused Livers

PURPOSE AND RATIONALE

Isolated perfused rat livers are used for physiological and pharmacological purposes to study intermediary metabolism of carbohydrates and lipids (e.g. gluconeogenesis, glycogenolysis, ketogenesis). This isolated organ preparation with separated cannulated bile duct can ideally be used for investigations of pharmacokinetic and of drug metabolism by elucidation of hepatic uptake and drug metabolic stability and its hepatobiliary elimination. The advantage of this perfused organ preparation over isolated hepatocytes is the maintained architecture of the hepatic structure (perivenous–periportal orientation).

PROCEDURE

Rats with a body weight between 250–300 g are anaesthetized with pentobarbital sodium (60 mg/kg i.p.). The liver is exposed by longitudinal midline and transverse subcostal incisions, and the bile duct is can-

ulated as well as the portal vein with a venous cannula. The liver is immediately infused via the portal vein at 37 °C with oxygenated saline containing heparin (70 units/ml). The Vena cava caudalis is opened to allow a continuous flow of the saline/heparin solution for about 2 minutes. Then the liver is transferred into a heated (37 °C) perfusion chamber and perfused via the portal vein in a recirculating manner at a constant flow rate of 35 ml/min with continuously oxygenated Krebs-Ringer-Bicarbonate buffer (100 ml total volume). The buffer consists of 137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.72 mM NaH_2PO_4, 1.8 mM $CaCl_2$ and 0.5 mM $MgCl_2$ with 10 mM glucose, and is supplemented with 30 % (v/v) washed bovine erythrocytes and 1.6 % (w/v) bovine serum albumin. Experiments are routinely carried out on four livers simultaneously, and they are perfused for 2 hours as total perfusion time for each liver.

The candidate compound is added to the perfusate before the isolated liver is transferred to the perfusion setup. After 5 minutes recirculation in the system without the organ a sample of the perfusate is collected for determination of the starting concentration of the candidate compound (value at time 0). Then the isolated liver is connected to the perfusion chamber and subsequently samples of the perfusate are taken in 10 to 15 minute intervals as well as bile samples are taken in 15 minutes intervals for the determination of compound levels. The volume of excreted bile per 30 minutes is determined gravimetrically (difference between tube weight without and with bile per collection period) with the assumption that 1 g is equivalent to 1 ml of bile. If radioactive-labelled candidate compounds are used the intervals for bile collection can be much shorter (1 to 2 minutes). The liver is perfused for 2 hours and at the end of the perfusion experiment the liver is removed and immediately frozen for determination of tissue level of the compound in liquid nitrogen and later on stored at –20 °C until analysis. For analysis of tissue levels of the candidate compound the frozen livers are homogenized in water (1 g tissue in 1 ml water (or solvent to extract the candidate compound from the tissue)) using an ultra-turrax.

Samples for determination of lactate dehydrogenase activity (LDH) are taken every 30 minutes and they are a measure for the viability and integrity of the isolated organ. A continuous bile flow throughout the perfusion time is the best measure of the functionally integrity of the organ (typically bile flow of four livers are shown in Fig. 2). Isolated livers without bile flow should be excluded from the final evaluation of the data.

EVALUATION

Compound levels in samples of the perfusate and bile are measured using appropriate analytical methods for detection of the candidate compound. The decline in the concentrations of the compound in the perfusate is a measure of the uptake, metabolism and elimination of the compound by the liver. The appearance of the candidate compound in the bile is a measure of the hepatobiliary elimination of the compound. The tissue level of the candidate compound is a measure for the uptake and accumulation of the candidate compound in the liver. Ideally the analytical method for the detection of the candidate compound included also the detections of known metabolites of the candidate compound in perfusate and bile.

From the total amount of the candidate compound and metabolites in the perfusate (100 ml) at the end of the perfusion experiment (after 2 hours) in relation to the total added amount of compound at time 0, the total uptake into the liver can be calculated. In addition the total hepatobiliary elimination of the candidate compound (sum of all collecting intervals (concentration × secreted volume per collecting interval)) can be calculated. From these values together with the remaining tissue concentration a balance calculation can be set up and the proportion of compound metabolism can be estimated. Representative data are shown for a candidate compound with a high first pass effect in Figure 3.

CRITICAL ASSESSMENT OF THE METHOD

Using an erythrocytes-containing medium for perfusion one has to take into account the putative involvement of the erythrocytes themselves with respect to uptake of the candidate compound. Therefore, not only the erythrocyte-free perfusate but also the erythrocytes fraction should be included in the analysis of the candidate compound, separately. In our hands the model of isolated perfused liver is metabolically active for up to 3 hours and during this time no decline in hepatic metabolic activity becomes obvious. However, bile flow declined during the perfusion experiments. Therefore, our total perfusion time of isolated livers in our standard experimental setup is limited to 2 hours. The tissue level of the candidate compound analyzed after 2 hours in the liver is a measure for the total amount of compound in the whole organ. This does not necessarily mean the presence of the candidate compound in hepatocytes but additionally in the capillary and biliary space of the liver.

MODIFICATIONS OF THE METHOD

This method has been widely used for studying carbohydrate and lipid intermediary metabolism (Herling et al. 1998) as well as drug metabolism (Milne et al. 1997 and Milne et al. 2000, Vuppugalla 2004). Many variations have been reported predominantly with respect to the animal species used. Chaib et al. (2004) compared isolated perfused livers of rats with those of guinea pigs. Den Butter (1994) used livers from rabbits. Further modifications are related to the direction of perfusion via hepatic artery or portal vein or both simultaneously or in connection with the isolated jointly perfused small intestine (Stumpel et al. 1997 and Stumpel et al. 2000) as well as the continuous perfusion in a recirculated (see above) or open (non-recirculated) manner (Lopez et al. 1998).

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EXAMPLE

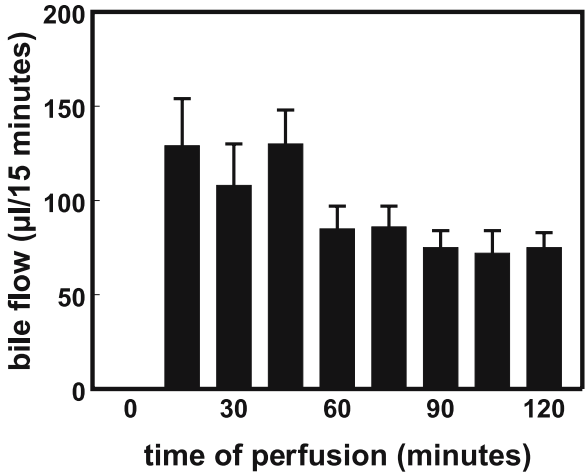

Fig. 2. Representative time-course of bile flow of isolated perfused rat livers. Values are mean +/– SEM, n = 4.

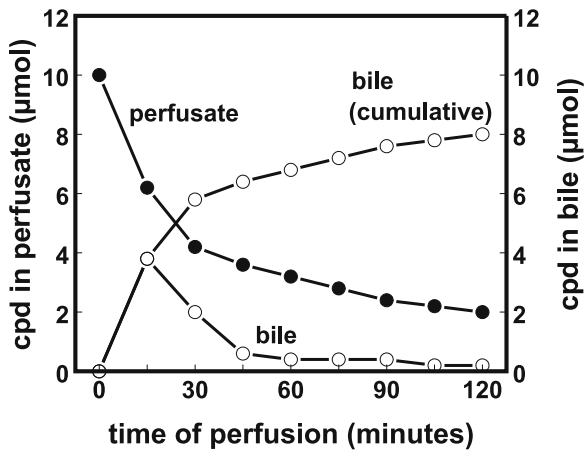

Fig. 3. Example for the time-course of a compound with a high first pass effect. The values for the perfusate represent the total amount of compound in the total volume of perfusate (100 ml; nmol/ml × 100). The values for the bile represent the total excreted amount of compound in the bile per sampling period of 15 minutes (µmol/ml × bile volume per 15 minutes) and were expressed as the values measured per sampling interval and as cumulative excreted into the bile.

II.G.3 Isolated Perfused Kidneys

PURPOSE AND RATIONALE

Isolated perfused rat kidneys are used for physiological and pharmacological purposes to study overall renal functions. For pharmacokinetic purposes this preparation is used to study renal excretion profiles of the candidate compound and/or its metabolites.

PROCEDURE

Karpf et al. (2003) used isolated kidney from rats. Rats are anaesthetised with an intraperitoneal injection of 60 mg/kg sodium pentobarbitone before surgery. The isolated kidneys are perfused in a recirculating manner in a thermostatically controlled cabinet maintained at 37 °C. An erythrocyte-free modified Krebs ± Henseleit bicarbonate buffer solution containing bovine serum albumin, glycine, L-cysteine, L-glutamic acid and glucose in a reservoir is pumped through the kidney via an in-line filter (8 μm), a membrane oxygenator, glass bubble trap, flow meter, and finally a glass arterial cannula inserted into the renal artery, and returned to the reservoir. Throughout the perfusion, carbogen is supplied to the membrane oxygenator to maintain a constant concentration of oxygen in the perfusate at a value greater than 0.6 mM and the pH was maintained at 7.4. The viability of the kidney is monitored by measuring glomerular filtration rate (GFR), and the reabsorption of water, sodium and glucose. The organs are considered viable if the respective values are greater than 0.4 mL/min, 75 %, 95 %, and 99 %, respectively.

Urine is collected over successive 10 min intervals (10–20, 20–30, 40–50, 50–60, 70–80 and 80–90 min) from the start of the perfusion; samples of the perfusate are collected from the reservoir at the midpoint times of these intervals.

EVALUATION

Compound levels in samples of the perfusate and urine are measured using appropriate analytical methods for detection of compound levels. The decline in the concentrations of the candidate compound in the perfusate is a measure of the uptake, metabolism and elimination of the compound by the kideny. The appearance of the candidate compound in the urine is a measure of the renal elimination (glomerular excretion and tubular secretion) of the compound. Ideally the analytical method for the detection of the candidate compound included also the detections of known metabolites of the compound in perfusate and urine.

CRITICAL ASSESSMENT OF THE METHOD

The isolated perfused kidney technique has been used for decades to investigate aspects of renal physiology, pharmacology, and pharmacokinetics. The model used most extensively is the isolated perfused rat kidney continuously perfused with medium containing electrolytes, glucose, amino acids and the oncotic agents, bovine serum albumin (BSA), or dextran. The oncotic agents included in perfusate create 'physiological' colloid osmotic pressure, which is one of the key factors to regulate glomerular filtration rate (GFR), tubular reabsorption of water and sodium, and water content in kidney interstitial tissue. BSA is the oncotic agent used most commonly in the isolated perfused rat kidney and affords reasonably stable isolated perfused kidney function. Perfusate BSA concentrations used in the past have varied from 20 to 80 g/l. Generally, the higher the BSA concentration in perfusate, the higher the extent of reabsorption of water and sodium, but at the expense of lower GFR and urine flow rate. Thus, it is necessary for investigators to weigh the balance between GFR and the extent of reabsorption of water and sodium. Practically, 60–65 g/l BSA in perfusate has been used by most investigators employing the isolated perfused kidney.

MODIFICATIONS OF THE METHOD

Wang et al. (2004a,b) used isolated kidneys from rats after removal from anaesthetized (sodium pentobarbitone (60 mg/kg)) animals. Briefly, a midline laparotomy incision was made from pelvis to sternum on the animal. Next, the right ureter was ligated immediately proximal to the bladder. A solution of mannitol (150 mg) and heparin (100 U) in 1 ml normal saline was injected into the penile vein. The right ureter was then cannulated by the introduction of a cannula consisting of a 200 mm length of tubing (od=0.61 mm, ID = 0.28 mm, Paton Scientific, Victor Harbour, Australia). The area around the anastomosis of the superior mesenteric artery, renal artery, and aorta was cleared of connective tissue and loose ligatures placed around the superior mesenteric artery and renal artery. After removing the capsule of the kidney, a right-angled glass cannula was inserted into the superior mesenteric artery via a small incision, and passed proximally along the superior mesenteric artery, across the aorta and into the renal artery where it was tied in place. Perfusate flow was commenced immediately. The cannulated kidney was then excised from the body of the rat and suspended within a thermostatically controlled cabinet at 34–37 °C. The recirculating perfusion medium (160 ml) was pumped at approximately 36 ml/min, which maintained renal artery perfusion pressure at 110 ± 25 mm Hg, which was monitored by a manometer, corrected for the intrinsic pressure of the apparatus. The functional viability of each kidney was assessed by the glomerular filtration rate (GFR), which was determined as the renal clearance of [^{14}C]-inulin, urine flow and the percentage tubular reabsorption of water, glucose, and sodium.

Urine samples were collected in 10 min intervals over the 10 to 130 min period after the bolus. Urine

volume was measured gravimetrically in preweighed collection vials. Perfusate samples were collected from the reservoir at the midpoint of each urine collection interval; appropriate amounts of perfusate and of urine were taken for further analysis.

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Wang J, Nation RL, Evans AM, Cox S (2004) Isolated rat kidney perfused with dextran and bovine serum albumin: a stable model for investigating renal drug handling. J Pharmacol Toxicol Methods 49:105–113

Chapter II.H
Metabolism Studies in vitro and in vivo

Angela Dudda
Gert Ulrich Kürzel

INTRODUCTION

In the era of combinatorial chemistry and high throughput screening, a huge number of hits and structural analogs potentially interesting as new chemical entities (NCEs) can be produced in a short period of time. Drug metabolism is a decisive determinant of the pharmacokinetic behavior of these compounds. Approximately three quarters of the top 200 prescribed drugs in the United States in 2002 are cleared by metabolism, one third are cleared via the kidney, while biliary clearance of unchanged drug plays only a minor role (Williams et al. 2004).

In vitro biotransformation tests are one piece of the puzzle to understand the pharmacokinetic characteristics of a given compound, to optimize PK parameters and to select the most drug like compounds that will progress into development (Eddershaw 2000; Li 2004; Masimirembwa et al. 2003). Relatively recent prospect of obtaining equivalent data from in vitro and in vivo studies has provided the pharmaceutical industry with an incentive to validate in vitro models with respect to increase throughput and/or to replace animal studies where appropriate. More over, in vitro test systems are the only humanized models in early development (Coleman et al. 2001). An early assessment using animal in vitro and in vivo data together with human in vitro data allows a qualitative prediction whether humans will act in similar (path-) ways as did the animal models (Figure 1).

In vivo biotransformation studies play a role later in development in both, animals and humans (Gupta and Atul 2000; Inskeep and Day 1999; Pool 1999). Use of transgenic animals facilitates understanding the role of drug metabolizing enzymes in the organism (Gonzalez and Kimura 2003). However, animal studies cannot entirely replace clinical studies in predicting all responses in human, but, for ethical reasons, the risk to human volunteers participating in early clinical studies should be minimized (Cross and Bayliss 2000). This is supported by a variety of in vitro metabolism studies.

Metabolic stability tests can be performed in higher through-put (White 2001). They allow ranking of compounds and ensure that the molecules resulting from the optimization process retain favorable metabolic properties. In addition, they give rise to set up computational models predictive for the in vitro test which helps to speed-up the selection and optimization processes although applications on the biotransformation of drugs are still limited (Li 2002; Gombar et al. 2003; Yu and Adedoyin 2003; Bugrim et al. 2004; see also chapter In silico approaches).

Toxicologically relevant biotransformation ("hot spots") and positions of metabolic attack ("soft spots") can be identified by structure elucidation of metabolites generated in vitro to support medicinal chemists in improving metabolic characteristics (Nassar and Talaat 2004b). In addition, structure elucidation of metabolites generated in vitro plays an important role because in vivo elucidation affords intense clean-up procedures besides the higher effort necessary to generate the samples.

Generation of metabolic profiles especially by use of radiolabeled test compounds in preclinical development makes sure that the animal model allows a qualitative and/or quantitative prediction to human. This is crucial for proof of validity of pharmacological and toxicological data obtained in animal models for humans.

In vitro metabolism studies are recognized as an important tool for predicting drug-drug interactions (FDA 1997 and 1999, EMEA 1997, Tucker et al. 2001) and

variability in exposure due to pharmacogenetic differences in the population. Besides studies on enzyme inhibition (see chapter Enzyme Inhibition) and induction (see chapter Enzyme Induction) information has to be generated on the enzymes involved in the biotransformation of a drug particularly for drugs which are subject to high metabolic clearance in the liver.

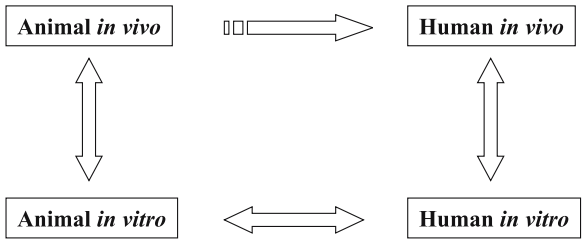

Fig. 1. Qualitative prediction of compound properties in humans from animal models using in vitro and in vivo data.

Essential part of submission dossiers is the elucidation of the specific enzyme(s) responsible for a certain metabolic step. A stepwise approach is recommended comprising of correlation analyses in individual livers, incubations in recombinant enzymes and incubation in human liver microsomes using inhibitors or antibodies specific for the particular isozyme (Wienkers et al. 2003; Lu et al. 2003).

Metabolism of many xenobiotics, including drugs and toxic compounds, occurs mainly in the liver (De Kanter 1999). Until now, in vitro studies on the metabolism of xenobiotics were usually performed using liver preparations such as isolated perfused livers, liver slices, liver homogenates, isolated hepatocytes, subcellular liver fractions (S9, cytosol, microsomes) or overexpressed recombinant metabolizing enzymes, particularly cytochrome P450 isozymes. The decreasing order of tissue organization goes in parallel with the technical effort of using these models (Figure 2).

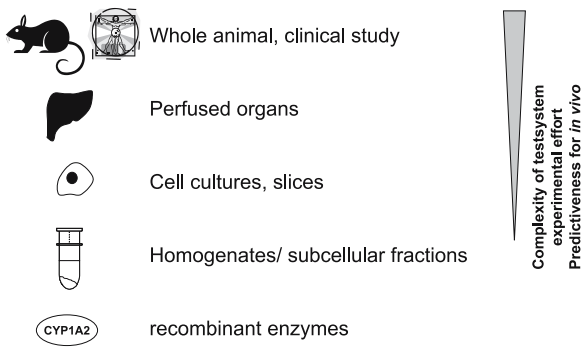

Fig. 2. Test systems for studying drug metabolism.

Each of these in vitro systems have their specific values and limitations regarding availability of tissues, costs, completeness of enzymatic capability, ease of handling, sensitivity to population differences (Plant 2004). Pros and cons have to be considered under the specific application intended e.g. if higher through-put even in an automated environment is needed at the early stage of drug discovery support or if regulatory aspects are concerned. A comparison between the various systems to study the metabolism of a drug in vitro is given in Table 1 based on a review recently published (Plant 2004; see also Brandon, 2003).

Whereas sophisticated studies require the technology to be available in-house in preparation e.g. of slices or performing an organ perfusion study (see also chapter on Perfused Organs), others can be performed with cells (Li 1999) or fractions commercially available or easily prepared. A typical preparation scheme for preparation of subcellular fractions by differential centrifugation is given in Figure 3.

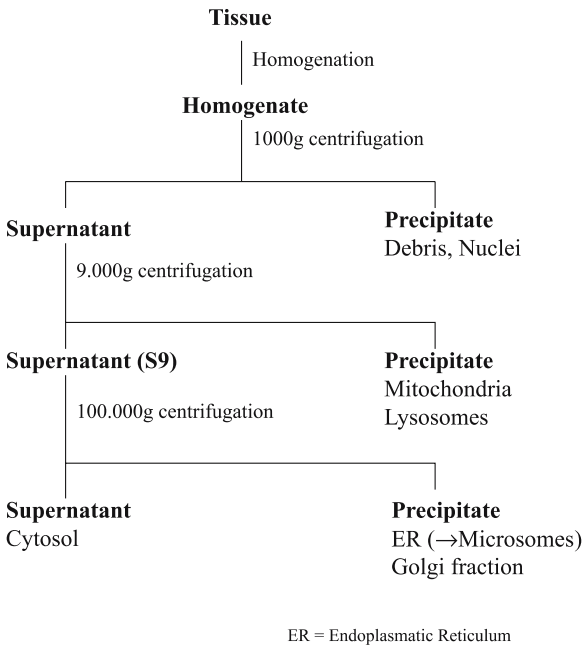

Fig. 3. Preparation of subcellular organ fractions by differential centrifugation (according to Ekins et al. 1999).

The biological function of metabolic transformation is to increase the water solubility of a drug. Typically, metabolism is divided into two steps: firstly, named Phase I, functionalization of the molecule takes place leading to introduction or liberation of polar groups like alcohols, phenols and amines by hydroxylation, desalkylation and heteroatom oxidation. Also, esters and amides are hydrolyzed.

Table 1 Comparison of in vitro test systems to study biotransformation (in part from Plant 2004).

In vitro System	Pros	Cons
Perfused organs	Phase I and II present, whole metabolic profile observed, best correlation to in vivo	expensive, ex vivo animal trial, complex methodology, high technical effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Slices	phase I and II present, whole metabolic profile observed, good correlation to in vivo	expensive, ex vivo animal trial, diffusion controlled, complex methodology, high technical effort, batch variability, more complicated than enzyme-only system, quality control, limited use for multiple compounds
Cells in primary culture (e.g. hepatocytes)	Phase I and II present, whole metabolic profile, Induction modeled, population pools for cryopreserved hepatocytes possible, good correlation to in vivo	expensive, batch variability, quality control, complex methodology, high technical effort, limited use for multiple compounds
S9 fraction	easy to use,cheap, phase I and II present, whole metabolic profile observed	addition of cofactors (complex mixtures), lower enzyme activity than microsomes/supersomes, induction not modeled
Microsomes	easy to use, cheap, Population pools	addition of cofactors (simple mixtures), Only membrane-bound metabolizing enzymes such as CYPs, FMOs and UGTs partial metabolic profile, induction not modeled
Cytosol	easy to use, cheap	addition of cofactors (simple mixtures), Only not membrane-bound metabolizing enzymes such as alcohol dehydrogenases, sulfotransferases, glutathione S transferase, N-acetyl transferases partial metabolic profile, induction not modeled
Supersomes	easy to use, moderately cheap, no addition of co-factors, single enzyme only	currently only CYPs, FMOs, UGTs, GSTs and SULTs, single enzyme only accuracy of kinetics?, induction not modeled

Phase I metabolites, together with unchanged parent compound, are excreted via bile and urine, if sufficient solubility and/or transporter specificity is given. In a second step, conjugation reactions often increase polarity even more by glucuronidation, sulfation, or glutathione conjugation (Phase II).

Enzymes responsible for these biotransformation reactions are present in many organs and tissues, the most important one in general being the liver. Under the enzymes involved in the biotransformation of drugs the cytochrome P450 superfamily plays the most important role followed by involvement of glucuronidases and esterases (Wrighton and Stevens 1992; Donato and Castell 2003; Kumar and Surapaneni 2001; Williams et al. 2004). The relative contribution of metabolic clearance pathways and the role of P450 isozymes to the biotransformation of drugs in shown in Figure 4 (Williams et al. 2004).

Cytochrome P450s are present in the endoplasmatic reticulum and therefore present in microsomal preparations. An overview on isoforms, polymorphisms, substrates, inhibitors, inducers and occurrence of cytochrome P450s is given in Table 2.

As total inhibitors of the P450 enzyme family, 1-Aminobenzotriazole (Lee and Slattery 1997) and Proadifen (SKF525A) (Lee et al. 1998) are suitable to distinguish from non-cytochrome P450 mediated pathways.

Also present in microsomes are flavin-containing monooxygenases (FMO) involved in oxygenation of heteroatoms like nitrogen and sulfur (Lang and Kalgutkar 2003). 5 different isoforms of FMO are currently characterized. In adult human liver FMO1 and FMO3 play an important role. Selective marker substrates for FMO1 are imipramine and orphenadrine. Methimazole inhibits FMO dependant pathways (Wynalda 2003) but also CYP2B6, CYP2C9 and CYP3A4.

Monoamine oxidases (MAO) are present in the mitochondria and are involved in oxidation of endogenous and exogenous amines (Lang and Kalgutkar 2003).

Carbonyl reductases and alcohol and aldehyde dehydrogenases are cytosolic enzymes being involved in the oxidation of alcohols and aldehydes and in the reduction of aldehydes and ketones (Lang and Kalgutkar 2003).

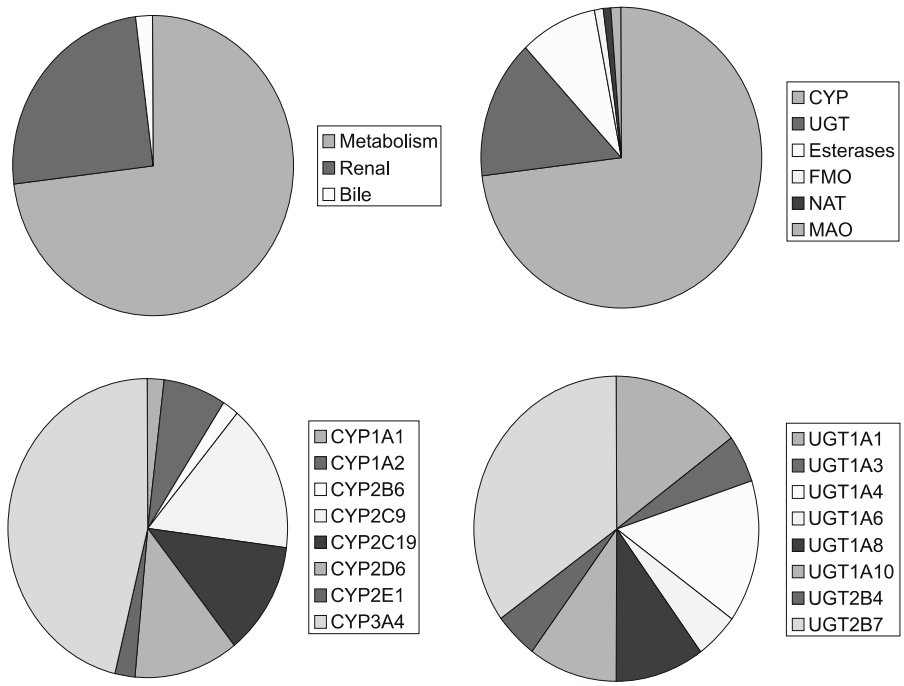

Fig. 4. Importance of clearance mechanism and the relative role of drug metabolizing enzymes (Williams et al. 2004).

Uridine diphosphoglucuronosyl transferases (UGT) are the most prominent enzymes within the phase II enzymes present in microsomes (Coughtrie and Fisher 2003). An overview on isoforms, substrates and tissue expression is given in Table 3. UGT2B7, UGT1A1 and UGT1A6 are responsible for glucuronidation of two third of the top 200 prescribed drugs in the United States in 2002. (Williams et al. 2004). Of clinical consequence for the conjugation of bilirubin are the deficiencies of UGT1A1 in patients suffering of hyperbilirubinemia (Crigler-Naijar and Gilbert's diseases). UGTs are involved in the formation of O-, N- and S-glucuronides. Formation of acylglucuronides is considered to be of potential risk as they can form reactive intermediates (Shipkova et al. 2003).

Sulfotransferases located in the cytosol are involved in the sulfation (Coughtrie and Fisher 2003). An overview on isoforms and their characteristics is given in Table 4.

Other important drug metabolizing enzymes are microsomal and soluble epoxide hydrolases (Hassett et al. 1997; Srivastava et al. 2004; Omiecinski et al. 2000), glutathione S-transferases (Igarashi and Satoh 1989; Strange et al. 2001), N-acetyltransferases (Price-Evans 1989) and methyltransferases (Weinshilboum et al. 1999). Genetic polymorphisms of these enzymes play a role in metabolism and toxicity of drugs (Wormhoudt et al. 1999; Hengstler et al. 1998)

It becomes evident, that organs other than liver, such as lung (Yost 1999), kidney and intestine (Roediger and Babidge 1997; Paine 2003; Kaminsky and Zhang 2003) can also contribute to the metabolism of xenobiotics (De Kanter 1999) and partly to organ specific toxicity.

Another important matrix for the metabolism of xenobiotics is blood. In addition to in vitro studies in whole blood, serum or plasma prepared from blood from humans or animals are used (Williams 1987). Under the enzymes present in blood, serum esterases have the most significant effect on drugs. These include cholinesterase (ChE), serum arylesterase (SA), carboxylesterase (CE) and red blood cell (RBC) esterases. These enzymes play a role in both, activation of prodrugs and deactivation of drugs (Williams 1987). Recent examples of marketed prodrugs are adefovir, tenofovir, valganciclovir, olmesartan, parecoxib, tamiflu, famciclovir and ximelagatran (Los et al. 1996; Bernardelli et al. 2002; Boyer-Joubert et al. 2003, Li et al. 1998; Vere-Hodge et al. 1989, Balimane et al. 2000; Clement et al. 2003; Powell et al. 1993).

In addition to the potential of liberating the active drug upon absorption, orally applicable prodrugs require a sufficient stability in the gastro-intestinal tract which is most importantly pH-dependant chemical stability. This can be studied with simulated gastrointestinal fluids (Balbach and Korn 2004). Also

Table 2 Characteristics of cytochrome P450 isozymes (Donato and Castell 2003; Brandon 2003).

Isoform	Occurrence	Major Polymorphic variant alleles	Model substrates	Inhibitor	Inducer	Abundance in human liver
CYP1A1	Mainly extrahepatic	*CYP1A1*2* *CYP1A1*3*	7-ethoxyresorufin *O*-deethylation	alpha-Naphthoflavone (acceptable[1], inhibits also CYP3A4)	Polycyclic hydrocarbons	< 1%
CYP1A2	Liver		Phenacetin *O*-deethylation (preferred[1]) Caffeine N3-demethylation (acceptable[1])	Furafylline (preferred[1])	Smoking 3-Methylcholanthrene Char-grilled meat Rifampicine	8–15 %
CYP2A6	Liver	*CYP2A6*2* *CYP2A6*3* *CYP2A6*4* *CYP2A6*5*	Coumarin 7-hydroxylation (preferred[1])	Coumarin (acceptable[1])	Pyrazole Barbiturates	5–12 %
CYP2B1/2			Pentoxyresorufin O-dealkylation			
CYP2B6	Liver		(S)-mephenytoin N-desmethylation (preferred[1]) Bupropion hydroxylation (acceptable[1])	Sertraline (acceptable[1], also inhibits CYP2D6)		1–5 %
CYP2C8	Liver Intestine		Paclitaxel 6-α-hydroxylation (preferred[1])	Glitazones (preferred[1])	Rifampicine Barbiturates	10 %
CYP2C9	Liver Intestine	*CYP2C9*2* *CYP2C9*3*	(S)-warfarin C6-, C7 hydroxylation (preferred[1]) Diclofenac 4'-hydroxylation (acceptable[1]) Tolbutamide para CH3-hydroxylation (acceptable[1])	Sulfaphenazole (preferred[1])	Rifampicine Phenobarbital	15–20 %
CYP2C18/ 2C19	Liver	*CYP2C19*2* *CYP2C19*3*	(S)-mephenytoin 4'-hydroxylation (preferred[1])	Ticlopidine (acceptable[1], also inhibits CYP2D6) Ketoconazole	Rifampicine Carbamazepine	< 5%
CYP2D6	Liver Intestine Kidney	*CYP2D6*2* x *n* *CYP2D6*4* *CYP2D6*5* *CYP2D6*10* *CYP2D6*17*	Bufuralol 1'-hydroxylation (preferred[1]) Dextromorphan O-demethylation (preferred[1]) Codeine O-demethylation (acceptable[1])	Quinidine (preferred[1]) Haloperidol		2 %
CYP2E1	Liver Intestine Leukocytes	*CYP2E1*2* *CYP2E1*3* *CYP2E1*4*	Chlorzoxazone 6-hydroxylation (preferred[1]) Lauric acid ω-1-hydroxylation (acceptable[1])	Diethyl-dithiocarbamate 4-methyl pyrazole (acceptable[1])	Ethanol	7–11 %
CYP3A4	Liver GI tract	*CYP3A4*2* *CYP3A4*3*	Midazolam 1'-hydroxylation (preferred[1]) Testosterone 6β-hydroxylation (preferred)	Ketoconazole (preferred[1]) Troleandomycin (preferred[1]) Cyclosporine (acceptable[1]) Grapefruit juice	Rifampicine Barbiturates	30–40 %
CYP4A11	Liver Kidney		Lauric acid ω-hydroxylation	17-Octadecynoic acid		

[1] Recommendation according to Tucker et al. 2001

Table 3 Characteristics of individual isozymes in the human UGT family (Coughtrie and Fisher 2003; Ritter 2000).

Isozyme	Probe Substrates	Tissues	Reported inducers
UGTIA1	Bilirubin β-estradiol (3-gluc) 17α-ethinylestradiol (3-gluc)	Liver, intestine	3-methylcholanthrene, phenobarbital, oltipraz; phenytoin
UGTIA3	Hyodeoxycholic acid (COO-gluc)	Liver	
UGTIA4	Imipramine Trifluoperazine	Liver	
UGTIA6	Serotonin	Liver, brain	TCDD, TBHQ
UGTIA7	Benzo[a]pyrene metabolites	Stomach	
UGTIA8	Not known	Intestine	
UGTIA9	Propofol	Liver, kidney	TCDD, TBHQ
UGTIA10	Not known	Stomach, intestine	
UGT2B4	Hyodeoxycholic acid (6-gluc)	Liver	
UGT2B7	AZT, morphine	Liver, kidney, intestine	TBHQ
UGT2B10	Not known	Liver, kidney, intestine	
UGT2B11	Not known	Liver, prostate, mammary	
UGT2B15	(S)-oxazepam	Liver, prostate	
UGT2B15	Dihydrotestosterone	Prostate	

TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; TBHQ, *ter*-butylhydroquinone; AZT, 3'-azido-3'-deoxythymidine

Table 4 Some Properties of the Human Sulfotransferase Enzyme Family (Coughtrie and Fisher 2003).

SULT Isoform	Probe Substrates	Known Drug Substrates	Major Sites of Expression
SULT1A1	4-Nitrophenol	Acetaminophen, Troglitazone, Minoxidil, 4-OH Tamoxifen, Apomorphone	Adult Liver, Adult GI Tract, Adult Platelets, Placenta
SULT1A2	No Selective Substrate Known	–	?
SULT1A3	Dopamine	Salbutamol, Dobutamine	Adult GI Tract, Adult Platelets, Adult Brain, Placenta, Fetal Liver
SULT1B1	No Selective Substrate Known	–	Adult Liver, Adult GI Tract, Fetal GI Tract
SULT1C2	No Selective Substrate Known	–	Fetal Kidney, Fetal Lung, Fetal GI Tract
SULT1C4	No Selective Substrate Known	–	Fetal Kidney, Fetal Lung
SULT1E1	17β-Estradiol	17α-Ethinylestradiol	Fetal Liver, Fetal Lung, Fetal Kidney, Adult Liver, Endometrium
SULT2A1	Dehydroepiandrosterone	Budenoside, Dehydroepiandrosterone, Pregnenolone	Fetal Adrenal, Fetal Liver, Adult Liver, Adult Adrenal
SULT2B1	Cholesterol (2B1b), Pregnenolone (2B1b)	–	Adult Skin, Prostate, Placenta
SULT4A1	No Selective Substrate Known	–	Brain

enzymatic or bacterial degradation of prodrugs in the GI tract can be a limiting factor. This can be studied in vitro e.g. in Caco-2 cells concomitant to permeability experiments (see also chapter Cell Based Absorption Tests), ex vivo or in vivo. The stability in blood is also of special importance for the administration of peptides due to the presence of endo-, carboxy- and amino-peptidases (Powell 1993).

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II.H.1 In vivo Biotransformation Studies

PURPOSE AND RATIONALE

In vitro studies can only give a limited, "mechanistic" picture of biotransformation in animals or humans. The quantitative importance of each individual metabolite can only be assessed in vivo. Also, samples collected from in vivo studies give rise to comprehensive metabolite identification work (Watt et al. 2003; Clarke et al. 2001) which is also required from a regulatory point of view (Baillie et al. 2002). Due to the labour-intense nature of these studies and the need of applying radiolabeled compounds in order to get a complete picture of biotransformation these studies are performed at a later stage of development during preclinical and clinical phase.

PROCEDURE

Samples for a biotransformation investigation are typically collected concomitant to mass balance and radiokinetic studies in animal and human employing radiolabeled test compound (Iyer et al. 2001; Iyer et al. 2003; Lantz et al. 2003; Hayakawa et al. 2003; Huskey et al. 2004; Cui et al. 2004; Cook et al. 2003; Bu et al. 2004; Rodrigues et al. 2003). Plasma prepared from blood, urine and feces samples serve as a basis. Additional matrices (e.g. tissues and bile) might complete the picture. Before analysis, samples, possibly after pooling, are processed in most instances to enhance sensitivity and to separate matrix components interfering with the analytical procedure e.g. proteins. Processing is mandatory for feces and tissue homogenates and is typically done by extraction with mixtures of organic solvents like acetonitrile or methanol with buffer in different ratios followed by evaporation of the extracts. Urine can be analyzed directly without processing if not necessary for sample concentration. Protein precipitation is normally applied to plasma followed by a concentration step if necessary. In addition to generation of metabolite pro-

files of the original samples information on glucuronic acid conjugates or sulfates (Walle et al. 1983) present as Phase II metabolites can be gained by enzymatic digestions of complete samples or isolated metabolite peaks.

Urine and feces homogenates might serve as a basis for structure elucidation of metabolites by LC-MS/MS directly or – if necessary – by more detailed NMR/MS investigation after isolation of the metabolites by preparative chromatography.

EVALUATION

Samples are analyzed by a suitable chromatographic system, typically HPLC with on-line radiodetection. Metabolite profiles in plasma and tissues are given as percent of radioactivity present in total. Profiles in urine, bile and feces are given as percent of the dose administered.

CRITICAL ASSESSMENT OF THE METHOD

Despite the elaborate effort necessary for using radioisotopes, mainly carbon-14, in metabolic profiling studies, theses studies still play a dominant role in this field. This is due to the fact that ultimately only quantification on the basis of radiodetection gives a reliable profile of structurally unknown metabolites formed from a given drug within a complex endogenous matrix. This is not achieved by other detection methods. A metabolite profile consisting of a quantitative comparison of the metabolites present in a given matrix is not possible to obtain by LC-MS/MS alone.

However, application of hyphenated techniques, in particular LC-MS/MS in combination to radiodetection or as a sole device in analysis of studies employing non-labeled test compounds give rise to rapid structural information (Watt et al. 2003; Clarke et al. 2001). The dominating role of LC-MS/MS is not reached by LC-NMR coupling despite of advances in this technique and the applications of LC-MS-NMR coupling (Dear et al. 2000). Other techniques applied in this context comprise of improvements of hyphenated MS techniques like capillary liquid chromatography (Sandvoss et al. 2004), or ion exchange liquid chromatography-MS for charged polar molecules (Dear et al. 2000).

In most instances classical approaches in isolation of metabolites followed by MS and NMR analyses are still necessary to obtain the definitive structural information. This is due for complex structures of metabolites as they are drugs in development in most instances with the need of multiple NMR experiments in addition to an initial one dimensional ^{1}H NMR experiment.

Techniques applying test compounds labeled with stable isotopes are applied as well (Browne et al. 1993). However, they have not found broad acceptance in substituting radioisotopes in this field due to their restrictions in quantification of unknown metabolites.

Accelerator mass spectrometry (AMS) might offer the possibility to perform metabolite profiling concomitant to microdosing study in humans applying ultralow amounts of radioactivity (Garner 2000a; Garner et al. 2000b; Combes et al. 2003). At least for a limited number of representative samples AMS might serve as an expensive offline radiodetector for HPLC eluates as well.

MODIFICATIONS OF THE METHOD

Due to the objective of the studies different analytical methodologies adapted to the particular test compound and its metabolites are used which have to be optimized case-by-case and cannot be generalized. Whereas high performance liquid chromatography (HPLC) is a method of choice for many pharmaceuticals, also other techniques like gas chromatography (GC) e.g. for profiling of steroids (Holland 1986) or capillary electrophoresis (CE) might play a role. In recent years the application of microplate scintillation counter for 96-well plates (TopCount) revolutionized the practice in offline radiodetection to enhance sensitivity and speed in analyzing low radioactivity level samples as an alternative to online radiodetection or offline radiodetection using the classical approach of liquid scintillation counting (Nassar 2004; Kiffe 2003; Boernsen 2000).

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EXAMPLE

Iyer et al. (2001) investigated the metabolism of [^{14}C]omapatrilat in humans with samples collected during a clinical study. Plasma samples were prepared from blood spiked with or without methylacrylate to trap compound free sufhydryl groups which was important for this particular compound. Samples were pooled over the 12 subjects enrolled in the study. Urine was pooled over time to give a 0–24 h pooled urine sample representing 92 % of the radioactivity excreted in urine and a 0–168 h sample. Feces was not analyzed.

For sample processing, pooled plasma samples (1 and 6 h, 2 ml) were mixed with acetonitrile (6 ml), vortexed vigorously, and centrifuged. The precipitates were again extracted with acetonitrile (2×) and the supernatants from previous extraction were combined. The pooled extracts were evaporated at room temperature under a stream of nitrogen and the residues were reconstituted in HPLC mobile phase. A portion of the sample was used for radioactivity determination, and the remaining sample used for HPLC profiling.

Pooled 0- to 24-h and 0- to 168-h human urine samples were concentrated on an Oasis HLB 3.0-ml cartridges. The cartridge was loaded with pooled urine (3 ml) and washed sequentially with water containing 0.1 % acetic acid (2 × 3 ml, pH 3.2) and methanol (2 × 3 ml). Liquid scintillation counting of the water and the methanol extracts showed quantitative recovery of radioactivity in the methanol fractions. The methanol extracts were combined, evaporated to dryness under a stream of nitrogen, and dissolved in HPLC mobile phase.

Selected urine samples were digested for cleavage of conjugates. For this, a solution of β-glucuronidase (0.2 ml, 2104 units) in water was added to a solution of 0- to 24-h pooled human urine (1.0 ml) in 0.2 M sodium acetate buffer (1.0 ml, pH 4.8). The mixture was incubated at 37 °C in a water bath for 24 h. In addition, control experiments were done in the absence of the enzyme (negative control), positive control in the presence of phenolphthalein glucuronide (1 mg), and incubation in the presence of the β-glucuronidase inhibitor 1,4-saccharolactone (10 mg). A 0.2 M glycine

buffer (8.0 ml, pH 10.5) was added at the end of the incubation to the positive control. The amount of phenolphthalein generated was quantified against a phenolphthalein standard curve with a UV-visible spectrophotometer operating at 550 nm. All other samples were centrifuged for 5 min in a bench top microcentrifuge and stored at –20 °C until further analysis.

Plasma and urine samples were profiled using a suitable HPLC system under specific conditions (gradient, column). Readout was done off-line by fraction collection of the HPLC run followed by liquid scintillation counting. Biotransformation profiles were prepared by plotting the base-line corrected radioactivity against time-after-injection.

For metabolite isolation, 1.5 liters of pooled urine were applied to a XAD-2 resin column first. The ethyl acetate extract obtained containing 85 % of the radioactivity was applied upon evaporation to semipreparative HPLC on a Zorbax RX C18 column (9.4 × 250 mm, 5 μm) using gradient elution. Fractions obtained were further separated by isocratic elution on the semipreparative column. The metabolite fractions obtained were finally purified by preparative thin-layer chromatography. Liquid chromatography/mass spectrometry (LC/MS) and LC/MS/MS analysis was applied to the isolated metabolite fractions for structure elucidation.

II.H.2 Perfused Organs

PURPOSE AND RATIONALE

In comparison to other in vitro systems for studying drug metabolism, metabolism studies with isolated organs allows a definitive conclusion about the contribution of a given organ to in vivo drug metabolism. Metabolism studies in perfused organs gives the best correlation to the in vivo situation since all metabolic pathways and cofactors are available. In comparison to in vivo animal studies, experiments in isolated organs can be performed under precisely defined conditions such as composition of the perfusion media or perfusion rate. Since the liver is the most active organ for drug metabolism, liver perfusion studies are frequently used. Additional tissue preparation for perfusion studies have been published in heart (e.g. Enser 1967; Scheuer 1967), lung (e.g. Baciewicz 1991; Kaneda 2001), kidney (Nizet 1975; Newton 1981), brain (e.g. Thompson 1968) and intestine (e.g. Pang 1985; Cong 2001).

Concentration of the drug and related metabolites are determined using appropriate analytical methods such as HPLC, LC-MS or LC-MS/MS techniques. The use of a radio-labeled drug allows are more easy and precise recognition of the drug and related metabolites.

PROCEDURE

As described in the section Perfused Organs.

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II.H.3 Organ Slices

PURPOSE AND RATIONALE

The slice technique was already introduced by Otto Wartburg in 1923 and commonly used in in vitro liver research until isolated hepatocytes and isolated perfused liver preparation were introduced and optimized (Olinga 1998). Previous methods of slicing using the Stadie Riggs (Stadie 1944), Vibratome (Smith 1985), or hand made slice technique (Forster 1948) suffered on a rapid preparation of thin tissue slices of uniform thickness and dimension under conditions that minimize trauma to the live tissue (Krumdieck 1980).

Since the introduction of the Krumdieck slicer (Krumdieck 1980) and a new incubation system for slices (Smith 1985) tissue slices are commonly used in drug metabolism and toxicity studies.

PROCEDURE

Cylindric piece of tissue of about 12 mm in diameter are placed into the Krumdieck microtome and

cut by means of a sharp punch. Slices are released into circulating buffer. The slice thickness is set by the screw-adjustable rest. The variability of the slice weight increase with increasing slice thickness (Krumdieck 1980). Afterwards, the slices were placed on the inside walls of stainless steel roller-equipped steel mesh scintillation vials containing 2 ml Waymouth's 752/1 media supplemented with 10 % fetal calf serum and Gentamycin (84 μg/ml). Vials were capped with silastic rubber septa (300 μM) to allow gas exchange and placed in an incubator set at 37 °C with constant aeration (O_2:CO_2 = 95:5). The vials were rotated at 10 rev/min and culture media replaced every 12 h (Smith 1985). Incubations of test compounds are done in media in comparison to a solvent control (Dogterom 1993).

EVALUATION

Thickness and viability of the slices (e.g. morphology, ATP content) are investigated in parallel. Metabolic capacity of various enzymes is ensured by positive and negative controls during the incubation procedure. Concentration of the drug and related metabolites are determined using appropriate analytical methods such as HPLC, LC-MS or LC-MS/MS techniques. The use of a radiolabeled drug allows are more easy and precise recognition of the drug and related metabolites.

CRITICAL ASSESSMENT OF THE METHOD

The use of liver slices as an in vitro tool for drug metabolism has various advantages compared to isolated hepatocytes, such as faster and easier preparation, the maintenance of an intact tissue architecture with cell-cell and cell-matrix interactions (except 1 or 2 cell layers which are always damaged because of the slicing procedure, so that even in very good slices, normally no more then approximately 80 % of the cells are intact (De Graaf 2002) and the presence of different cell types. In general, the complete cellular machinery is available. Since mostly all organs are applicable to the slice technology, organ selective drug toxicity or the relative contribution of the organs to the total body clearance can be investigated.

Organ slices are applicable to long-term cultures, viability up to 72 h has been described for rat liver slices (Fisher 1995). On the other and, investigation of enzyme activity revealed changes in the metabolic capacity of phase I and phase II enzymes in long-term cultures (Vandenbranden 1998). De Kanter (1995) established a general method for cryopreservation of slices. The best viability of rat liver slices was found by exposure for 30 minutes to 12 % dimethyl sulfoxide at 2 °C before rapid freezing in liquid nitrogen (De Kanter 1995). Post-thaw viability, especially phase I and phase II biotransformation activity maintained for at least 4 h (De Graaf 2002). Generally, cryopreservation allows a more universal use of liver slices since the method becomes independent from the availability of tissue, especially for human tissue. In addition, using slices from the same donor simplifies a comparison of compounds in the early stage of drug discovery.

MODIFICATIONS OF THE METHOD

Vandenbranden (1998) used additional antibiotics to reduce bacterial impurities during long-term cultivation.

The slicing technique can be adapted to various organs and allows tissues specific comparison of metabolism and toxicity within animals and humans. Stefaniak (1988)used the method for metabolism studies in lung. Therefore, lung trachea or bronchiole (rat/human) was cannulated and lungs were instilled with 1.5 % (w/v) low melting agarose solution containing 0.9 % (w/v) NaCl at 37 °C and allowed to gel on ice. Tissue cylinders with an 8 mm diameter were cut as described from Krumdieck (1980). Ruegg et al. (1987) adapted the method to investigate cell specific toxicity in kidney cells.

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II.H.4
Primary Hepatocytes

PURPOSE AND RATIONALE

Isolated hepatocytes provide a convenient link between the complex architecture of the intact organ and subcellular fractions. The latter approach suffers from lack of relevance to the in vivo situation, while the former one, as close as possible to the liver in vivo, suffers from complex methodology and limited use for multiple compounds (Mandan 2002).

Cryopreserved hepatocytes in suspension were successfully applied in short-term metabolism studies and as metabolizing system in mutagenicity assays (Hengstler 2000), providing qualitative metabolic information and quantitative pharmacokinetic parameters from key animal species and human at the early stage of drug discovery and drug development.

PROCEDURE

Hepatocytes – both freshly prepared or cryopreserved – are commercially available e.g. Gentest, IVT, Xenotech or prepared in-house. Isolation of the animal hepatocytes follows after a two step collagenase perfusion of the liver via the vena portae in situ (Seglen 1976) or via several blunt-end cannulae inserted into vessels available on the cut surface of pieces of human liver obtained from resection. Liver cells are gently scraped out into suspension buffer and washed twice to three times by centrifugation to remove cell fragments and non-vital cells. Hepatocytes are used immediately or cryopreserved for further use.

Isolation of hepatocytes according to the standard operation procedures mentioned below results generally in a viability of the hepatocytes $\geq 80\,\%$. Incubations with test compounds is performed in various drug concentrations and incubation time point(s).

EVALUATION

Viability of the hepatocytes is usually determined by trypan blue exclusion rate. Viability over incubation time can be determined by LDH retention or albumin secretion (Gebhardt 2003). For metabolism purposes, positive controls with well known phase I and/or phase II metabolism are incubated in parallel to test compounds to assure metabolic capacity of the hepatocytes e.g. ethoxyresorufin, ethoxycoumarin, testosterone for phase I metabolism and 4-hydroxybiphenyl, 4-methylumbelliferone, 1-chloro-2,4-dinitro-benzene and 2-naphthol for phase II metabolism (Gebhardt 2003). Concentration of the drug and related metabolites are determined using appropriate analytical methods such as HPLC, LC-MS or LC-MS/MS techniques. The use of a radiolabeled drug allows are more easy and precise recognition of the drug and related metabolites.

CRITICAL ASSESSMENT OF THE METHOD

Human hepatocytes (fresh or cryopreserved) are now commercially available e.g. from BD/Gentest, In Vitro Technology or Xenotech. However, the quality, stability and availability of the commercial preparation remain questionable (Mandan 2002). Isolation (and cultivation) of hepatocytes is still time- and lab-intensive and needs to be optimized for livers of every different animal species (De Graaf 2002). Metabolism studies in hepatocytes might be a good compromise between perfused livers and subcellular fractions such as microsomes, since the complete cellular machinery is available. Nevertheless, some pitfalls have to be taken in account:

Hepatocytes lose their polarity during isolation (Olinga 1998). A modulation in metabolic capacity and transport internalization of hepatocytes in suspension and in long-term cultures have been described (Hengstler 2000; Olinga 1998). The loss of cofactors such as NADPH are probably the reason for a stronger decrease of metabolic capacity of phase I and phase II metabolism in cryopreserved hepatocytes compared to fresh hepatocytes or liver homogenate (Hengstler 2000). An increase of metabolism of cryopreserved hepatocytes of benzo[a]pyren equally to freshly isolated hepatocytes was reported after Percoll centrifugation (Hengstler 2000). Hence, Percoll centrifugation is recommended before using cryopreserved hepatocytes in metabolism studies. Due to the variability of the enzyme activity with time, the maximum incubation time in suspension should be limited up to 4–8 h (Hengstler 2000; Olinga 1998).

MODIFICATIONS OF THE METHOD

Since enzyme activities of hepatocytes in suspension decrease markedly with longer incubation time, hepatocytes in culture are required if longer incubation time is necessary, particularly phase II metabolism.

To achieve longer incubation times, cultivation of freshly isolated hepatocytes in culture on monolayer (Maslansky 1982; Wang 2002), sandwich culture on variable matrixes (e.g. Maurel 1996; Kern 1997; Wang 2002), in co-culture (Hengstler 2000; Gebhardt 2003) or in bioreactors perfusion culture system or 96 well bioreactor (e.g. Bader 1998; Wang 2002; Gebhardt 2003) are described.

Immobilization of liver cells in alginate beats has facilitated commercialization of isolated hepatocytes (Guyomard 1996; Rialland 2000) but has not succeeded in a broad acceptance.

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EXAMPLE

1. Isolation of Human Hepatocytes

The isolation of human hepatocytes is described according to Hengstler et al. (Hengstler 2000).

Preparation of buffer solutions:

Suspension buffer:

- 620 ml of glucose solution (9 g/L D-glucose)
- 100 ml of KH buffer (60 g/L NaCl, 1.75 g/L KCl, 1.6 g/L KH_2PO_4 adjusted to pH 7.6 with NaOH)
- 100 ml of HEPES buffer (60 g/L HEPES adjusted to pH 7.6; without carbogen-equilibration)
- 150 ml of amino acid solution (0.27 g/L L-alanine, 0.14 g/L L-aspartic acid, 0.4 g/L L-asparagines, 0.27 g/L L-citrulline, 0.14 g/L L-cysteine, 1 g/L L-histidine, 1 g/L L-glutamic acid, 1 g/L L-glycin, 0.4 g/L L-isoleucine, 0.8 g/L L-leucine, 1.3 g/L L-lysine, 0.55 g/L L-methionine, 0.65 g/L L-ornithine, 0.55 g/L L-phenylalanine, 0.55 g/L L-proline, 0.65 g/L L-serine, 1.35 g/L L-threonine, 0.65 g/L L-tryptophan, 0.55 g/L L-tyrosine, 0.8 g/L L-valine. Dissolve the amino acids that cannot be dissolved at neutral pH by addition of 10 N NaOH and thereafter adjust pH to 7.6 by 37 % HCL)
- 5 ml of insulin solution (2 g/L insulin dissolved I 1N NaOH, adjusted to pH 7.6 by 1 N HCl)
- 8 ml of $CaCl_2$ solution (19 g/L $CaCL_2 \times 2\ H_2O$)
- 4 ml of $MgSO_4$ (24.6 g/L $MgSO_4 \times 7\ H_2O$)
- 2 g BSA/L; dissolved in the mixture of the above-mentioned solutions

Buffer A:

- 498 ml of washing buffer (8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, adjust to pH 7.4 with 4 N NaOH)
- 2 ml of EGTA solution (47.5 g EGTA/L; dissolved by addition of NaOH, adjusted to pH 7.6 by HCL)

Buffer B:

- 498 ml of washing buffer (8.3 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, adjust to pH 7.4 with 4 N NaOH)

Collagenase buffer (Buffer C):

- (3.9 g/L NaCl, 0.5 g/L KCl, 2.4 g/L HEPES, 0.7 g/L $CaCl_2 \times 2\ H_2O$ adjust to pH 7.2 with 4 N NaOH), prewarm to 37 °C before use

- Dissolve 100 mg of Collagenase[1] in 200 ml of buffer C immediately before perfusion.

Human liver samples e.g. from liver resection should be immediately transferred into ice-cold suspension buffer (resected tissue can be stored in ice-cold suspension buffer for at least 4 h). Liver samples of approx. 100 g should be cut off in such a way, that they only present one cut surface. Perfuse with buffer A for 20 min at 37 °C (3 drops/s). Perfusion should be performed by several blunt-end cannulae inserted into vessels of the cut surface. The number of the cannulae depends on the number of large vessels available on the cut surface. Perfuse with buffer B for additional 20 min at 37 °C. Thereafter, perfuse with collagenase buffer for 30 min in a circulating way at 37 °C. Transfer the tissue into a large Petri dish with suspension buffer, scrape liver cells gently out with spatula. Filter the liver cell suspension through gauze and centrifuge for 5 min at 50 × g. Wash twice with suspension buffer, centrifuge again and resuspend the final pellet in 30 ml suspension buffer. Determine Trypan Blue exclusion rate after a 1:1 dilution of the hepatocyte suspension with Trypan blue solution (4 g/L Trypan Blue). A similar procedure is described for the isolation of rat hepatocytes (Hengstler 2000).

A standard operation procedure for the isolation of rat hepatocytes is published from Gebhardt et al. (Gebhardt 2003).

2. Cryopreservation

The cryopreservation of hepatocytes is described according to Hengstler et al. (2000).

Adjust hepatocytes to 3 Mio cell/ml in suspension buffer in an Erlenmeyer flask. Incubate for 30 min at 37 °C during gentle shaking and carbogen equilibration. Determine the volume (original volume) and centrifuge the suspension for 5 min at 50 × g at 4 °C. All further steps are performed at 4 °C. Discard a volume equal to two-third of the original volume from the supernatant and resuspend the cell pellet in the remaining suspension buffer (one third of the original volume) by shaking gently. Add ice-cold suspension buffer containing 12 % (v/v) of DMSO to the cell suspension up to 50 % of the original volume, resulting in a DMSO concentration of 4 % and approx. 6 Mio/ml hepatocytes. After 5 min on ice, add suspension buffer containing 16 % DMSO (v/v) of DMSO up to the original volume of the cell suspension, resulting in a DMSO concentration of 10 % and 3 Mio/ml hepatocytes. After 5 min n ice, transfer the hepatocyte suspension to cold cryovials with 1.5 ml/vial. Start the freezing program within 5 min. The time period between the second addition o DMSO and initiation of the cryopreservation should not exceed 10 min. The freezing procedure should be performed as follows (can be performed by any computer-controlled freezing machine):

- Cooling in 10 min down to 0 °C
- 8 min at 0 °C
- in 4 min down to –8 °C
- in 0.1 min down to –28 °C
- in 2 min down to –33 °C
- in 2 min up to –28 °C
- in 16 min down to –60 °C
- in 4 min down to –100 °C

The temperature in the chamber and in one cryovial should be monitored by a chart record to control whether crystallization heat was sufficiently compensated. Transfer the cryovials into liquid nitrogen immediately after the freezing program has been finished.

3. Thawing

Thawing of hepatocytes is described according to Hengstler et al. (2000).

Thaw the frozen hepatocytes quickly by gentle shaking in a 37 °C water bath. The hepatocytes should thaw but not become warm. Transfer the hepatocyte suspension into an ice-cold Erlenmeyer flask immediately after thawing and dilute DMSO gradually by the addition of ice-cold not carbogen-equilibrated suspension buffer, 0.5-, 1-, 2- and 3-fold of the volume of the thawed hepatocyte suspension. Suspension buffer should be added drop wise and hepatocytes should be on ice for 3 min before the next dilution step takes place. After centrifugation at 50 × g for 5 min at 4 °C and resuspension in 10 ml suspension buffer, the hepatocytes can be purified by Percoll centrifugation.

4. Percoll Centrifugation

The Percoll centrifugation of hepatocytes is described according to Hengstler et al. (2000).

Add the following into an ice-cold 50 ml Falcon tube:

- 10 ml of hepatocytes in suspension buffer (max. 20 Mio cells)

[1] Selection of an adequate batch of collagenase is the key critical step for successful isolation of human hepatocytes. Whereas the majority of all collagenases allows successful isolation of rat hepatocytes, selection of a good batch for human liver is more critical. Mostly, the collagenase concentration has to be optimized for an individual batch of collagenase.

- 22 ml of suspension buffer (without carbogen equilibration)
- 18 ml Percoll solution

Mix gently and centrifuge at 250 × g for 20 min at 4 °C. The pellet contains intact hepatocytes. Resuspend the cell pellet in suspension buffer and wash twice in suspension buffer (centrifugation at 50 × g for 5 min at 4 °C).

5. Incubation of Test Compounds with Hepatocytes in Suspension

Transfer 1 ml of suspension buffer containing 1 Mio hepatocytes (fresh or cryopreserved) into glass vials (the diameter of the hepatocyte suspensions in the glass vial should be at least twice its height). Transfer the glass vials into a shaking water bath or in an air-conditioned rotating incubator (37 °C, approx. 40 rpm). Add test compound in the appropriate concentration and incubate up to 2 h (Gebhardt 2003), 4 h (Olinga 1998) or 8 h (Hengstler 2000), respectively. Organic solvent should be minimized as much ($\leq 0.2\,\%$ of DMSO or $\leq 1\,\%$ methanol or acetonitrile) as possible to circumvent inhibitory effects of the solvents (Busby 1999). The reactions is stopped by adding ice-cold acetonitrile and a sharp centrifugation step afterwards. The supernatant is directly applied to HPLC or LC/MS analytics for quantification of the remaining compound and related metabolites. Note, activities of xenobiotic metabolizing enzymes decrease within the incubation time (Hengstler 2000).

II.H.5 Homogenates

PURPOSE AND RATIONALE

Metabolism studies in tissue homogenate are probably one of the oldest methods and already published as early as in 1930s (Potter 1936). Drug metabolism studies in homogenate are usually used to investigate potential species-species difference early in the drug metabolism research in view of selecting the appropriate species for toxicology studies. In comparison to subcellular fractions e.g. S9 or microsomes, tissue homogenate contains most of the enzymes and sometimes cofactors necessary for function.

PROCEDURE

The tissue is homogenized at low temperature in buffer media using a mincer or a mixer such as a Waring blender or Ultra Turax followed by grinding in a tissue grinder equipped with a motorized, serrated Teflon pestle, to produce uniform suspension, that is immediately used or deep-frozen until use. Incubations with test compounds are performed in various drug and cofactor concentrations with different incubation time point(s) and extraction procedures.

EVALUATION

Concentration und structure characterization of the drug and related metabolites are usually determined with HPLC, LC/MS or LC/MS/MS techniques. The use of a radiolabeled drug allows are more easy and precise recognition of the drug and related metabolites.

CRITICAL ASSESSMENT OF THE METHOD

Due to interindividual variation of the animals and differences in the enzyme panel of each individuum, a quantitative comparison of two different compounds seems to be difficult. The same is true for reproducibility. Determination of the protein concentration should be performed and kept constant in the incubation.

MODIFICATIONS OF THE METHOD

Numerous application using liver homogenates are published with variable drug and cofactor concentration, incubation time points and workup procedures.

Kinetic but also dynamic responses are investigated in various other tissue homogenate such as lung (e.g. Zhang 1996; Manautou 1992), kidney (e.g. Gergel 1992; Knudsen 1996) and brain (Yagen 1991; Aragon 1992; Hornykiewicz 2002).

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EXAMPLE

Tissues were immediately frozen on dry ice after sacrifice until use. The tissue is homogenized at 0–4 °C in a 5-fold volume (tissue wt/buffer vol) of 0.1 M potassium phosphate buffer containing 5 % sucrose, pH 7.4 using a 15 sec spin in a Waring blender followed by grinding in a tissue grinder equipped with a motorized, serrated Teflon pestle, to produce uniform suspension. The suspension was immediately used or deep-frozen until use (Norton 1992). Incubation with test compounds are usually performed in 1 ml suspension and at a drug concentration of 1 μM or 10 μM and glucose-6-phoshate dehydrogenase, glucose-6-phosphate, 0.4 mM triphosphopyridine nucleotide and $MgCl_2$ (3.5 U/ml, 1 mM, 0.4 mM and 10 mM, respectively) for 30 min at 37 °C (Norton 1992). Reaction was stopped by adding 0.1 ml of 1 N HCl and the drug and related metabolites are extracted with organic solvent.

According to Norton et al. (Norton 1992), the drug BW1370U87 was incubated in 1 ml suspension and at a drug concentration of 1 μM or 10 μM and glucose-6-phoshate dehydrogenase, glucose-6-phosphate, 0.4 mM TPN and $MgCl_2$ (3.5 U/ml, 1 mM, 0.4 mM and 10 mM, respectively) as cofactors for 30 min at 37 °C. Reaction was stopped by adding 0.1 ml of 1 N HCl and the drug and related metabolites are extracted with methyl-3°-butyl ether. Iley (1999) demonstrated the biotransformation of various tertiary amidomethyl ester prodrugs at a concentration of 200 μM in 300 μl liver homogenate using the following cofactor concentrations: 6.25 mM glucose-6-phosphate, 1.25 mM $NADP^+$, 6 mM $MgCl_2$ and 2.5 U/ml glucose-6-phosphate dehydrogenase at 37 °C. The reaction was stopped at various time points by adding 10 volumes of acetonitrile (ice cold) and centrifuged. 3-Methylcholanthrene induced rat liver homogenates were used to study the metabolism of (+)-trans-benzo[a]pyrene at a concentration of 20 μM and various volumes of rat homogenate at 37 °C (Sindhu 1995). The incubation medium consisted of 50 mM Tris-HCl, pH 7.4 containing 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM NADPH in a total volume of 1 ml. After various time points, the reaction was stopped by adding H_2O saturated ethyl acetate. For additional application see also Zhang et al. (1996); Otsuka et al. (1996); Guillouzo (1995); Poon et al. (1995) or Lan et al. (1989).

II.H.6 9000g Supernatant (S9) Fractions

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations the procedures described here focus on liver 9000g fractions exemplarily. 9000g supernatants (S9 fractions) contain both, microsomal and cytosolic proteins. 9000g fractions are commercially available and more easily to prepare than microsomes (Testa 1976; Wrighton 1992; Jones 1998; Linget 1999; Li 2004) since 9000g centrifugation is an intermediate step in the preparation of microsomes (Figure 3). In combination with the high storage stability, S9 fractions also give rise to high-throughput applications using S9 fractions. 9000g liver fractions from various species are available from various commercial suppliers like Xenotech, Invitro Technologies and others.

PROCEDURE

The 9000g fraction might either be of commercial source or prepared in-house. The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation. The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or a NADPH generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethylsulfoxide most often as solubilizer. Incubation is carried out at 37 °C over incubations time of up to about 1 h. To stop the reaction icecold acetonitrile or aqueous trifluo-

roacetic acid is added in equal amounts. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

Due to the presence of both, microsomal and cytosolic enzymes present in 9000g fractions these preparations offer the advantage of the most complete picture of biotransformation compared to other subcellular fractions (Plant 2004; Brandon 2003) if cofactors in the appropriate concentrations are added. On the other hand, enzyme activity in the total protein content is lower compared to microsomal preparations causing lower turnover of a substrate in general. Because of these characteristics the 9000g fraction is applied in high-throughput applications for screening of metabolic stability to a lesser extent – in general – compared to the application of microsomes.

The same considerations as for other subcellular systems with respect to solvent influences have to be considered in tests in 9000g fractions because organic solvents deactivate cytochrome P450 isozymes and others concentration dependently (Busby 1999; Easterbrook 2001). Solvent influences are lowest for methanol and acetonitrile (< 10 % inhibition at 0.3 % solvent) but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used.

MODIFICATIONS OF THE METHOD

Depending on the matter of interest in use of 9000g fractions modifications in use of cofactors are necessary. With respect to microsomal proteins, an NADPH regenerating system has to be present to cover cytchrome P450 dependant metabolism, UDPGA as a cofactor for glucuronidation in combination with alamethizin as a modifier. Indeed these cofactors are in use in most instances as by Wittman (2000, 2001), Rajanikanth (2003); Epperly et al. (2001) Mae (2000) and Hewawasam (2002). Incubation time and substrate concentrations are highly subject to modifications.

Besides applications in studying the metabolism of compounds the S9 liver fractions of human or aroclor induced rat are in use as "metabolic activation" of the Ames Test for mutagenicity of chemicals, in most instances without addition of Phase II cofactors (Maron and Ames 1983). S9 fractions are also used for activation in reportergene assays (Sumida et al. 2001).

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www.xenotechllc.com (accessed in October 2004)

EXAMPLE

A standard procedure is taken from Mandagere 2002: Substrates are incubated at 37 °C and pH 7.4. The reaction mixture consists of 4 mL of a 5 mg protein/mL suspension of liver S9 (equivalent to approximately 1 mg/mL of microsomal protein in the final reaction mixture), 4 mL of an NADPH generating cofactor (6.4 mM glucose-6 phosphate, 1.1 mM NADP and 1.3 mM $MgSO_4$), 0.32 mL of glucose-6-phosphate dehydrogenase, 7.58 mL of 0.1 N K_2HPO_4 and 0.1 mL of substrate (6–8μg/mL) such that the concentration was 10 μM in the final reaction volume of 16 mL. Aliquots are taken at 1, 3, 5, 10, 15, 30 and 60 min and snap frozen for deactivation of enzymes. Samples are stored at –70 °C until assayed.

II.H.7 Microsomes

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations the procedures described here focus on liver microsomes exemplarily. Microsomes are a widely used test system in studying metabolic stability and profiles both of large number of compounds in the early part of the value chain during drug discovery and development (Brandon 2003). Commercial availability (Xenotech, Gentest, In Vitro Technologies, others) or ease of preparation (Testa 1976; Raucy 1991; Wrighton 1992; Jones 1998; Linget 1999; Li 2004) of the microsomal liver fraction in combination with the capability for automation of the incubation gives rise to high-throughput applications. High storage stability (Meier 1983; Pearce 1996; Yamazaki 1997) also provides the possibility to generate a human liver bank (von Bahr, 1980) in order to study the huge interindividual differences in enzyme activities especially in the human population. The latter is applied in the correlation analysis to study metabolic pathways in the context of enzyme typology (Beaune 1986).

PROCEDURE

Procedures in use are numerous and depend strongly on the objective of the application. The microsomal protein might either be of commercial source or prepared in-house. A comprehensive overview on preparation of subcellular fractions is given by Ekins et al. (1999). The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation. The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or a NADPH generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethylsulfoxide most often as solubilizer. Incubation is carried out at 37 °C over incubation time of up to about 1 h. To stop the reaction icecold acetonitrile or aqueous trifluoroacetic acid is added in equal amounts. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

The high batch-to-batch variability of enzyme activities affords usage of pooled microsomes if metabolic stability has to be screened. Individual donors are used only for specific applications like correlation analysis. Since no cytosolic enzymes are present, the screening in liver microsomes might sometimes lead to misleading results if compared to in vivo data. On the other hand, the enzyme activities are higher in microsomal

preparations than in 9000g fractions giving rise to higher metabolic turnover (Brandon 2003). However, this might lead also to overestimation of metabolic instability in microsomes compared to systems like hepatocytes or liver slices (Sidelmann 1996).

The same considerations as for other subcellular systems with respect to solvent influences have to be considered in tests in 9000g fractions because organic solvents deactivate cytochrome P450 isozymes and others concentration dependently (Busby 1999; Easterbrook 2001). Solvent influences are lowest for methanol and acetonitrile (< 10 % inhibition at 0.3 % solvent), but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used. Published applications of microsomal stability tests use DMSO amounts of up to 1 % which clearly can lead to enzyme-specific inhibition.

MODIFICATIONS OF THE METHOD

Numerous modifications are in place at each lab regarding incubation conditions like substrate concentration, incubation time, cofactor use and concentration, protein concentration etc. These modifications depend on the purpose of the method, e.g. as a high through-put screen in early drug discovery or as a tool to characterize a development compound in vitro for regulatory files. Besides these modifications on the biological part of the assay, specific protocols on pipetting robots and conditions used for evaluation by LC-MS/MS also in combination with the instrumental equipment available are applied which are typically not published. Besides LC-MS/MS also LC-UV (Shearer 2002; Stratford 1999) and capillary electrophoresis (Clohs et al. 2002) are described.

Typically the reaction is started by addition of NADPH (Plobeck et al. 2000; Stratford et al. 1999) or the NADPH regenerating system (Bloomer et al. 2001; Wei 2000).

MacKenzie et al. (2002) added 5 μM MnCl2 in addition to $MgCl_2$.

Linget (1999) incubated in the presence of 3 % bovine serum albumin to assist dissolution of compounds with poor solubility.

Different incubation time, substrate and protein concentration used are published (e.g. Kling et al. 2003; Mitsuya et al. 2000; Diana 1995).

For extrapolation of in vitro metabolism data to in vivo more detailed in vitro investigations are necessary as a first step. In this either enzyme kinetics is determined to calculate Km and Vmax and finally the intrinsic metabolic clearance as the quotient of both (Houston 1994; Iwatsubo et al. 1996). Alternatively the in vitro $t_{1/2}$ method is used (Obach 1997; 2002, Jones 2004).

In order to cover glucuronidation reactions in incubations in microsomal fractions several modifications have been applied in order to optimize conditions. These comprise longer incubation times than necessary for oxidative reactions by cytochrome P450s, and use of modifiers, both to overcome the latency in activity due to the diffusional barriers of the endoplasmatic reticulum (Coughtrie and Fisher 2003; Csala et al. 2004). Modifiers used are detergents or the pore-forming peptide alamethicin (Fisher 2000). Also disruption of cells by sonication is applied (Ethell 1998).

For elucidating the specific enzyme responsible for a certain metabolic step occurring in liver microsomes the addition of enzyme specific inhibitors is useful. If suicide inhibitors are used, preincubation of the inhibitor is needed. Selective or total inhibitors are used for cytochrome P450s in particular (Donato and Castell 2003; Newton 1995; Rettie 1995; Birkett 1993). Also antibodies raised against specific enzymes are used for studying the involvement of these enzymes in the biotransformation. These are mainly in use for cytochrome P450s (e.g. Wang 1997) due to their commercial availability (BDGentest, Invitrogen).

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EXAMPLES

Example 1: High-throughput Application for Metabolic Stability Tests

Di et al. (2003) described in detail an automated application on a Packard Multiprobe robot for high through-put screening. The following final concentrations are applied:

1.3 mM NADPH
3.3 mM Glucose-6-phosphate
3.3 mM $MgCl_2$
0.4 U/ml Glucose-6-phosphate dehydrogenase
1 mM EDTA
88 mM Phosphate buffer, pH 7.4
0.5 mg/ml Microsomal protein
1 μM Test compound
0.2 % DMSO

The incubation is performed in 96 multititerplates for 0 and 5 minutes. The pipetting steps for the 5 minute incubation plate are:

1. 990 μl of dilution buffer was added to each well of the 2-ml deep well dilution plate
2. 10 μl of sample stock (0.5 mM) was added to the corresponding well of the dilution plate and mixed by aspiration/dispensation 3 times at high speed to form the diluted sample
3. 100 μl of NADPH cofactor (10.938 mL of phosphate buffer, 1.609 mL of regenerating solution A and 0.322 mL of solution B, obtained from BD Gentest, Woburn, MD) was added to each well of the incubation plate
4. 50 μl of each diluted sample was added to the 1 ml deep well incubation plate in duplicate and warmed at 37 °C for 10 min
5. 100 μl of the prewarmed microsomes were added to each well of the incubation plate
6. the plate was incubated on the robot at 37 °C, 70 rpm for 5 min on a shaker (Armalab, Bethesda, MD)
7. at the end of the incubation the plate was immediately returned to the Packard robot, and 500 μl of cold acetonitrile was added to each well. The plate was gently mixed on a vortexer (VWR, So. Plainfield, NJ).

Subsequently, precipitated protein is removed by centrifugation.

Samples obtained are analyzed by LC-MS/MS under conditions optimized with an optimization plate specifically prepared for this purpose

A general generic LC comprises of application of a trapping cartridge to improve efficiency and peak shape and subsequent elution of the trapped components with a high amount of organic modifier. The following analytical conditions are used:

Column: Keystone Aquasil C18 10 × 2.1 mm, 5 μm, guard cartridge

Mobile Phase: Loading = 0.1 % formic acid/water, Elution = 0.1 % formic acid (95 % acetonitrile/water)

Flow rate: Optimization = 0.3 ml/min, 0.8 min, Loading = 3.0 ml/min, 5 sec, Flash/analysis = 1.0 ml/min, 0.5 min

Gradient: isocratic

Injection volume: 20 μl

Detection: electrospray (positive or negative mode), multiple reaction monitoring.

A backup generic LC method is used for compounds failed by method 1 using the conditions as follows:

Column: Keystone Aquasil C18 50 × 2.1 mm, 5 μm, column

Mobile Phase: A = 0.1 % formic acid/water, B = 0.1 % formic acid/acetonitrile

Flow rate: 0.8 ml/min

Gradient: 0.1 min/10 %B, 2.5 min/90 %B, 4.0 min/90 %B, 4.1 min/10 %B, 5.5 min/10 %B

Injection volume: 20 μl

Detection: atmospheric pressure chemical ionisation (positive or negative mode), selected ion mode.

Final results are given as percent remaining after incubation by dividing peak area of parent compound in the 5 min sample by the area of the time 0 sample and multiplication by 100.

Example 2: Enzyme Mapping Studies by Use of P450 Isozyme Selective Inhibitors

An example for applying specific inhibitors for cytochrome P450 isozymes in incubations with human liver microsomes is given by Wójcikowskia et al. (2004). Objective of the study was to elucidate the enzymes involved in the metabolism of perazine.

Human liver microsomes from 3 patients were used to optimize the conditions of perazine metabolism. On the basis of the obtained results, perazine metabolism in liver microsomes was studied in respect of the linear dependence of product formation on the time and concentrations of protein and substrate. Microsomal protein, 500 μg, was resuspended in 500 μl of 20 mM Tris/HCl buffer (pH = 7.4). For inhibition studies, 25 μM perazine was chosen as a therapeutic concentration in the liver, which did not saturate the enzyme. Perazine was incubated with liver microsomes and the specific CYP inhibitors: 2 μM α-naphthoflavone (a CYP1A2 inhibitor), 10 μM sulfaphenazole (a CYP2C9 inhibitor), 5 μM ticlopidine (a CYP2C19 inhibitor), 10 μM quinidine (a CYP2D6 inhibitor), 200 μM DDC (a CYP2A6 + CYP2E1 inhibitor) and 2 μM ketoconazole (a CYP3A4 inhibitor). After 3-min preincubation at 37 °C, the reaction was initiated by adding NADPH to a final concentration of 1 mM. After 15-min incubation, the reaction was stopped by adding 200 μl of methanol. Perazine and its metabolites were analyzed by HPLC. The reduction of formation of the two main metabolites formed by N-desmethylation and sulfoxidation was determined in comparison to control incubations without inhibitor.

II.H.8 Cytosol

PURPOSE AND RATIONALE

Since liver is the most important organ for metabolism investigations the procedures described here focus on liver cytosol exemplarily. Liver cytosol fraction contains soluble Phase I and Phase II enzymes which play an important role in drug metabolism (Brandon 2003). These are alcohol and aldehyde dehydrogenases, epoxide hydrolases, sulfotransferases, glutathione S transferase, N-acetyl transferases, and methyl transferases. Therefore, in cytosolic preparations these biotransformation steps can be studied. Cytosolic fractions are commercially available (BDGentest, Invitro Technologies, Xenotech and others) or easy to prepare, alternatively.

PROCEDURE

In a typical procedure to study sulfation reactions (Tabrett 2003) the reaction mixtures consists of a 6.25 mM potassium phosphate buffer (pH 7.4), containing 60 μM adenosine 3'-phosphate 5'-phosphosulfate (PAPS) as a cofactor, 75 μg liver cytosolic protein and 0.125–100 μM substrate (4-nitrophenol in the example cited here). Reaction is started by the addition of PAPS and incubated for 10 min at 37 °C. PAPS not utilized in the reaction is precipitated with 200 μL 0.1 M barium acetate, 200 μL 0.1 M barium hydroxide and 200 μL 0.1 M zinc sulphate and centrifuged for 5 min at 14 000 g.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

The conduct of stability/profiling studies in liver cytosol is most often fine-tuned to the specific biotransformation reaction which is intended to be studied (Favetta et al. 2000; Frandsen and Alexander 2000; Long et al. 2001). A generalized procedure, e.g. with fortification of the cytosol with all relevant cofactors to cover the entire cytosolic enzyme activities is uncommon. This might also be due to the fact that utilization of cytosol as a screening tool for metabolic stability is rather rare (Linget 1999) and may only come into consideration if an in vitro-in vivo correlation cannot be established on the basis of microsomal tests. On the other hand, the enzyme activities of NAT, SULT and GST are higher in cytosol preparations than in 9000g fractions giving rise to better cover the metabolites derived by these routes (Brandon 2003).

MODIFICATIONS OF THE METHOD

Conditions for cytosolic incubations depend on the aim of the assay e.g. to cover specific enzymatic activity present in the cytosol. For this purpose it is essential to fortify the incubation medium with the specific cofactor for the reaction-if needed (Ekins 1999). β -Nicotinamide adenine dinucleotide (NAD) is needed for alcohol and aldehyde dehydrogenases, flavin adenine dinucleotide (FAD) for polyamine oxidase, β-nicotinamide adenine dinucleotide phosphate (NADPH) for Dihydropyrimidine dehydrogenase. Phase II reactions depend on PAPS (sulfotransferases,

Xu 2001), glutathione (glutathione S-transferases, Raney et al. 1992; Slone 1995), acetyl-coenzyme A (N-acetyltransferases) or S-adenosylmethionine (methyltransferases). NADPH as a cofactor has to be added if cytosolic reductases are the aim of interest (Inaba 1989).

Obach (2004) set up a method for inhibition studies in cytosol using phthalazine as a substrate of aldehyde oxidase. In this system 0.05 mg protein/ml was used in 25 mm potassium phosphate buffer ph7.4 containing 0.1 mm ethylendiaminetetraacetic acid. Incubation is terminated after 2.5 min.

Chen et al. (1999, 2003) used cytosol prepared from various sections of the human intestine to study the occurrence and distribution of sulfotransferases in the gastrointestinal tract. They fortified the cytosol with PAPS. They utilized the sulfuryl group transfer from p-nitrophenol sulphate to PAP to generate PAPS for measurement of the phenol sulfotransferase activity by measurement of the colored product p-nitro-phenol. Cytosolic incubation were stopped by addition of Tris buffer, pH 8.7.

Antibodies against SULT can be incorporated into the assay (Lewis 2000; Thomas et al. 2003).

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www.xenotechllc.com (accessed in October 2004)

www.invitrotechnologies.com (accessed in October 2004)

EXAMPLE

In an automated assay developed by Linget (1999) incubations were performed using 2.5 μM substrate and cytosolic protein concentrations between 0.26 and 2.6 mg/mL. Incubations were done on a 215 Gilson liquid handler. The assay was set up for the screening of glutathione adducts. Therefore, 1,2 epoxy-3-(p-nitrophenoxy) propane was used as a model substrate and 1 mM glutathione was added. Substrate was pipetted as a solution in 0.1 M Tris HCL buffer containing 5 mM of magnesium chloride and 3 % bovine serum albumin. The BSA was added to assist dissolution of compounds with poor solubility. Incubations are done on a vibrating device. Samples are taken after incubation times of 0, 1, 2, 3, 4 and 5 min. At each of these time points an aliquot of the incubation mixture was transferred from the incubation tube into a well in a 96 deep well plate containing an equal volume of acetonitrile for quenching by protein precipitation followed by centrifugation of the plates. Supernatants were analyzed by HPLC for metabolic screening. Half-life and rate were determined for intra and in-

terassay variability showing standard deviations of less than 5 %.

II.H.9 Recombinant Enzymes

PURPOSE AND RATIONALE

Recombinant enzymes are used to elucidate the enzymes involved in certain biotransformation steps and to determine the relative contribution if more than one enzyme is involved (Rodrigues et al. 1999; Friedberg et al. 1999; Brandon et al. 2003; Donato et al. 2003). This is particularly of importance for metabolic steps which are catalyzed by polymorphic enzymes. In combination with correlation analyses from data obtained with individual human liver microsomes and inhibition experiments in liver microsomes employing either specific chemical inhibitors or antibodies a solid statement on the enzymes involved in the metabolism of a drug can be obtained in many instances.

PROCEDURE

Experimental conditions are similar to those applied in human liver microsomal incubations. In case of human P450 isozymes microsomal protein is derived from CYP transfected insect cells (Supersomes, Baculosomes) or bacteria (Bactosomes). The microsomal protein is reconstituted in phosphate buffer pH 7.4 for the incubation. The reaction requires reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for electron donation or a NADPH generating system consisting of glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PD). Magnesium chloride is used for stimulation. Test compounds are added in solution using dimethylsulfoxide most often as solubilizer in as low as possible amounts. Incubation is carried out at 37 °C over incubations time of up to about 1 h. To stop the reaction icecold acetonitrile or aqueous trifluoroacetic acid is added in equal amounts. Subsequently, precipitated protein is removed by centrifugation or filtration.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds. Determination of a full enzyme kinetics (V_{max}, K_m) is possible using various time points and concentrations.

CRITICAL ASSESSMENT OF THE METHOD

Besides expression in insect cells and bacteria (E. coli) a variety of other expression systems are used like mammalian cells, V79 hamster cells, systems in yeast (Friedberg 1999 and references cited therein). High expression level and/or high yields in producing cells are achieved in baculovirus and E. coli. Therefore, these systems are nowadays in broad application in the industrial environment, also because of their commercial availability.

Supersomes are available containing cDNA-expressed cytochrome b5 or not. Since insect cell microsomes do not contain significant amounts of cytochrome b5 the incorporation of this enzyme increases the respective cytochrome P450 activity which is of importance if the substrate specific turnover is increased.

The availability of allelic variants gives rise to genotyping studies with recombinant enzymes (Coller et al. 2002).

Organic solvents deactivate cytochrome P450 isozymes and others concentration dependently (Busby 1999). Moreover, this effect is different between the isoforms and depends also on the solvent used. Solvent influences are lowest for methanol and acetonitrile (< 10 % inhibition at 0.3 % solvent), but higher for ethanol and DMSO. Therefore, as low as possible solvent amounts should be used.

MODIFICATIONS OF THE METHOD

Commercially available (BDGentest, Invitrogen, Cypex) are a wide variety of cytochrome P450 isozymes including allelic variants expressed in insect or mammalian cells. Additional Phase I and Phase II isozymes are available. They comprise of uridine diphosphoglucuronosyl transferases (UGT), flavin monooxygenases (FMO), monoamine oxidases (MAO), microsomal epoxide hydrolases, arylamine N-acetyltransferases (NAT), glutathione transferases (GST) and sulfotransferases (SULT). According to the isozyme to be studied specific cofactors have to be incorporated: an NADPH regenerating system for P450 isozymes and FMOs, uridine diphosphoglucuronic acid (UDPGA) for UGTs in combination with alamethizine as a modifier to assist membrane transport (Ethell et al. 2003; Kuehl and Murphy 2003), acetyl-coenzyme A for the cytosolic N-acetyl trans-

ferases, glutathione for GST's and PAPS for SULTs. If radiolabeled cofactors are available ([^{3}H]GST, [^{14}C]UDPGA) their use facilitates in some instances the detection of the metabolites formed even from non-labeled substrates.

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www.gentest.com (accessed in October 2004)

www.invitrogen.com (accessed in October 2004)

www.cypex.co.uk (accessed in October 2004)

EXAMPLE

Example 1

The example for applying cDNA expressed P450 isozymes is given by Wójcikowskia et al. (2004). Objective of the study was to elucidate the enzymes involved in the metabolism of perazine.

Microsomes from baculovirus-infected insect cells expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Supersomes) were obtained from Gentest. All the Supersomes were coexpressed with human P-450 reductase.

CYP2E1 was additionally coexpressed with the human cytochrome b-5. Microsomal protein, 500 μg, was resuspended in 500 μl of 20 mM Tris/HCl buffer (pH = 7.4). Study into perazine metabolism in Supersomes was carried out at the neuroleptic concentration of 750 μM (3 K_m) allowing to reach the velocity of reaction of about V_{max} to show the maximum ability of cDNA expressed enzyme to metabolize perazine. After 3-min preincubation at 37 °C, the reaction was initiated by adding NADPH to a final concentration of 0.1 mM. After 2 h incubation, the reaction was stopped by adding 200 μl of methanol. Perazine and its metabolites were analyzed by HPLC.

Example 2

A fully automated assay for the determination of the intrinsic metabolic clearance in cytochrome P450 isozymes has been developed by McGinnity (2000) using the major drug-metabolizing human hepatic cytochrome P450s (CYP1A2, -2C9, -2C19, -2D6, and -3A4) coexpressed functionally in E. coli with human NADPH-P450 reductase, to predict the CYPisoform(s) involved in the oxidative metabolism of NCEs.

CYP CL_{int} determination assays were performed by a robotic sample processor (RSP) (Genesis RSP 150; Tecan, Reading, UK). Assays performed by the RSP were done using a program created by the user and not by a default program supplied with the hardware. The primary stock of all probe substrates was prepared manually in dimethyl sulfoxide or acetonitrile at 100-fold final incubation concentration. The final concentration of organic solvent in the incubation was 1 % v/v. At this concentration dimethyl sulfoxide has been shown to reduce the activities of CYP2C9/19, although this effect appears to be substrate-dependent. All substrates were incubated at 3 μM except tolbutamide (CL_{int} calculated by determining V_{max} and K_m), ibuprofen (10 μM), and testosterone (10 μM). The RSP was programmed to add chilled quenching solvent (100 μl of acetonitrile) to 96-well refrigerated blocks, and compound stocks were then prediluted in 100 mM potassium phosphate buffer, pH 7.4. *E. coli* membranes and microsomes prepared from baculovirus coexpressing individual CYPs and NADPH reductase were added to incubation tubes (100 pmol of CYP/mL final concentration) located in a 96-well heated block (37 °C). A subaliquot was removed to produce a 0-min

time point, and the assay was initiated via addition of NADPH (1 mM final concentration). Aliquots (50 µl) were removed at 5, 10, 15, and 20 min and quenched in acetonitrile. Samples were subsequently removed from the RSP, frozen for 1 h at –20 °C, and then centrifuged at 3500 rpm for 20 min. The supernatants were removed and transferred into HPLC vials using the RSP. Test compounds used for the validation of the method (diltiazem, testosterone, dextromethorphan, propranolol, metoprolol, diazepam, tolbutamide, ibuprofen, verapamil, omeprazole) were analyzed by HPLC under compound specific conditions.

II.H.10
Blood, Plasma and Serum

PURPOSE AND RATIONALE

Mainly due to esterase activities many drugs reveal degradation in blood (Williams 1987). In some instances, namely if a prodrug concept (Huryn-DM 2004) is applied e.g. for enhancing the absorption of a drug, cleavage of the ester prodrug in blood is intended for liberation of the pharmacologically active compound. Therefore, stability studies in blood are important. In most instances, these studies are performed in plasma or serum derived from human and/or animal blood. However, also red blood cells are accountable for metabolic activity.

PROCEDURE

In a typical procedure the drug to be investigated is spiked to serum or plasma in a concentration of 25 µM and subsequently incubated at 37 °C over a time of up to several hours (typically 5 min to 1 h). After addition of acetonitrile, denatured proteins are removed and the supernatant is analyzed appropriately.

EVALUATION

Samples obtained are analyzed by chromatographic means, e.g. LC-MS/MS, HLPC using UV detection or radiodetection, if radiolabeled substrates are applied. Metabolic stability as final result is given as percent remaining after incubation by dividing peak area of parent compound in the sample by the area of the time 0 sample and multiplication by 100. Also evaluation of parent and metabolites detected is possible for generating a metabolic profile, in particular using radiolabeled compounds.

CRITICAL ASSESSMENT OF THE METHOD

In general, serum as well as plasma reflect the enzymatic status in blood in a similar manner. However, it may become relevant that the coagulation cascade is suppressed during preparation of plasma by addition of citrate, heparin or EDTA whereas in serum those enzymes are present in non-physiological concentrations. Since these enzymes, e.g. thrombin reveal proteolytic activity (Lafleur 2001), this has to be considered if studying the stability of peptides.

Stability tests in plasma and serum are fixed part in the validation procedures of bioanalytical assays (FDA 2001).

Besides modifications of time and concentrations incubations in serum or plasma appear to be rather simple and reliable in predicting the in vivo relevance of the in vitro data.

MODIFICATIONS OF THE METHOD

It can be appropriate to coincubate the compound of interest in the presence of inhibitors of serum esterases. Used is sodium fluoride, physostigmin or ecothiophate iodide (Chien 1990; Quon et al. 1993). In case of carboxy- or aminopeptidase cleavage of peptides specific peptidase inhibitors like amastatin, bestatin, phenylmethylsulphonylfluoride, 1,10-phenanthroline or ethylenediamine tetra acetic acid (EDTA) are useful (Lee 1995).

An esterase-like activity of human serum albumin (HSA) might also contribute to the serum instability of esters which can be studied in buffer preparations containing albumin (Ohta 1987).

Quon et al. (1985) investigated the stability of esmolol in blood, plasma, red blood cells, and purified enzymes (human serum pseudocholinesterase, human and dog serum albumin, acetyl choline esterase, carbonic anhydrases A and B and human haemoglobin). Udata et al. (1999) studied the hydrolysis of propranolol ester prodrugs in purified acetylcholine esterase.

Some authors reported the use of serum or plasma diluted to 80 % or less instead of the native matrix (Di-Stefano 2001, Mahfouz 1999; Scriba 1993).

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EXAMPLE

An example for an automated stability test in plasma is described by Linget and du Vignaud (1999). Incubations are performed on a 215 Gilson liquid handler. Incubation was done at substrate concentrations of 50 μM on 96 deep well plates. Each incubation tube contained 375 μL of a 200 μM test compound solution (in 0.1 M Tris buffer with 3 % BSA, added to assist dissolution of compounds with poor solubility) and 1125 μL of plasma. Samples are taken after incubation times of 0, 1, 2, 3, 4 and 5 min. At each of these time points an aliquot of the incubation mixture was transferred from the incubation tube into a well in a 96 deep well plate containing an equal volume of acetonitrile for quenching by protein precipitation followed by centrifugation of the plates. Supernatants were analyzed by HPLC for metabolic screening.

Chapter II.I
Distribution – Across Barriers

Tanja Eisenblaetter

INTRODUCTION

Distribution of drugs across barriers plays a predominant role in the processes of absorption (A), distribution (D) and excretion (E) and is thus a major determinant of a drugs pharmacokinetic profile. The barrier at the site of absorption is in most cases built by the enterocytes of the small intestine (see section on Absorption). Apart from lung, heart, muscle and brain, main target organs are kidney and liver, which are also the major elimination pathways of drugs. Cell membranes of liver hepatocytes and kidney cells have to be passed in these cases. The brain plays a special role in the distribution of drugs because drugs normally should not enter the central nervous system to avoid severe adverse effects. It is, therefore, protected by the very tight blood-brain barrier. However, when the target is located in the brain, the drug needs to cross this barrier. Meanwhile, there exist several in vitro systems to study drug permeation across this special blood-brain barrier. They cover labor-intensive primary cultures of brain endothelial cells, more or less characterized immortalized cell lines and surrogate models, which allow a very high throughput but hardly resemble the blood-brain barrier phenotype.

In the future, specific drug transporters will alternatively be addressed for a successful drug delivery to or exclusion from target organs, because drug transporters are expressed in specific cell membranes of various tissues, where they have pivotal roles in the distribution and thus determining the blood-concentration-time profiles and therefore the area under the curve (AUC) of particular drugs. Major membrane transporters have been classified into the solute carrier (SLC) transporter family and the ATP-binding cassette (ABC) transporter family by the HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/). The SLC family consists of 43 gene subfamilies and a total of ~ 300 family members, including ion-coupled transporters, facilitated transporters and exchangers (http://www.bioparadigms.org/slc/). In the ABC transporter family, 56 genes have been identified and classi-

fied into seven subfamilies (http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html). Some of these transporters accept not only physiological or endogenous substrates, but also xenobiotics, including drugs, and are therefore referred to as drug transporters. Typical drug transporters are members of the families of organic solute transporters SLC22A (OCTs, OCTNs and OATs), organic anion transporting polypeptides SLC21A/SLCO (OATPs), oligopeptide transporters SLC15A (PEPTs) and ABC transporters ABC (e.g. Pgp, MRPs). By knowing the substrate specificity and the expression profile of each transporter (Table 1 summarizes the most important drug transporters and their localization at targt organs), efficient drug delivery to, or exclusion from, the target organs can be accomplished by appropriately modifying the structure of the drug. Another aspect that demands careful consideration is drug-drug interaction, because the pharmacokinetics of drugs that are transporter substrates might be influenced by co-administered drugs that work as inhibitors or enhancers of the transporter function.

To study these drug transporter interactions, in vitro systems expressing single transporters or transporter combinations can successfully be used. Drug uptake mediated by SLC transporters can be measured by using transfected eukaryotic cells like HEK, CHO or injected oocytes. Drug efflux processes mediated by ABC transporters are investigated using membrane vesicles from either eukaryotic cell lines like HEK, CHO, MDCK or insect cells. By using double transfected MDCK cells, drug uptake and efflux can be measured in one single set-up. For liver specific drug transport sandwhich-cultured hepatocytes can be used.

II.I.1 Blood-Brain Barrier (BBB)

II.I.1.1 Primary Cultures of Brain Capillary Endothelial Cells

II.I.1.1.1 Primary Cultures of Porcine Brain Microvascular Endothelial Cells

PURPOSE AND RATIONALE

Franke et al. (1999) have described an in vitro model for screening of drug entry into the brain using primary cultures of porcine brain capillary endothelial cells (PBCEC). By using serum-free culture conditions transport studies are highly reproducible and sample analysis (HPLC, LC-MS) is facilitated.

PROCEDURE

Porcine brain microvessel endothelial cells are prepared following a method of Bowman et al. (1983) using modifications described by Tewes et al. (1997) and Franke et al. (2000). Hemispheres of porcine brains of freshly slaughtered animals (female, about 6 months) are collected in ice-cold ethanol (70 % v/v) and stored in PBS (4 °C; containing penicillin/streptomycin (200 U/ml each)) for transport and during the preparation procedure. After flaming the hemisphere shortly the meninges and adhering larger vessels are completely removed. Secretory brain areas are dissected and the remaining cerebral grey and white matter is homogenized mechanically. The homogenate is supplemented with preparation medium (Earle's Medium M199 containing 0.7 mM L-glutamine, 1 % (v/v) penicillin/streptomycin (10 mg/ml) and 1 % (v/v) gentamicin (10 mg/ml)) to a final volume of 50 ml per brain hemisphere and 1 % (w/v) dry powdered unspecific protease/dispase II (Sigma) is added for an incubation period of 2 h at 37 °C with magnetic stirring. 150 ml of 18 % (w/v) dextran solution per 100 ml of digested homogenate is then added, shaken and spun with 6,800 × g for 10 min at 4 °C. After dextran density gradient centrifugation capillary fragments are obtained as pellet and are resuspended in culture medium. This microvessel-fragment suspension is filtered through a nylon mesh (180 μm poresize) and then triturated up and down 3 to 5 times in a 10 ml pipette with the pipette tip placed directly onto the bottom of a petri dish. For the second digestion the suspension is incubated with 0.1 % (w/v) collagenase/dispase (Boehringer) in 10 ml plating medium (preparation medium + 10 % (v/v) ox serum) per brain equivalent for 1 h at 37 °C using a flask with hanging stirrer. A discontinuous percoll gradient is prepared from 15 ml percoll solution (δ = 1.07 g/ml) and 20 ml percoll solution (δ = 1.03 g/ml). 10 ml of the cell suspension is placed onto the top of the gradient and centrifuged in a swing-out bucket rotor (1 250 × g, 10 min, room temperature). Released endothelial cells are collected from the interface of the gradient and washed with an excess of plating medium (110 × g, 10 min, room temperature). The cells are resuspended very gently in plating medium and seeded onto collagen-coated culture surfaces (seeding density: 450 cm^2 per brain). Cultures are kept at 37 °C in a humidified atmosphere with 5 % CO_2. 24 h after initial plating, cells are washed with

Table 1 Members of transporter families and their expression profiles.

Organic Solute Transporter Family (SLC22A)		Organic Anion Transporting Polypeptide Family (SLC21A/SLCO)		ABC Transporter Family (ABC)	
Names (Gene Symbols)	Expression Profile[1]	Names (Gene Symbols)	Expression Profile[1]	Names (Gene Symbols)	Expression Profile[1]
Organic cation transporters		OATP-A (SCL21A3, SLCO1A2)	b (bbb), k, l	MDR1 (ABCB1)	l, b (bbb), k, i
OCT1 (SLC22A1)	l	OATP-B (SLC21A9, SLCO2B1)	l	BSEP (ABCB11)	l
OCT2 (SLC22A2)	k, b	OATP-C (SLC21A6, SLCO1B1)	l	MRP1 (ABCC1)	ubi
OCT3 (SLC22A3)	l, k, h, lu	OATP-D (SLC21A11, SLCO3A1)	ubi	MRP2 (ABCC2)	l, k, i
Organic cation/ carnitine transporters		OATP-E (SLC21A12, SLCO4A1)	ubi	MRP3 (ABCC3)	l, k, i
OCTN1 (SLC22A4)	k, h	OATP-F (SLC21A14, SLCO1C1)	b	BCRP (ABCG2)	l, i, b (bbb)
OCTN2 (SLC22A5)	k, lu, h, i, l	OATP-8 (SLC21A8, SLCO1B3)	l		
Organic anion transporters					
OAT1 (SLC22A6)	k, b	Oligopeptide Transporter Family (SLC15A)			
OAT2 (SLC22A7)	l, k	Names (Gene Symbols)	Expression Profile[1]		
OAT3 (SLC22A8)	k, b (cp)	PEPT1 (SLC15A1)	i, k		
OAT4 (SLC22A11)	k	PEPT2 (SLC15A2)	k, lu, b (cp)		

[1]Expression in kidney (k), brain (b), choroid plexus (cp), blood-brain barrier (bbb), liver (l), intestine (i), lung (lu), heart (h), ubiquitously (ubi).

PBS containing Ca^{2+} (0.9 mM) and Mg^{2+} (0.5 mM) and supplied with culture medium (plating medium without gentamicin). For further purification and to obtain a homogenous monolayer, primary cultures of PBCEC are subcultured at second day of culture by gentle trypsination (1:250 trypsin solution in PBS) at room temperature. The displacement of endothelial cells is followed with a reverse phase-contrast microscope and stopped by addition of ox serum when the majority of endothelial cells are removed from the culture surface. The cells are collected and centrifuged with 110 × g for 10 min at room temperature and plated in culture medium with a density of 30 000 cells/cm^2 on rat tail collagen coated Transwell or Transwell-Clear filter inserts (24 mm diameter, 0.4 mm pore size, Corning Costar). Medium volumes are 1.5 ml apical and 2.5 ml basolateral. On the second day after passage the culture medium is replaced by a serum-free assay medium (DME/Ham's F12; 1:1) containing 550 nM hydrocortisone. Within two to four days after this medium exchange the PBCEC have built up a very tight monolayer and are ready for BBB in vitro-transport experiments.

Coating with Rat-tail Collagen

Collagen fibers are collected, as described by Bornstein et al. (1958). Fibers of each tail are incubated in 50 ml 0.1 % (v/v) acetic acid at 4 °C for 24 h. Centrifugation for 2 h at 5000 × g separates the fiber residues from solubilized collagen which is aspirated. Total protein content of the collagen solution is determined and adjusted to 0.1 mg/ml. Transwell or Transwell-Clear filters are coated by adding 100 µl of rat-tail collagen solution per

filter and allowed to dry for at least 2 days at 37 °C. No further washing steps are occurred (Franke et al. 1999).

Transport Measurements

Directly prior the experiment the quality, that is the tightness, of the PBCEC monolayer is assessed by determination of the transendothelial electrical resistance (TEER) of each filter. TEER measurements can be easily performed with the ENDOHM-24 chamber and the EVOHM voltometer (World Precision Instruments, Inc,). The resistance data, expressed in Ω, are multiplied with the filter surface (4.52 cm^2), corrected for blank filter resistance and expressed in $\Omega \times cm^2$.

To measure the transport of drugs across the BBB in vitro 2.5 μCi of ^{3}H-labelled drug and ^{14}C-sucrose are applied to each Transwell (in case of ^{14}C-labeled substances, permeability studies are performed with ^{3}H-sucrose). This concentration is high enough to ensure sufficient excess to neglect the decrease of tracer in the donor (apical) compartment during the experiments. Volumes of 1.5 ml in the donor (apical) and 2.5 ml in the acceptor (basolateral) compartment avoid hydrostatic pressure. After addition of the radiolabeled compound, samples of 50 μl are taken in duplicate from the basolateral acceptor compartment every 20 min and replaced by 100 μl of fresh assay medium. Cells are kept under culture conditions during the whole transport experiment. Radioactivity is measured after addition of liquid scintillation cocktail in a counter.

Uptake Experiments

To determine the uptake of test substances, filters are gently washed 90 min after drug application with PBS and cells are solubilized with a solution containing 0.1 M NaOH, 1 % (w/v) sodium-dodecylsulfate and 2 % (w/v) Na_2CO_3. Radioactivity of the solubilized cells is counted and compared with the radioactivity of the medium in the donor compartment at 90 min.

Calculation of Permeability Coefficients

Following the majority of publications, permeability coefficients can be calculated according to Pardridge et al. (1990). The permeability coefficient of the endothelial cell layer P_e is calculated as follows:

$$1/P_e = 1/P_t + 1/P_f$$

(P_t permeability coefficient of the total system and P_f permeability coefficient of the cell-free filter)

$$P[cm/s] = \frac{cpm_t^a \times volume^d[cm^3]}{\Delta t[s] \times filter\text{-}area[cm^2] \times cpm_0^d}$$

(cpm_t^a total radioactivity in the acceptor compartment after t seconds; cpm_0^d total radioactivity added at time 0 to the donor compartment; Δt time measured in seconds; filter-area here 4.52 cm^2; $volume^d$ here 1.5 ml)

EVALUATION

Under these culture and assay conditions PBCEC displayed sucrose permeabilties as low as 2×10^{-7}cm/s. This tightness becomes close to in vivo-permeabilities of 3×10^{-8}cm/s (Ohno et al. 1978) and 1.2×10^{-7}cm/s (Levin et al. 1980). Data are expressed as mean ± standard deviation (n = 3–6).

CRITICAL ASSESSMENT OF THE METHOD

The absence of serum and presence of hydrocortisone in this in vitro model ensures the appropriate differentiation of brain endothelial cells with a characteristic BBB phenotype including a) impermeable intercellular tight junctions (Nitz et al. 2001, 2003 and Eisenblaetter et al. 2001), b) low pinocytotic activity, c) expression of specific transporters like SGLT1 (Elfeber et al. 2004), neutral amino acid transporter, P-glycoprotein (Eisenblaetter et al. 2003), ABCG2 (Eisenblaetter and Galla 2002) and the transferrin, scavenger and LDL receptors (Nitz et al. 2001), d) expression of specific enzymes like γ-GT (Mischeck et al. 1989) and alkaline phosphatase (Meyer et al. 1990), and finally e) an efficient endothelial barrier function with low paracellular permeability (Hoheisel et al. 1998; Franke et al. 1999, 2000; Nitz et al. 2003).

As described above, TEER measurements can be performed with the ENDOHM-24 chamber and the EVOHM voltometer. Use of the STX-2 “chopstick” electrodes (World Precision Instruments, Inc) leads to inaccurate data and cannot be recommended for TEER measurements. Very much in favor is a technique called impedance spectroscopy (Wegener et al. 1999).

MODIFICATIONS OF THE METHOD

Prof. H-J Galla (University of Muenster, Germany) has already established an in vitro BBB model with murine brain capillary endothelial cells based on this procedure (not yet published). Thus, this procedure might easily be adapted to brain endothelial cells of other relevant species in drug discovery like rat, dog, rabbit.

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EXAMPLE

The industrial application of ranking of CNS penetration was nicely shown by Lohmann et al. (2002). In this work different in vitro assays were evaluated in order to predict the BBB permeability.

II.I.1.1.2
Cocultures of Bovine Brain Microvascular Endothelial Cells and Rat Astrocytes

PURPOSE AND RATIONALE

The passage of substances across the blood-brain barrier (BBB) is regulated in the cerebral capillaries, which possess certain distinct different morphological and enzymatic properties compared with the capillaries of other organs. To provide an in vitro system with the functional characteristics of a BBB Cecchelli et al. (1999) developed an in vitro system with cloned bovine brain capillary endothelial cells on one side of a filter and rat astrocytes on the other side.

PROCEDURE

The cloning of endothelial cell islands is purchased according to Méresse et al. (1989). In brief, microvessels are isolated by mechanical homogenisation from one cerebral hemisphere and then seeded onto dishes coated with an extracellular matrix secreted by bovine corneal endothelial cells. Only capillaries adhere on the extracellular matrix, whereas arterioles and venules can easily be discarded. Five days after seeding, the first endothelial cells migrate out of the capillaries and start to form microcolonies. When the colonies are sufficiently large, the five largest islands are trypsinized and seeded onto 35 mm diameter gelatin coated dishes (one clone per dish) in the presence of Dulbecco's modified Eagle's medium supplemented with 15 % calf serum, 2 mM glutamine, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and basic fibroblast growth factor (bFGF, 1 ng/ml added every other day). Endothelial cells from one 35 mm diameter dish are then harvested and seeded onto 60 mm diameter gelatin-coated dishes. After 6–8 days, confluent cells are subcultured at the split ratio of 1:20. Cells at the third passage are stored in liquid nitrogen.

For experiments, cells are rapidly thawed at 37 °C and seeded onto two 60 mm diameter gelatin coated dishes. Once they reach confluence, cells are subcultured up to passage eight. The life span of the endothelial cell cultures is about 50 cumulative population doublings. At each passage, the cells are seeded in stock gelatin coated dishes and on microporous membranes. At this stage, care needs to be given in selecting the matrix on the microporous membrane

which will support cell attachment, growth and differentiation. Different cell culture inserts and coatings were evaluated by Cecchelli et al. (1999) and they found self-made rat-tail collagen on Millicell-CM inserts (Millipore, 0.4 μm pore size, 30 mm diameter) to give well organized and confluent monolayers with typical junctions between them.

Preparation of Rat Astrocytes

Primary cultures of astrocytes are made from newborn rat cerebral cortex according to Dehouck et al. (1990). The meninges is cleaned off and the brain tissue is forced gently through a nylon sieve. DMEM supplemented with 10 % fetal calf serum, 2 mM glutamine, and 50 μg/ml of gentamicin is used for the dissociation of cerebral tissue and development of astrocytes.

Cocultures of Bovine Brain Capillary Endothelial Cells (BBCEC) and Astrocytes

Astrocytes are plated at a concentration of 2.5×10^5 cells/ml on the bottom side using the filter upside down. After 8 days, filters are properly positioned, and the medium is changed twice a week. Three weeks after seeding, cultures of astrocytes become stabilized. Then, frozen BBCEC are recultured on a 60 mm diameter gelatin-coated dish. Confluent cells are trypsinized and plated on the upper side of the filters at a concentration of 4×10^5 cells. The medium used for the coculture is DMEM supplemented with 15 % calf serum, 2 mM glutamine, 50 μg/ml of gentamicin and 1 ng/ml of bovine fibroblast growth factor added every other day. Under these conditions, BBCEC form a confluent monolayer in 8 days (Dehouck et al. 1990).

EVALUATION

Cecchelli et al. (1999) compared the extraction ratios in vivo to the permeability of the in vitro model and showed a strong correlation between both values.

CRITICAL ASSESSMENT OF THE METHOD

The relative ease with which such cocultures can be produced in large quantities facilitates the screening of new CNS drugs. This model provides an easier, reproducible and mass-production method to study the blood-brain barrier in vitro.

Using this model one has to consider that two different species are used and that cow is a species not typically used in pharmaceutical development. Species differences according transporter expression and lipid membrane composition has to taken into account.

Most of the laboratories use enzymatic digestion to isolate endothelial cells. Often cells of capillary, arteriolar and venular origin as well as pericytes lead to a heterogenous mixture of these different cellular types. The subculture technique enables circumvention of the limitations of primary cultures and to provide large quantities of these monolayers. Endothelial cells can be cultured from passage three after thawing to passage seven, each passage generating at least 75 cocultures.

A complete characterization is summarized by Dehouck et al. (1990).

MODIFICATIONS OF THE METHOD

Instead of using cococulteres with rat astrocytes, some laboratories are also using astrocytic conditioned medium in order to reinduce blood-brain barrier properties (Dehouck et al. 1994). They have found that induction does not require direct cell-cell interactions.

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EXAMPLE

Cocultures of bovine capillary endothelial cells and astrocytes can be used to study the permeability of drugs (Dehouck et al. 1990). Culture plate inserts are set into six-well plates with 2 ml of buffer added to the upper chamber and 2 ml addded to the plate coating the inserts. Radiolabeled compounds are added to the upper chamber and 100 μl is removed from the lower chamber at various times. The radioactivity can be determined and the permeability can be calculated. Using such techniques ranking of CNS drugs concerning their permeability into the brain can be achieved.

II.I.1.2 Immortalized Cell Lines

II.I.1.2.1 Immortalized Human Cerebromicrovascular Endothelial Cells

PURPOSE AND RATIONALE

Primary cultures of cerebromicrovascular endothelial cells (CEC) derived from rat, bovine, porcine, mouse and human can rapidly lose key phenotypic markers of the blood-brain barrier (BBB) and undergo cellular senescence after a limited number of divisions in vitro. Furthermore, expression of BBB markers varies considerably among BCEC obtained from different species. These issues, compounded with the problems associated with very limited availability of human brain biopsies, small initial yield of cells and short proliferative life span of human cells, greatly restrict the utility of primary human BCEC as a reliable in vitro BBB model. Therefore, Muruganandam et al. (1997) developed an immortalized human cerebromicrovascular endothelial cell line as an in vitro model of the human blood-brain barrier.

PROCEDURE

Human CEC are isolated by using a modification of the procedure described by Gerhart et al. (1988). Briefly, the brain samples are excised surgically from patients treated with idiopathic epilepsy (Stanimirovic et al. 1996) and cleaned of meninges, surface vessels, cortical grey matter. The tissue is minced and homogenized and passed once though 350 μm and twice through 112 μm mesh nylon nets. The filtrate is then centrifuged at 1000 × g for 10 min and the pellet is resuspended in 20 % dextran (mol wt. 70 000) and again centrifuged at 3000 × g for 15 min. The microvessels and capillaries contained in the pellet are collected on a 20 μm nylon mesh, dislodged, and exposed to 1 mg/ml type IV collagenase for 15 min at 37 °C. The dissociated microvessels are collected by centrifugation and resuspended in growth media containing 65 % medium M199 (Earles's salts, 25 mM HEPES, 4.35 g/l sodium bicarbonate, and 3 mM L-glutamine), 10 % fetal bovine serum, 5 % human serum, 20 % murine melanoma cell-conditioned media (mouse melanoma, Cloudman S91, clone M-3, melanin-producing cells), 5 μg/ml of insulin, 5 μg/ml transferrin, 5 ng/ml selenium and 10 μg/ml endothelial cell growth supplement (ECGS). Dissociated microvessels are seeded in 0.5 % gelatin-coated plastic tissue culture dishes and maintained at 37 °C in an atmosphere of 5 % CO_2 in air. Endothelial cell colonies emerging from attached microvessels at days 4–5 after seeding are isolated using cloning rings; two or three of these cloned colonies are pooled and grown to confluence (10–14 days). The cells are further propagated at 1:3 split ratio every 7–10 days (Muruganandam et al. (1997): human cerebromicrovascular endothelial cells (HCEC).

Transfection of Human CEC with pSV3-neoplasmid

Human CEC are transfected with the pSV3-neoplasmid encoding for the SV40 large T antigen and the neomycin resistance gene by using a calcium phosphate coprecipitation method (Graham et al. 1973). Transfection solution containing 2.5 M $CaCl_2$ (65 μl), 30 μg of pSV3-neo DNA, 500 μl HBSS buffer (275 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 4.2 mM HEPES, 11 mM dextrose; pH 7.19) and 400 μl water is vortexed briefly and allowed to precipitate for 30 min at room temperature. Human BCEC grown to near confluence in 100 mm dishes are exposed to the transfection solution for 6 h at 37 °C. After removing the transfection solution, the cells are subjected to a short (90 s) glycerol shock (20 % v/v) and washed with HEPES buffer, pH 7.1. Fresh growth media is added and the cells are allowed to grow for 48 h at 37 °C. Transfected cell cultures are then split (1:3) and seeded in the media containing the neomycin analog G418 (400 μg/ml). Cell colonies that survive 72 h of G418 treatment are isolated using cloning rings and passaged three times in G418-containing medium. The colonies that yielded cultures with cobblestone morphology should be used for initial genotypic and phenotypic analyses. They are called SV-HCEC.

Transport Studies

The barrier-like properties of SV-HCEC are developed in cells grown on a 0.5 % gelatin-coated Falcon tissue culture insert (1 μm pore size; 0.83 cm^2 surface area) in 1 ml growth medium. The bottom chamber of the insert assembly contains 2 ml of growth medium either alone or supplemented with the fetal human astrocyte (FHAS)-conditioned medium in a 1:1 (v/v) ratio. The FHAS-conditioned medium is obtained by incubating confluent FHAS in serum-free M199 for 72 h. The transendothelial electrical resistance (TEER) of the endothelial cell monolayers can be measured with the ENDOHM-24 chamber voltometer (Worl Precision Instruments, Inc.). Paracellular passage of ^{3}H-sucrose (2 μCi/ml) and ^{14}C-inulin (0.5 μCi/ml) across triplicate SV-HCEC monolayers and across the 0.5 % gelatin-coated inserts without cells is determined from clearance values (Pardridge et al. 1990). Samples are collected from the bottom chambers every 15 min over

120 min period. Clearance volume [μl] was calculated as:

$$CL[\mu l] = (C_b \times V_b)/C_a,$$

where C_b is the basolateral tracer concentration, V_b the volume of the basolateral (lower) chamber, and C_a is the initial apical tracer concentration. The slopes of the clearance curves for membranes alone (PS_m) and membranes with cell monolayers (PS_{m+e}) are calculated using linear regression analysis to give PS [μl/min]. The PS_e value for the SV-HCEC monolayer is calculated from:

$$1/PS_e = 1/PS_{m+e} - 1/PS_m.$$

The permeability coefficient P_e [cm/min] for the endothelial monolayer is derived by dividing PS_e by surface area (0.83 cm^2) of the membranes.

EVALUATION

Permeability coefficients for paracellular diffusion of ^{3}H-sucrose and ^{14}C-inulin across monolayers of HCEC and immortalized SV-HCEC and transendothelial resistances have been compared to human lung microvascular endothelial cells to evaluate the BBB phenotype of the cerebromicrovascular endothelial cell cultures (Muruganandam et al. 1997).

CRITICAL ASSESSMENT OF THE METHOD

Immortalization of especially human cerebromicrovascular endothelial cells is an invaluable tool for identifying specific aspects of the human BBB physiology and to analyse drug penetration into the human CNS. Nevertheless, D. Stanimirovic uses in all her following publications the primary cultures of HCEC and not the immortal cell line SV-HCEC to study transporter and receptor expression at the human BBB and to investigate the complicated brain inflammation processes. Possibly the phenotype of HCEC is not retained in the Simian virus 40 large tumor antigen transfected SV-HCEC cell line. One should probably think of a milder immortalization method when developing an immortalized human brain capillary endothelial cell line.

MODIFICATIONS OF THE METHOD

Rat brain capillary endothelial cells have been immortalized by the entire E1A region of adenovirus 2 by Roux et al. (1994) which was used for a number of cell types without oncogenic transformation. Bovine umbilical vein endothelial cells have been immortalized by transfection with an expression vector containing the human papillomavirus type 16 E6E7 oncongenes (Cajero-Juarez et al. 2002).

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EXAMPLE

Human CEC cultured on filter inserts can be used to measure brain penetration of drugs to make in vitro–in vivo correlations. They can further be used to study for example brain inflammation that has been implicated in the development of brain edema and secondary brain damage in ischemia and trauma. Zhang et al. (2000) studied leukocyte infiltration across the blood-brain barrier and showed the expression of adhesion molecules and leukocyte chemoattractants under ishemia in vitro conditions.

II.I.1.2.2
Immortalized Rat Brain Microvascular Endothelial Cells

PURPOSE AND RATIONALE

To overcome the large variability in the starting material and the rapid senescence observed after passages of pure cerebromicrovascular endothelial cells from primary cultures, Roux et al. (1994) produced an immortalized cellular clone displaying a stable, non-transformed phenotype. The aim was to constitute a valuable in vitro model of the rat blood-brain barrier

in order to make mechanistic studies and to avoid pharmaceutical drug screening for CNS penetration in vivo.

PROCEDURE

Primary cultures of rat brain microvessel endothelial cells are prepared according to the method of Bowman et al. (1982) and Roux et al. (1989). Briefly, two brains from 3-month-old Sprague Dawley rats are removed, cleaned of meninges and white matter, minced, and incubated for 1 hour at 37 °C in 25 ml of minimal essential medium (MEM) containing 1 mg/ml collagenase/dispase, 1 % bovine serum albumine (BSA), and 15 mM Hepes, pH 7.4. After enzymatic treatment, brain tissue is suspended in 20 ml medium containing 25 % BSA. Microvessels are pelleted by centrifugation at 1000 × g for 10 minutes to eliminate contaminating cells and debris, passed through a 120-μm nylon mesh, and incubated in 10 ml MEM containing 1 mg/ml collagenase/dispase for 3 hours. Clumps of cells are layered over 7 ml Percoll gradients prepared by centrifuging 50 % isotonic Percoll at 25 000 × g for 1 hour. The band containing mainly endothelial cells is removed and seeded onto extracellular matrix (ECM)-coated dishes (Gospodarowicz et al. 1986). Cultures are maintained in Alpha MEM/Ham's F10 (1:1) supplemented with 10 mM Hepes, 2 mM glutamine, 100 μg/ml gentamycin, 20 % heat-inactivated, rat plasma-derived serum and 1 ng/ml basic fibroblast growth factor, in humidified 5 % CO_2/95 % air at 37 °C. After 3 days, contaminating pericytes surrounding endothelial cell colonies are removed with a modified glass Pasteur pipette. Experiments can be performed on confluent once-passaged 3-week-old cultures (Régina et al. 2001).

Transfection of Rat Brain Capillary Endothelial Cells

The plasmid pE1A-neo carries the entire E1A region of adenovirus 2 and the neo gene for resistance to the aminoglycoside G418 (Roux et al. 1994). Transfection is carried out using the calcium phosphate precipitation procedure. Endothelial cells are transfected 24 hours after the second passage, at ~50 % confluence, with 1–10 μg plasmid DNA. After 3 hours in the presence of DNA, cells are submitted to a 2 minute DMSO (10 %) shock, then rinsed and refed with complete medium. They are trypsinized 25 hours later and seeded at various densities in 6 cm dishes in complete medium supplemented with 300 μg/ml G418. The selective medium is changed every 3–4 days. The clones appear ~2 weeks later; they are individually trypsinized within cloning cylinders and transferred into collagen-coated 24-well plates. For experiments immortalized rat brain microvessel endothelial cells are plated on rat tail collagen I-coated plates and maintained in a-MEM/Ham's F10 (1:1 v/v) with glutamax containing 10 % heat-inactivated FCS, 300 μg/ml geneticin (G418) and 1 ng/ml basic fibroblast growth factor (bFGF) in humidified 5 % CO_2/95 % air at 37 °C.

Induction of BBB Phenotype by Astrocyte-conditioned Medium (ACM)

For endothelial cell treatment, the medium is changed to a 1:1 (v/v) mixture of standard endothelial cell culture medium and ACM (Régina et al. 2001). ACM is obtained by incubating 3–5-week-old confluent astrocytes for 48 h in serum-free medium supplemented with 2 mM glutamine and 100 μg/ml gentamicin. Primary cultures of astrocytes are prepared from the cerebral cortex of three newborn rats. After removing meninges, the brain tissue is forced gently through a nylon sieve. Astrocytes are plated at a concentration of 1.2×10^5 cells/ml in 75 cm^2 flasks in αMEM/Ham's F10 (1:1 v/v) with glutamax containing 10 % FCS and 100 μg/ml gentamicin. Two weeks after seeding, astrocytes are purified by overnight shaking at 37 °C, and treatment with cytosyl arabinoside and 3-estermethylleucine according to the method of Meyer et al. (1989). Three weeks after seeding, astrocytes are used for preparation of ACM.

EVALUATION

The BBB model system of RBE4 cells (Roux et al. 1994) is used in many publications to study brain transport (carnitine: Mroczkowska et al. 2000; P-glycoprotein and Mrp1: Begley et al. 1996 and Régina et al. 1998) as well as drug metabolising enzyme activities (Chat et al. 1998).

CRITICAL ASSESSMENT OF THE METHOD

Immortalization of brain capillary endothelial cells is a difficult process and phenotype characterization must be performed very carefully and always in comparison to primary cultured cells. However, an immortal cell line promises higher reproducibility of results, easier handling and finally to be less time-, material- and labour-consuming.

MODIFICATIONS OF THE METHOD

Another immortalized rat brain capillary endothelial cell line is used in some labs, called GPNT. This cell line is obtained from GP8 cell line (Greenwood et al. 1996) by re-transfection with the plasmid pcDNA3-RSV and repeated limiting dilution cloning of the

parent line (GPNT: GP8 and the French Company NeuroTech SA). GPNT cells are repeatedly treated with 5 μg/ml puromycin. They are plated on collagen I-coated plates and grown in α-MEM/Ham's F-10 (1:1) containing 10 % heat-inactivated foetal calf serum, 0.5 ng/ml basic fibroblast growth factor, 5 μg/ml transferring, 5 μg/ml insulin, 5 ng/ml selenium and 5 μg/ml puromycin, in humidified 5 % CO_2/95 % air at 37 °C (Théron et al. 2003). P-glycoprotein expression can selectively be increased by repeated puromycin treatment (Demeuse et al. 2004).

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EXAMPLE

Begley et al. (1996) demonstrated the use of RBE4 cells for the assessment of P-glycoprotein activity at the blood-brain barrier. They showed that cellular accumulation of ^{3}H-colchicine and ^{3}H-vinblastine is enhanced in the presence of azidothymidine, chlorpromazine, verapamil, cyclosporin A and PSC833. Vinblastine uptake was measured in RBE4 cells grown on collagen-coated 24- or 48-well plates. Cells were washed several times with DMEM containing 0.1 % BSA and then incubated with 200 nM ^{3}H-vinblastine alone or together with 50 μM of the inhibitors. At several time points (15, 30, 60 and 90 min) the incubation medium was aspirated, and the cells washed with ice-cold medium and then solubilized in 1 N NaOH containing 0.1 % SDS. Solubilized cells were pipetted into vials containing scintillation liquid for β-scintillation counting.

II.I.1.3 A Surrogate BBB Model: MDCK-MDR1 Cells

PURPOSE AND RATIONALE

Early assessment of the ability of a drug candidate to penetrate the CNS is critical during the drug discovery selection process, especially for therapeutic indications that require delivery to a CNS site of action. Equally important is the ability to design drugs for non-CNS indications that have minimal brain penetration to avoid undesirable CNS side effects. In vitro BBB models using primary and immortalized brain capillary endothelial cells are described in the previous sections. The Madine Darby canine kidney (MDCK) cell-line is increasingly used as a substitute for the more labor-intensive in vitro BBB models in passive permeability and membrane transport studies.

PROCEDURE

Multidrug resistance-transfected MDCK type II (MDR1-MDCKII) cells were firstly described by Evers et al. (1997). They are maintained in Minimum Essential Medium (MEM) containing 10 % FBS and 2 mM L-glutamine and are cultured at 37 °C in an atmosphere of 5 % CO_2 and 95 % relative humidity. Cells are passaged at 80–90 % confluence (every 3–4 days) at a ratio 1:10 using trypsin-EDTA solution according to Irvine et al. (1998). They are grown in the absence

of any selection agent to maintain Pgp (MDR1) expression (Mahar Doan et al. 2002). For transport studies, cells are seeded onto polycarbonate Transwell filter membranes at a density of 300 000 cells/cm^2. MDCK monolayers are washed and fed with fresh medium 1 h post-seeding and again 24 h post-seeding, and monolayers are ready for studies 3 days later.

Permeability Studies

Drugs are tested at 10 μM concentration and in two directions (apical to basolateral (a-b) and basolateral to apical (b-a)). Monolayer efflux studies are conducted at 37 °C in a humidified incubator with shaking (90 rpm) for 60 min. Transendothelial electrical resistance is measured with an Endohm Meter (World Precision Instruments, New Haven, CT). Reference drugs for paracellular transport (^{14}C-mannitol), transcellular transport (^{3}H-propranolol), and Pgp efflux (amprenavir) should be included in each experiment. Concentrations of ^{14}C-mannitol and ^{3}H-propranolol are measured by liquid scintiallation counting. Amprenavir is analyzed by cassette LC/MS/MS analysis along with the test drugs.

Calculations

The apparent permeability (P_{app}) is calculated with the equation:

$$P_{app}[nm/s] = 1/(S \times C_0) \times (dQ/dt),$$

where S = membrane surface area, C_0 = donor concentration at time 0, and dQ/dt = amount of drug transported per time. A ratio of the b-a and a-b P_{app} value is calculated. Involvement of a Pgp-mediated efflux mechanism is concluded if the b-a/a-b ratio is > 1.5 (Mahar Doan et al. 2002).

EVALUATION

Mahar Doan et al. (2002) used the assay to classify 93 CNS and non-CNS drugs. The CNS set of drugs had a 7-fold lower incidence of passive permeability values < 150 nm/s compared with the non-CNS set. The majority of drugs (72 %) were not Pgp substrates. The CNS drug set had a 3-fold lower incidence of Pgp-mediate efflux than the non-CNS drug set. For CNS delivery, they concluded that drugs should ideally have an in vitro passive permeability > 150 nm/s and not be a good (b-a/a-b ratio < 2.5) Pgp substrate.

CRITICAL ASSESSMENT OF THE METHOD

The MDR1-MDCK transport assay was evaluated by Polli et al. (2001). The scientist compared the Pgp transport assay with the calcein-AM and ATPase assays. They tested 66 compounds and yielded two categories of compounds. Category I (n = 35) exhibited concordance across the assays. Category II (n = 31) revealed differences among the assays that related to the apparent permeability (P_{app}) of the compounds. Within category II, two groups are discerned based on the absence (group IIA, nontransported substrates) or presence (group IIB, transported substrates) of monolayer efflux. Detection of efflux (group IIB) is associated with compounds having low/moderate P_{app} values (mean = 16.6 nm/s), whereas inability to detect efflux (group IIA) is associated with compounds having high P_{app} values (mean = 535 nm/s). The calcein-AM and ATPase assays revealed Pgp interactions for highly permeable group IIA compounds but are less responsive than monolayer efflux for low/moderate P_{app} compounds of group IIB. All assays detect substrates across a broad range of P_{app}, but the efflux assay is more prone to fail at high P_{app}, whereas the calcein-AM and ATPase assays are more prone to fail at low P_{app}. When P_{app} is low, efflux is a greater factor in the disposition of Pgp substrates. The MDR1-MDCK assay is more reliable at low/moderate P_{app} and is therefore the method of choice for evaluating drug candidates despite its much lower throughput and more complicated read out.

However, the user needs to take into account that MDCK cells are derived from kidney. Thus, kidney specific transporters are expressed which do not play any role at the BBB. Furthermore, membrane composition is distinct in epithelial cell type (MDCK) and endothelial cells lining the brain capillaries influencing membrane partitioning of drug candidates.

MODIFICATIONS OF THE METHOD

To confirm that drugs are Pgp substrates, drugs can also be tested in the presence of a specific Pgp inhibitor, e.g. 2 μM GF120918 (Polli et al. 2001). Inclusion of a Pgp inhibitor reduces the b-a/a-b ratio to ~1 for Pgp substrates.

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EXAMPLE

As stated earlier, Mahar Doan et al. (2002) demonstrated in a very convincing paper the use of MDCK-MDR1 cell line to classify CNS and non CNS drugs. They investigated 93 drugs and found that a drug should ideally have an in vitro passive permeability > 150 nm/s and not be a good (B-A/A-B ratio < 2.5) Pgp substrate for CNS delivery. Together with physical chemical properties like molecular weight, solute McGowen volume, solute dipolarity/polarizability, hydrogen bond acidity and basiscity, charges and number of aromatic rings a good prediction of a drugs permeability into the CNS should be possible. In 2004 (Gombar et al.) they published a first quantitative structure-activity relationship (QSAR) model predicting Pgp substrates that takes into account the ability to partition into membranes, molecular bulk, and the counts and electrotopological values of certain isolated and bonded hydrides. The training set consisted of 95 compounds classified as substrates or non-substrates based on the results from MDR1-MDCK efflux assays (Mahar Doan et al. 2002). The model fit the data with sensitivity of 100 % and specificity of 91 % in the jackknifed cross-validation test. A prediction accuracy of 86 % was obtained on a test set of 58 compounds.

II.I.2 Drug Uptake by SLC Transporters

II.I.2.1 Drug Transport Mediated by SLC Transporters Using Eukaryotic Cells

PURPOSE AND RATIONALE

Endogenous compounds like bilirubin, bile salts, steroid conjugates, thyroid hormones, prostaglandins as well as pharmaceutical drugs are taken up from the blood circulation into the liver in order to be eliminated from the body by metabolism and/or excretion into bile. Sinusoidal hepatic uptake transporters like OATP2, OATP8, OATP-B, OAT2 and NTCP are involved in such liver uptake processes. For example, OATP2 seems to play a key role in the prevention of hyperbilirubinemia by facilitating the selective entry of unconjugated bilirubin and its glucuronate conjugates into human hepatocytes.

PROCEDURE

Cell Culture and Cell Lines

HEK293 cells are cultured in minimum essential medium supplemented with 10 % foetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 95 % humidity and 5 % CO_2. HEK cells can be transfected by the polybrene (hexadimethrine bromide) method (Brewer 1994). Briefly, exponential growing cells are incubated in a 10 cm petri dish with 10 μg plasmid DNA and 30 μg polybrene in 3 ml of complete medium for 8 h under normal culture conditions. Cells are then incubated with 5 ml of 30 % DMSO in complete medium at room temperature for 5 min. The DMSO mixture is then removed, and cells are washed twice with complete medium and cultured overnight before starting Geneticin (G418) selection. After 3 weeks of G418 selection (600 μg/ml), single colonies are screened for transporter expression by immunoblot and immunofluorescence microscopy. Cell clones expressing high levels of human recombinant transporters are used. Expression of recombinant SLC transporters is further enhanced by culturing transfected cells with 10 mM sodium butyrate for 24 h (König et al. 2000a,b) if cDNA was cloned into pcDNA3.1 (Invitrogen) before.

Uptake Studies with Radiolabeled Substrates

For uptake assays according to Cui et al. (2001), cells are seeded in 6-well plates (coated with 0.1 mg/ml poly-D-lysine) at a density of $1.5–2 \times 10^6$ cells/well and cultured with 10 mM sodium butyrate for 24 h (for cells which are transfected with the pDNA3.1 expression system). Before the uptake experiments, cells are washed with uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3). The uptake assay is started by the addition of 1 ml uptake buffer containing ^{3}H-labeled substrate (18.5–37 MBq/ml) to the cells. For inhibition studies, inhibitors are included in the uptake buffer. After incubation at 37 °C, transport is stopped at different time points by the addition of 1 ml ice-cold uptake buffer. Cells are subsequently washed three times with uptake buffer and lysed with 1 ml of 0.2 % SDS in water. Aliquots (250 μl) of the lysate are counted for radioactivity. Protein content is determined by the Lowry method using 100 μl lysate. The net uptake rates are calculated by subtracting values obtained with HEK cells from those obtained with transporter transfected HEK cells.

EVALUATION

Cui et al. (2001) studied the hepatic uptake of bilirubin and its conjugates by the human organic anion transporters OATP2 and OATP8 using transfected HEK293 (human embryonic kidney) cells. They demonstrated a high affinity uptake of ^{3}H-monoglucuronosyl bilirubin and ^{3}H-bisglucuronosyl bilirubin by human OATP2 with K_m values of 100 and 280 nM, respectively. High-affinity uptake of unconjugated ^{3}H-bilirubin by OATP2 occured in the presence of albumin and was not mediated by the second sinusoidal hepatic uptake transporter OATP8. OATP2 and OATP8 differed by their capacity to extract substrates from albumin before transport. In comparison to the high-affinity transport by OATP2, OATP8 transported ^{3}H-monoglucuronosyl bilirubin with lower affinity. Thus, human OATP2 may play a key role in the prevention of hyperbilirubinemia by facilitating the selective entry of unconjugated bilirubin and its glucuronate conjugates into human hepatocytes.

CRITICAL ASSESSMENT OF THE METHOD

HEK293 cells are perfectly used to express and study liver specific uptake transporters like organic anion transporting polypeptides (OATPs). One should consider the endogenous expression of OATs if using HEK293 cells for the kidney specific uptake transporter. In these cases transfection of CHO (chinese hamster ovarian) cells might be preferred.

MODIFICATIONS OF THE METHOD

Due to high background binding of some radiolabeled substrates (e.g. ^{3}H-bilirubin) to poly-D-lysine-coated plastic dishes, uptake of these compounds into transfected cells is measured in cell suspension (Cui et al. 2001). For uptake assays, cells are detached from culture flasks by knocking, washed twice with uptake buffer, and resuspended in uptake buffer at a density of 3×10^{6} cells/ml. ^{3}H-Bilirubin is diluted with human serum albumin in uptake buffer (75 000–100 000 dpm/ml). Unlabeled bilirubin is added to give the desired final concentrations. Uptake is started by mixing 1 ml of cell suspension with 1 ml bilirubin/albumin solution to give a final radioactivity of 37 500–50 000 dm/ml and stopped at different time points by centrifugation of the mixture at 13 000 rpm for 10 s. Cell pellets are washed twice with 1 ml of uptake buffer containing HSA and lysed in 2 ml of 0.2 % SDS in water. Aliquots (300 µl) of the lysate are counted for radioactivity. To determine the non-specific binding of ^{3}H-bilirubin, cells are incubated with ^{3}H-bilirubin in the presence of HSA for 1 min at 4 °C. Cell associated radioactivity measured under these conditions is used as a blank and subtracted from all other values.

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EXAMPLE

Coadministration of statins and fibrates is associated with an increased risk for myopathy, which may be due in part to a pharmacokinetic interaction. In case of the drug-drug interaction between cerivastatin and gemfibrozil involvement of both metabolism and transporters are discussed. Shitara et al. (2004) have nicely presented the interaction of gemfibrozil and gemfibrozil-glucoronide on the ^{14}C-cerivastatin uptake by the liver specific OATP2 uptake transporter. Using transfected MDCK cells they showed that gemfibrozil and gemfibrozil-glucoronide significantly inhibited the OATP2-mediated uptake of cerivastatin with IC_{50} values of 72 and 24 µM, respectively. These IC_{50} values of gemfibrozil and gemfibrozil-glucuronide were lower than their total plasma concentrations. However, because of the high plasma protein binding, the unbound concentrations of gemfibrozil and gemfibrozil-glucuronide were at most 0.97 and 2.3 µM, respectively, i.e. less than the IC_{50} values obtained in the presented study. Because only unbound drugs interact with transporters, this result suggests that it is unlikely to cause the reported serious drug-drug interaction between cerivastatin and gemfibrozil. However, it is likely that gemfibrozil or its glucuronide inhibit the metabolism of cerivastatin in the liver, because they are actively transported to the liver and accumulate there (Sallustio et al. 1996). The estimated unbound

concentration in the liver of gemfibrozil-glucuronide is about 90 μM (Shitara et al. 2004).

II.I.2.2 Drug Transport Mediated by SLC Transporters Using Xenopus Laevis Oocytes

PURPOSE AND RATIONALE

To study liver (or kidney) specific uptake of endogenous compounds or pharmaceutical drug oocytes expressing single uptake transporters at a very high expression level can easily be used. K_m and K_i constants can be calculated and compared with in vivo PK data (e.g. saturation levels).

PROCEDURE

Xenopus laevis frogs can be purchased from the African Xenopus Facility, Knysna, Republic of South Africa, and are kept in a constant alternating 12-hour light (6:30 am to 6:30 pm)/dark cycle.

The procedure described is based on the references Vavricka et al. (2002), Hagenbuch et al. (1990) and Kullak-Ublick et al. (1995). In vitro synthesis of SLC transporter complementary RNA (cRNA) can be performed according to Kullak-Ublick et al. (1995) using T7 RNA polymerase (Promega Corp, Madison, WI). Xenopus laevis oocytes are prepared according to Hagenbuch et al. (1990): Frogs are anaesthetized by immersion for 15 min in a 0.17 % solution of ethyl m-aminobenzoate (MS-222). Oocytes are removed (Colman 1984) and incubated at room temperature for 3 h in Ca^{2+}-free OR-2 solution (82 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.6) supplemented with 2 mg/ml collagenase (Sigma type I) and 10 units/ml penicillin and 10 μg/ml streptomycin. They are then washed in modified Barth's solution consisting of 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES-NaOH (pH 7.6), 0.3 mM $Ca(NO_3)_2 \times 4H_2O$, 0.41 mM $CaCl_2 \times 6H_2O$, 0.82 mM $MgSO_4 \times 7H_2O$ and containing 10 units/ml penicillin and 10 μg/ml streptomycin. Stage V and VI oocytes are selected. After an overnight incubation at 18 °C modified Barth's solution, healthy oocytes are injected with 5 ng of cRNA, cultured for 3 days in a medium containing 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.05 mg/ml gentamicin, and 15 mM HEPES (pH 7.6) and washed once with sodium-free buffer consisting of 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.5), trace amounts of radiolabeled substrate ^{3}H-BSP or ^{3}H-rifampicin, and appropriated substrate and inhibitor concentrations. Water injected oocytes are used as controls for unspecific substrate uptake. After an incubation period of 20 minutes the uptake experiment is stopped by adding 6 ml of ice-cold solution consisting of sodium-free choline buffer and 1 mM unlabeled substrate. Oocytes are washed twice with 6 ml ice-cold stop solution. Each oocyte is dissolved in 0.5 ml of 10 % sodium dodecyl sulfate and 5 ml scintillation fluid and the oocyte associated radioactivity is determined.

Calculations

Inhibition constants (K_i) are estimated by Dixon plot analysis and linear regression using ordinary least squares. Apparent K_m values are estimated by nonlinear regression (Vavricka et al. 2002).

EVALUATION

Standard substrate bromosulfophthalein (BSP) was used with OATP-C, 8, -B and -A expressing oocytes (Vavricka et al. 2002) and its K_m values were determined. For OATP-C they measured a K_m of 300 nM, for OATP8 400 nM, for OATP-B 700 nM and 20 μM for OATP-A.

CRITICAL ASSESSMENT OF THE METHOD

Oocytes are in favor over mammalian cells like HEK because of higher expression levels and less endogenous background. However, glycosylation of transport proteins as well as the membrane composition differs and might influence transport activity. For standard substrate BSP Vavricka et al. (2002) measured different K_m values for OATP2 and OATP8 compared to Cui et al. (2001): 300 nM (OATP2) and 400 nM (OATP8) compared to 140 nM (OATP2) and 3.3 μM (OATP8) in HEK293 cells. Future comparative studies will show the meaning of these differences.

MODIFICATIONS OF THE METHOD

Alternatively, pre-injected Xenopus oocytes are commercially available by BD Biosciences, Woburn, MA. Standard protocols and oocytes for following human transporters OCT1, OATP1, OATP2, NTCP, PEPT1, PEPT2, OAT1, OAT3 and rat OATP1 could be obtained in 2004. Protocols for LC-MS analytics instead of radioactivity are also supplied.

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Vavricka SR, Van Montfoort J, Ha HR et al. (2002) Interactions of rifamycin SV and rifampicin with organic anion uptake systems of human liver. Hepatology 36:164–172

EXAMPLE

The antibiotics rifamycin SV and rifampicin reduce Sulfobromophthalein (BSP) elimination in humans. Using injected oocytes, Vavricka et al. (2002) demonstrated that rifampcin is transported by OATP-C and OATP8 and that both rifampicin and rifamycin SV inhibit OATP-C, 8, -B and -A mediated BSP uptake. These results show that rifamycin SV and rifampicin interact with OATP-mediated substrate transport to different extents. Inhibition of human liver OATPs can explain the previously observed effects of rifamycin SV and rifampicin on hepatic organic anion elimination.

II.I.3
Drug Efflux by ABC Transporters

II.I.3.1
Drug Transport Mediated by ABC Transporters Using Eukaryotic Membrane Vesicles

PURPOSE AND RATIONALE

Efflux transporters are often accounted for by the transport of endogenous compounds and pharmaceutical drugs out of the body into bile, urine or gut in order to protect the cell. To study drug efflux, membrane preparations are necessary. Eukaryotic cell lines, tissues as well as insect cells can be used for this application.

PROCEDURE

Preparation of Plasma Membrane Vesicles from Eukaryotic Cell Lines

Selection of the cell line or tissue type influences not only the technique for membrane vesicle preparation but also the resulting percentage of inside-out-oriented plasma membrane vesicles. A sufficient amount of inside-out-oriented vesicles is essential, since only this fraction, with the ATP-binding domains oriented to the outer surface, mediates ATP-dependent transport of a labeled substrate into the vesicle.

The procedure described is based on Jedlitschky et al. (1994, 1996), Leier et al. (1994a,b) and Keppler et al. (1998).

Eukaryotic cells expressing ABC transporters (e.g. HEK-MRP1) (about 3×10^9) are harvested by centrifugation (1200 × g, 10 min, 4 °C) and washed twice in ice-cold phosphate-buffered saline (PBS: 0.15 M NaCl, 50 mM KH_2PO_4, pH 7.4). Hypotonic lysis of the cell pellet (about 5 ml) is induced by 40-fold dilution with hypotonic buffer (0.5 mM sodium phosphate, pH 7.0, 0.1 mM EDTA) supplemented with proteinase inhibitors [0.1 mM phenylmethylsulfonyl fluoride (PMSF), 2.8 μM E64 [trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane], 1 μM leupeptin, and 0.3 μM aprotinin]. After gentle stirring on ice for 1.5 hr, the cell lysate is centrifuged at 100 000 × g for 40 min at 4 °C. The pellet is then resuspended in 20 ml of hypotonic buffer and homogenized with a Potter-Elvehjem homogenizer (500 rpm, 2 strokes/min, 30 strokes, 4 °C). The homogenate is diluted with incubation buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) and centrifuged for 10 min at 12 000 × g at 4 °C. The resulting postnuclear supernatant is stored on ice. The corresponding pellet is resuspended in 20 ml of incubation buffer supplemented with proteinase inhibitors and homogenized and centrifuged again as described earlier. Both postnuclear supernatants are combined and centrifuged at 100 000 × g for 40 min at 4 °C. The pellet is resuspended in 20 ml of incubation buffer and homogenized manually by 50 strokes with a tightly fitting Dounce B (glass/glass) homogenizer (Fisher Scientific, Pittsburgh, PA) on ice. The homogenate is diluted by addition of 10 ml of incubation buffer to give a crude membrane fraction which is then layered on top of a 38 % (w/v) sucrose solution in 5 mM HEPES-KOH, pH 7.4 and centrifuged at 280 000 × g for 2 h at 4 °C in a swing-out rotor. The turbid layer at the interface is collected and diluted in 20 ml of incubation buffer, resuspended and homogenized by 20 strokes with a Dounce B homogenizer on ice and finally washed by centrifugation at 100 000 × g for 40 min at 4 °C. The resulting pellet is diluted in 1 ml of incubation buffer. Vesicles are formed by passing the suspension 20 times through a 27-gauge needle with a syringe. Aliquots of the membrane vesicle suspension are frozen and stored in liquid nitrogen.

Measurement of ATP-dependent Transport of Leukotriene C_4(LTC4)

Various labeled substrates for the conjugate export pumps encoded by MRP1 and MRP2 genes may serve to assess the transport rate in membrane vesicle preparations (e.g. Leukotriene C4, 17β-glucuronosyl estradiol, 6α-glucuronosylhyodeoxycholate, 3α-sulfatolithocholyltaurine, glucuronosyl-etoposide, dox-

orbicin, daunorubicin, vinblastine). Because of its high affinity and specificity for MRP1 and MPR2 and commercial availability, ^{3}H-LTC4 is a preferred substrate for transport measurements. This method is based on Böhme et al. (1993), Büchler et al. (1994) and Leier et al. (1994a).

Using LTC_4 as substrate, nitrocellulose filters (0.2 μm pore size, 25-mm diameter) are soaked in incubation buffer (0.25 mM sucrose, 10 mM Tris-HCl, pH 7.4). A rapid filtration apparatus from Millipore (Bedford, MA) is prepared. A final volume of 110 μl of transport assay mixture (4 mM ATP (potassium salt), 10 mM creatine phosphate (Tris salt), 10 mM $MgCl_2$, 10 mM Tris/HCl (pH 7.4), 5 mM glutathione (reduced), 0.25 mM sucrose, 100 μg/ml creatine phosphokinase (2 units/110 μl), 50 nM ^{3}H-LTC_4 (50 nCi/110 μl); pH 7.4) is preincubated at 37 °C for 1 min. Blanks are prepared by replacing ATP by 5'-AMP.

Membrane vesicle suspension should be thawed quickly at 37 °C and stored on ice for about 40 min before use. The transport assay is initiated at 37 °C by addition of membrane suspension (30 μg protein) to the transport assay mixture (110 μl). 20 μl aliquots are removed after 30 or 60 sec intervals, immediately diluted with 1 ml of ice-cold incubation buffer and immediately filtered through nitrocellulose membrane using the vacuum of the filtration apparatus (200 mbar). Filter membranes are rinsed twice with 5 ml of cold incubation buffer, dried and dissolved in 10 ml scintillation fluid to count for radioactivity.

The transport rate of ^{3}H-LTC_4 into the membrane vesicle is calculated by subtracting the corresponding values in the presence of 5'-AMP (blank sample) from those in the presence of ATP.

EVALUATION

The protein concentration of the membrane vesicle preparation is determined by standard protocols like the Lowry method. The measurement of the Na^+,K^+-ATPase activity may serve to assess the enrichment of plasma membranes relative to the original homogenate (Scharschmidt et al. 1979). This enrichment should be 15- to 30-fold. The sidedness of the membrane vesicle preparation may be estimated by the activity of an ectoenzyme, nucleotide pyrophosphatase (EC 3.6.1.9), in the presence or absence of Triton X-100 for solubilization of the membrane vesicle (Böhme et al. 1994). The percentage of inside-out-oriented plasma membrane vesicles from HL60 cells (Jedlitschky et al. 1994), mastocytoma cells (Leier et al. 1994b), or HeLa (Leier et al. 1994a) cells should range from 30 to 40 %.

CRITICAL ASSESSMENT OF THE METHOD

Inside-out vesicles can be perfectly used to study single efflux processes, because preloading of cells is not necessary and because of well-defined study conditions with transporter directly facing compound concentration in the incubation medium in contrast to double-transfected cell lines (3.1.2.4.1.). Therefore, these studies allow establishing structure activity relationships (SAR) for one efflux transporter only.

MODIFICATIONS OF THE METHOD

Preparation of Plasma Membrane Vesicles

The preparation of plasma membrane vesicles from liver canalicular membrane is highly enriched with the canalicular (apical) isoform MRP2 (Büchler et al. 1996). Methods for the isolation of hepatocyte canalicular membranes from liver tissue have been described in detail (Böhme et al. 1994 and Boyer and Meier et al. 1990). The percentage of inside-out-oriented vesicles in these preparations amounts to 32 %. Alternatively, transfected HEK and MDCK cells are often used to study ATP-dependent transport into inside-out vesicles (Cui et al. 1999; Leier et al. 2000).

Measurement of ATP-dependent Transport of Leukotriene C_4(LTC4)

Alternatively, the rapid filtration through nitrocellulose filters described above can be replaced by centrifugation of the vesicles through a gel matrix. For substrates more hydrophobic than LTC_4 which binds strongly to nitrocellulose filters (e.g. glucuronosyl-etoposide, glutathione S-conjugates of melphalan and chlorambucil), small Sephadex G-50 columns should be employed (Jedlitschky et al. 1996; Böhme et al. 1993; Büchler et al. 1994). For this procedure, NICK spin columns (Pharmacia, Uppsala, Sweden) (0.2 g Sephadex G-50/3.3 ml) are prepared, rinsed with incubation buffer and centrifuged at 400 × g for 4 min at 4 °C. Aliquots of the incubations are diluted in 80 μl of ice-cold incubation buffer and loaded immediately onto the columns. After rinsing with 100 μl of incubation buffer, the columns are centrifuged at 400 × g for 4 min 4 °C. The effluents are collected and counted for radioactivity.

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EXAMPLE

Para-aminohippurate (PAH) is widely used as model substrate for organic anion transport in proximal tubule epithelia (kidney). Leier et al. (2000) investigated PAH as a substrate for the apical multidrug resistance protein MRP2 (ABCC2) which is expressed in kidney, as well as in liver and small intestine. Membrane vesicles from HEK-MRP2 cells and from control cells were incubated with various concentrations of ^{3}H-PAH, and the net ATP-dependent transport into inside-out vesicles was determined. Transport rates at 10 μM PAH were 22 pmol/mg protein/min and 1.6 pmol/mg protein/min with membrane vesicles from HEK-MRP2 and HEK-control cells, respectively. The K_m value for PAH was 880 μM. MRP2 substrate leukotriene C4 and the MRP1-inhibitor MK571 inhibited MRP2 mediated PAH (100 nM) transport with IC_{50} values of 3.3 and 4.0 μM, respectively. The nephrotoxic mycotoxin ochratoxin A inhibited MRP2-mediated PAH transport with an IC_{50} value of 58 μM. Ochratoxin A itself was a substrate of MRP2 (Leier et al. 2000).

II.I.3.2 Drug Transport Mediated by ABC Transporters Using Membrane Vesicles from Insect Cells

PURPOSE AND RATIONALE

It has been suggested that multidrug resistance proteins (MRPs) play an important role in the transport and detoxification of a wide range of endogenous compounds and xenobiotics. They are predominantly expressed at the apical membrane of the small intestine, proximal tubules of the kidney and canalicular membrane of hepatocytes involved in intestinal, renal and hepatobiliary excretion of compounds.

PROCEDURE

Preparation of Plasma Membrane Vesicles

According to Chu et al. (1997, 2004), spodoptera frugiperda (Sf9) cells in suspension are grown in Sf-900 II SFM medium in the absence of serum (Invitrogen, Carlsbad, CA). About 4×10^7 Sf9 cells are seeded in 175 cm^2 tissue culture flasks. After the cells become attached, the medium is removed and 3 ml of medium and 3 ml of virus stock containing e.g. MRP1, MRP2 or MRP3 (about $5–8 \times 10^7$ recombinant baculovirus/ml) is added to infect the cells. One hour after addition of the virus to the cells, cell culture medium is added up to a final volume of 30 ml. After incubation for 72 h at 26 °C, the cells are harvested and washed twice in ice-cold washing buffer (50 mM Tris/HCl, 300 mM Mannitol, 0.5 mM PMSF, pH 7.0) and centrifuged at 800 × g for 5 min at 4 °C. The cell pellet is resuspended in ice-cold TMEP buffer (50 mM Tris, 50 mM Mannitol, 2 mM EGTA-Tris, 2 mM DTT, Aprotinin (8 μg/ml), Leupeptin (10 μg/ml), PMSF (50 μg/ml), pH 7.0) and homogenized for 10 min on ice using a tight-fitting Dounce homogenizer. After centrifugation at 800 × g for 10 min at 4 °C, the supernatant is collected and centrifuged at 100 000 × g for 1 hr at 4 °C. The pellet is resuspended in TMEP buffer and passed 20 times through a 27-gauge needle. The vesicles are dispensed in aliquots, frozen in liquid nitrogen, and stored at –80 °C until use.

Vesicular Uptake Studies

Vesicular uptake studies are performed using the rapid filtration technique as reported by Chu et al. 1997. The transport medium contained the radiolabeled substrate (e.g. ^{3}H-ethinylestradiol-3-O-sulfate or -3-O-glucuronide, ^{3}H-estradiol-17β-D-glucuronide, ^{14}C-ethacrynic acid glutathione), 250 mM sucrose,

10 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM adenosine 5'triphosphate (ATP), or 5 mM adenosine monophosphate (AMP), and an ATP-regenerating system (10 mM creatinine phosphate and 100 μg/ml creatine phosphokinase). The uptake study is performed at 37 °C. After preincubation for 3 min at 37 °C, the uptake study is started by the addition of vesicle suspension (10 μg protein). The final incubation volume is 20 μl. In the inhibition study, the inhibitors are dissolved in transport buffer and preincubated with radiolabeled substrates for 3 min. At designated time points, transport is terminated by adding 1 ml of ice-cold stop solution containing 10 mM Tris/HCl (pH 7.4), 250 mM sucrose and 100 mM NaCl. The stopped reaction mixture is filtered through 0.45 μm HA Millipore filters (Millipore Corporation, Bedford, MA) and subsequently washed twice with 5 ml ice-cold stop solution. For the uptake study with ^{3}H-estrone-3-sulfate, glass fiber (type A/E) filters (Gelman Sciences, Dorval, Quebec, Canada) are used to minimize non-specific binding to membrane filters. The radioactivity retained on the filter and in the reaction mixture is measured in a liquid scintillation counter. ATP-dependent uptake is determined as the difference in uptake in the presence and absence of ATP.

ATPase Measurements

ATPase activity of the isolated Sf9 cell membranes is estimated by measuring inorganic phosphate liberation as described by Sarkadi et al. (1992). The differences between ATPase activities measured in the absence and presence of vanadate (100 μM) can be plotted.

EVALUATION

Membrane Preparations

The membrane protein concentrations of membrane vesicles are determined as described by Sarkadi et al. (1992).

Data Analysis

Kinetic parameters for the ATP-dependent uptake are obtained by fitting the data to the following equation:

$$V_c = V_{max} \times S/(K_m + S) \,, \qquad (1)$$

where V_c is the initial uptake rate of substrate (pmol/min/mg protein), S is the substrate concentration in the medium (μM), K_m is the Michaelis constant (μM) and V_{max} is the maximum uptake rate (pmol/min/mg protein).

The inhibition constant (K_i) values for evaluating the inhibitory effect of e.g. ethinylestradiol-3-O-glucuronide on the uptake of ^{14}C-ethacrynic acid glutathione by MRP2 is obtained by fitting the following equation to the data as described by Chu et al. (1997):

$$V_{(+I)}/V_{(-I)} = 1/[1 + (I/K_i)] \,. \qquad (2)$$

$V_{(+I)}$ and $V_{(-I)}$ represent the transport velocity in the presence and absence of inhibitor, respecitvely, and I is the inhibitor concentration. This equation is derived based on the assumptions that firstly inhibition is competitive or non-competitive and secondly that the radiolabeld substrate concentration used (e.g. ^{14}C-ethacrynic acid glutathione (2 μM)) is much lower than its K_m value (15 μM for MRP2).

CRITICAL ASSESSMENT OF THE METHOD

Using insect cells instead of eukaryotic cell lines has following advantages: a reduced transporter background and higher expression levels. However, one should also consider that the lipid membrane composition and glycosylation mechanism differ in insect cells from eukaryotic cells.

MODIFICATIONS OF THE METHOD

Ready to use membrane vesicles for the ABC transporters MDR1, MRP1, MRP2, MRP3, MXR are commercially available from Solvo Biotechnology (Hungary, Budapest; www.solvo.com) and can be used for uptake measurements. Membranes and protocols for ATPase measurements are also available.

Recombinant baculoviruses can be prepared as described by Bakos et al. (1998) by using the Baculo-Gold Transfection Kit according to the manufacturer's recommendations (Pharmingen, San Diego, CA) or by using the pAcUW21 plasmid (Invitrogen, San Diego, CA).

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EXAMPLE

Bakos et al. (2000) studied the interactions of the human multidrug resistance proteins MRP1 and MRP2 with the organinc anions probenecid, furosemide, sulfinpyrazone, penicillin G and inomethacin. Firstly, they determined the concentrations at half-maximum uptake rates ($K_{1/2}$) for N-ethylmaleimide glutathione (NEM-GS) with $K_{1/2}$ 200 μM for MRP1 and 2.5 mM for MRP2. The effect of organic anions on the relative rate of NEM-GS uptake by MRP1 and MRP2 was then analyzed: sulfinpyrazone and probenecid efficiently inhibited ATP-dependent active vesicular NEM-GS uptake by MRP1. In case of indomethacin low concentrations produced a significant stimulation and only indomethacin concentrations of more than 100 μM inhibited the NEM-GS uptake by MRP1. For MRP2 probenecid inhibited NEM-GS uptake, whereas low concentrations of sulfinpyrazone and indomethacin strongly stimulated the transport of this GS conjugate. Higher sulfinpyrazone and indomethacin concentrations were inhibitory again. The addition of methotrexate caused a slight inhibition of the NEM-GS uptake in both MRP1 and MRP2. Penicillin G caused a major stimulation of MRP2 transport, whereas benzbromarone or furosemide was inhibitory. These tracer uptake experiments clearly suggested that various organic anions acted differently on the two transportes.

II.I.4 Drug Uptake and Efflux

II.I.4.1 Drug Transport Mediated by SLC and ABC Transporters Using Double Transfected Cells

PURPOSE AND RATIONALE

Hepatobiliary excretion is an important pathway for the elimination of endogenous and xenobiotic substances. In order to study the hepatobiliary transport of organic anions and drugs in cellular model systems Prof. Keppler (DKFZ, Heidelberg, Germany) established a double-transfected Madin-Darby canine kidney (MDCK) cell line expressing the human hepatocyte basolateral uptake transporter SLC21A8 (OATP8) and the apical conjugate export pump ABCC2 (MRP2) (Cui et al. 2001). This in vitro model will be a powerful alternative to animal studies for the pharmaceutical industry in order to optimize the compounds against hepatobiliary elimination.

PROCEDURE

Cell Lines

Double-transfected MDCKII cells expressing the human uptake transporter SLC21A8 (OATP8) and the apical export pump ABCC2 (MRP2) can be generated by stepwise transfection of MDCK cells with pcDNA3.1-hMRP2 and -hOATP8 using Effectene Transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's instructions. After three weeks of selection (zeocin: 1 mg/ml; G418 disulfate 800 μM; hygromycin B 500 μg/ml), single colonies can be screened for transporter expression by immunoblot analysis and immunofluorescence microscopy.

Cell Culture

MDCKII cells are cultured according to Cui et al. (2001). In brief, cells are cultured in minimum essential medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified 5 % CO_2/95 % air at 37 °C. For selection antibiotics are added (1 mg/ml zeocin, 800 μg/ml G418 disulfate, 500 μg/ml hygromycin B). Cells are passaged every 3 to 4 days at 1:10 to 1:15 ratios.

Transport Measurements

According to Cui et al. (1999, 2001), for transport assays, MDCKII cells are grown on Transwell Clear polyester membrane inserts (pore size 0.4 μm; Corning Costar #3450, Cambridge, MA) for 8 days in culture medium without antibiotics. Medium is replaced twice. Butyrate, at a final concentration of 10 mM, is added to the medium 24 h before use of the cells. Cells are first washed with transport buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3). Subsequently, ^{3}H-labeled substrates (^{3}H-BSP, 50 to 100 nM, 12.3 Ci/mmol) or non-labeled drug candidates (50 μM) are added in transport buffer either to the apical compartments (1.5 ml) or to the basolateral compartments (2.5 ml). After 90 minutes, samples from the opposite compartments are taken and analysed either by liquid scintillation spectrometry or by LC-MS/MS.

The transcellular leakage is determined by incubating cells with 50 μM ^{14}C-inulin carboxylic acid in the basolateral compartments for 30 min and measuring the radioactivity in the apical compartments. The transcellular leakage should be less than 1 %.

EVALUATION

Expression and localization of OATP8 and MRP2 in MDCK cells growing on Transwell membrane

inserts was demonstrated by immunoblotting and confocal laser scanning microscopy (Cui et al. 2001). ^{3}H-labeled sulfobromophthalein (BSP) was a substrate for both transport proteins and was transferred from the basolateral to the apical compartment at a rate at least six times faster by double-transfected MDCK-OATP8/MRP2 cells than by single-transfected MDCK-OATP8 or MDCK-MRP2 cells. They also demonstrate transcellular transport for leukotriene C4, 17β-glucuronosyl estradiol, dehydroepiandrosterone sulfate, fluo-3 and rifampicin.

CRITICAL ASSESSMENT OF THE METHOD

The user of this kind of in vitro model should consider that for drugs which are highly membrane permeable the permeation rate across the cell-monolayer on the Transwell membrane insert might overcompensate the active transport of the drug. In these cases the transport rate does not differ between double-transfected, single-transfected and wild-type cells. However, a drug-substrate interaction study might help in these cases. For example, the transport of ^{3}H-labeled sulfobromophthalein (BSP) might be measured in the absence and presence of various concentrations of a drug candidate and the inhibition konstant K_i might be calculated (see modifications of the method).

One further point has to be taken into account which is generally valid for cell-based assays: the background expression of endogenous canine transporters. In case of MDCK cells especially the efflux transporter MRP2 and Pgp (MDR1) are strongly expressed. Kidney specific organic anion transporters (OATs) will be present as well.

MODIFICATIONS OF THE METHOD

For inhibition studies, the inhibitors are preincubated in both apical and basolateral compartments for one hour and then added simultaneously with ^{3}H-BSP into the basolateral compartment and without ^{3}H-BSP into the apical compartment. After 90 minutes the radioactivity is measured in the apical compartment. Using different inhibitor concentrations the K_i (IC_{50}) value can be calculated. This technique can also be used for drug-drug interaction studies on the level of transporters.

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EXAMPLE

Sasaki et al. (2002) established the double-transfected MDCK cell line, which expresses both SLC21A6 (OATP2) and ABCC2 (MRP2) on basal and apical membranes. They measured the basolateral-to-apical transport of the lipid-lowering drug pravastatin. The K_m value of pravastatin was 24.3 +/– 10.4 μM, which was in the same magnitude to the K_m of estradiol-17β-glucuronide and comparable with the K_m reported for OATP2 only (33.7 μM, Hsiang et al. 1999). Because pravastatin is a low affinity substrate of MRP2 (220 μM, Yamazaki et al. 1997), the rate-determining step in the transcellular transport of this compound is the uptake mediated by OATP2. This hypothesis is consistent with the previous observations that uptake is the rate-determining step for the biliary excretion of pravastatin in rats (Yamazaki et al. 1997).

II.I.4.2 Liver Specific Drug Transport in Sandwich-cultured Hepatocytes

PURPOSE AND RATIONALE

The use of in vitro systems to evaluate hepatic drug uptake and efflux is an essential part of the drug development process. Primary hepatocyte culture is one technique to address this issue.

PROCEDURE

Animals

Male Wistar rats (250–280 g) can be obtained from Charles River. They are allowed free access to food (laboratory rodent diet 5001, PMI Feeds, St. Louis, MO) and water, and are housed in a constantly alternating 12-h light (6:00 am to 6:00 pm) and dark cycle.

Preparation of Culture Dishes

According to Liu et al. (1999b), plastic culture dishes (60 mm) are precoated with rat tail collagen at least 1 day before the hepatocyte cultures are prepared. To obtain a simple (rigid) substractum, collagen solution (0.1 ml, 1.5 mg/ml) is added to each dish. Coated dishes are stored overnight in a sterile hood. Immediately before use, fresh medium is added to neutralize the collagen. To obtain a gelled collagen substractum, ice-cold neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) is spread onto each culture dish. Freshly coated dishes are placed at 37 °C in a humidified incubator for ~1 h to allow the matrix material to gel, followed by addition of 3 ml DMEM to each dish and storage in a humidified incubator.

Isolation and Culture of Rat Hepatocytes

Hepatocytes are isolated with a two-step perfusion method according to LeCluyse et al. (1996), Anneart et al. (2001) and Chandra et al. (2001): After the rat is anesthetized (60 mg/kg ketamine + 12 mg/kg xylazine intraperitoneal), the portal vein is cannulated, and the liver is perfused with a Ca^{2+}-free buffer (118.1 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM glucose, 1 mM EGTA, 4.7 mM KCl, 1.2 mM KH_2PO_4, pH 7.4) equilibrated with 95 % O_2/5 % CO_2 at a rate of 30 ml/min. The inferior vena cava is cannulated to establish a recirculating system (100 ml Ca^{2+}-free buffer). After 10 min of perfusion, 0.05 to 0.075 g of collagenase and 4 mg of soybean trypsin inhibitor are added to the perfusate reservoir to obtain a final collagenase concentration of ~200 U/ml; 1 min later, 1 ml of $CaCl_2$ is added. The liver is perfused for ~10 min with the collagenase buffer, removed from the rat, and immersed in ice-cold medium (DMEM containing 5 % FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 4 mg/l insulin, and 1 μM dexamethasone). The capsule surrounding the liver is torn gently in order to release the hepatocytes. The cells are filtered through a 70 μm mesh filter and then centrifuged (50 × g) for 2 min at 4 °C. The pellet is resuspended in equal parts of medium and isotonic Percoll and centrifuged (70 × g) for 5 min at 4 °C to separate out the nonviable cells. The pellet is resuspended in medium and centrifuged (50 × g) for 2 min at 4 °C. Hepatocytes are counted in a hemocytometer and viability is determined using the trypan blue exclusion method. Viability is always > 90 % with a yield of 2–3 × 10^8 cells. Cells are resuspended in medium and diluted to a final concteration of 1 × 10^6 cells/ml.

Hepatocyte suspension is added to the precoated dishes at a density of 2 × 10^6 cells/60 mm dish. Approximately 1 h after the cells are plated, the medium is aspirated and 3 ml fresh DMEM is added. For hepatic transport studies, hepatocytes that have been seeded for 3–5 h without collagen overlay are defined as day 0 or short-term cultured hepatocytes.

To prepare sandwich-cultured hepatocytes, neutralized collagen solution (0.1 ml, 1.5 mg/ml, pH 7.4) is added to the monolayers 24 h after the cells are seeded. Cultures with collagen overlay are incubated for 45 min at 37 °C in a humidified incubator to allow the collagen to gel before addition of DMEM. Medium is changed on a daily basis until the fifth day after the cells are seeded. These hepatocytes are referred to as day 5 or long-termed cultured hepatocytes.

Efflux Studies in Sandwich-cultured Hepatocytes

According to Liu et al. (1999a,b) hepatocytes cultured in a collagen-sandwich configuration are incubated in 3 ml of standard buffer (Hanks' balanced salt solution: 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 136.9 mM NaCl, and 5.6 mM D-glucose) at 37 °C for 10 min. Each dish received 3 ml of standard buffer containing 1 μM ^{3}H-taurocholate or 3.6 μM ^{14}C-salicylate, followed by incubation at 37 °C for 10 min. Subsequently, the incubation buffer is removed and cultures are washed four times with 3 ml ice-cold standard buffer to quench the transport processes and remove extracellular substrate. Efflux is initiated by addition of 3 ml of standard buffer or Ca^{2+}-free buffer to each dish. Aliquots of efflux buffer (0.1 ml) are removed at designated times and analysed by liquid scintillation spectrometry.

Uptake Studies in Sandwich-cultured Hepatocytes

According to Liu et al. (1999a,b) hepatocytes cultured in a collagen-sandwich configuration are incubated in 3 ml of standard buffer or Ca^{2+}-free buffer at 37 °C for 10 min. After the incubation buffer is removed, uptake is initiated by addition of 3 ml of standard buffer containing 1 μM ^{3}H-taurocholate or 0.9 μM ^{14}C-salicylate to each dish. After incubation for designated times, cumulative uptake is terminated by aspirating the incubation solution and rinsing four times with 3 ml of ice-cold standard buffer to remove extracellular substrate. Each rinse lasted 10 s. After washing, 2 ml of 1 % Triton X-100 solution are added to culture dishes to lyse cells by shaking the dish on a shaker for 20 min at room temperature. An aliquot (1 ml) of lysate is analysed by liquid scintillation spectrometry. All values for taurocholate uptake into cell monolayers are corrected for non-specific binding to the collagen

by subtracting taurocholate uptake determined in the appropriate control dishes in the absence of cells.

EVALUATION

Efflux or uptake data are normalized to the total solubilized protein content. Bio-Rad DC protein assay kit (Bio-Rad Laboratories) can be used to determine the protein concentration in the culture extracts using BSA as standard. Triton X-100 (1 %) does not interfere with this assay.

CRITICAL ASSESSMENT OF THE METHOD

Using primary cultures of hepatocytes the retention of transporter expression and activity needs to be guaranteed strictly speaking after each preparation. Cryopreserved hepatocytes from one preparation could be a powerful alternative for industrial applications in the future, because transporter expression and activity could only be characterized once. Houle et al. (2003) compared the transporter activities in cropreserved and freshly isolated hepatocytes and found no significant difference in the transport rates of ^{14}C-taurocholate, ^{3}H-estrone sulfate and ^{3}H-estradiol-17β-D-glucuronide.

MODIFICATIONS OF THE METHOD

For MDR1 mediated in vitro biliary excretion rhodamine 123 and ^{3}H-digoxin can be used in accumulation studies according to Annaert et al. (2001). Cells are rinsed twice with 2 ml of warm standard HBSS and incubated in 3 ml of the same buffer for 10 min at 37 °C. for experiments in which the effect of a Pgp modulator (e.g. GF120918; GlaxoSmithKline) is investigated, cells are preincubated for 60 min with the appropriate concentration of the Pgp modulator. Subsequently, cells are incubated in 3 ml of 1 μM substrate dissolved either in standard or Ca^{2+}-free HBSS for 1 to 30 min and subsequently rinsed for times with 3 ml of ice-cold HBSS. For rinsing of ^{3}H-digoxin-treated hepatocytes, 10 μM unlabeld digoxin is added to the rinsing buffer to reduce non-specific binding. Hepatocytes are lysed with 3 ml of 0.5 % Triton X-100 solution by placing plates on a rotator for 20 min at room temperature. Cell lysates are analysed by fluorescence spectroscopy for Rh 123 analysis, and by liquid scintillation spectroscopy for ^{3}H-digoxin. Accumulation is normalized to the protein content of the hepatocytes in each well. All accumulation data are corrected for non-specific binding to collagen-coated, hepatocyte-free culture dishes.

An exclusive sandwich-cultured hepatocyte system for hepatobiliary disposition is also commercially available by Qualyst, Inc (www.qualyst.com), named B-CLEAR.

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EXAMPLE

Liu et al. (1999) studied the hepatic uptake and biliary excretion of one model compound 264W94 and its O-demethylated metabolite 2169W94 as well as the O-glucuronide conjugate of 2169W94 in freshly prepared hepatocytes. By comparing the uptake of the model compound and its metabolites in sandwich-cultured hepatocytes in the presence of Ca^{2+} and in the absence of Ca^{2+} they have shown that the model compound itself (264W94) was taken up but not excreted in bile either in vitro or in vivo. However, the O-demethylated metabolite (2169W94) and its glucuronide conjugate were excreted into bile in vitro, when the O-demethylated metabolite (2169W94) was incubated with the hepatocytes. In vivo 2169W94-glucuronide also underwent extensive biliary excretion after administration of 2169W94. Thus, biliary excretion in long-term sandwich cultured rat hepatocytes (96 h) correlated with in vivo biliary excretion.

Chapter II.J
Drug–Drug Interaction – Enzyme Induction

Albert P. Li

PURPOSE AND RATIONALE

Enzyme induction can be considered a natural defense mechanism. Xenobiotics that enter the human body via ingestion are subjected to metabolism firstly by the drug metabolizing enzymes (DME) in the intestinal epithelium, and may be removed by the p-glycoprotein (Pgp) from the epithelial cells back into the lumen. The xenobiotics that escape intestinal metabolism and efflux enter into the portal circulation are subjected to metabolism by the liver – the organ specializing in xenobiotic metabolism, before entering the system circulation. As a defense mechanism, the DME and Pgp can be induced, leading to a higher rate and capacity of xenobiotics elimination.

Enzyme induction is generally considered an adverse drug property. The major consequences of enzyme induction are pharmacokinetic drug–drug interactions and liver toxicity.

Adverse drug–drug interactions occur mainly due to pharmacokinetic drug–drug interactions – the alteration of the rate of metabolism of one drug by a co-administered drug, or pharmacological drug–drug interactions – toxicity occurring due to the combined pharmacological effects of the interacting drugs. There are two major mechanisms of pharmacokinetic drug–drug interactions: inhibitory and inductive drug–drug interactions (Li 1998):

Inhibitory drug–drug interactions: One drug may inhibit the metabolism of a co-administered drug, leading to a higher than expected level of the affected drug. Drug toxicity is the major consequence of inhibitory drug–drug interactions, as the affected drug may exceed the safe level.

Inductive drug–drug interactions: When a drug induces DME, it has the potential to cause inductive drug–drug interactions. A drug that induces a DME may lead to faster metabolic elimination of a co-administered drug which is a substrate of the affected DME, which may result in a loss of efficacy. The known human enzyme inducers are tabulated in Table 1.

Enzyme induction in laboratory animals is general associated with liver enlargement which is considered a toxic endpoint. A large percentage of drugs that are associated with idiosyncratic liver toxicity are also enzyme inducers (Li 2002). The co-existence of enzyme induction and hepatotoxicity for drugs is illustrated in Table 1. The mechanistic link between enzyme induction and liver toxicity, if any, is yet to be established. There may be a true link as enzyme induction represents a disturbance of the homeostasis of the liver. The induced enzyme may activate certain environmental or endogenous toxicants. Alternatively, as the drugs that are associated with severe liver toxicity are administered at relatively high doses, the enzyme induction may be related more to the high dosages and is independent of the liver toxicity. However, because of the apparent relationship between enzyme induction and hepatotoxicity, some researchers in drug development view enzyme induction potential as an indicator of hepatotoxic potential.

The mechanism of P450 induction is an area of intensive research activities. There is a general finding, with some exceptions, that enzyme induction occurs via the binding of the inducers to nuclear receptors that subsequently leads to increased gene expression and protein synthesis (Waxman 1999; Honkakoski and Negishi 2000; Tirona et al. 2003; Wang et al. 2004). The key receptors for enzyme inducers are tabulated in Table 2. These receptors are as follows:

- Aryl hydrocarbon receptor (AhR): AhR is a basic helix-loop-helix protein belong to the Per-ARnt-Sim family of transcription factors. It has been found to be the key receptor for the activation of CYP1A and CYP1B genes. AhR ligands include aryl hydrocarobons (e.g. 3-methylcholanthrene) and dioxins. AhR resides in the cytoplasm, and, upon ligand binding, translocates to the nucleus. In the nucleus AhR forms a heterodimer with the AhR nuclear translocator (ARNT) which then binds to the xenobiotic response elements (XRE)

Table 1 Examples of known human enzyme inducers, the drug metabolizing enzymes (DME) induced, induction in vitro and in vivo (in humans) and their association with liver toxicity in humans. Although no clear mechanistic link is yet developed, almost all known enzyme inducers appear to possess hepatotoxic effects.

Inducer	Major DME Induced	In vivo induction	In vitro induction	Clinical Hepatotoxicity
Carbamazepine	CYP3A4, UGT	Yes	Yes	Yes
Felbamate	CYP3A4	Yes	No report	Yes
Isoniazid	CYP2E1	Yes	Yes	Yes
Omeprazole	CYP1A2	Yes	Yes	No
Phenobarbital	CYP2B6, CYP2C8, CYP2C9, CYP3A4, UGT	Yes	Yes	Yes
Phenytoin	CYP2B6, CYP2C8, CYP2C9, CYP3A4, UGT	Yes	Yes	Yes
Rifampin	CYP2B6, CYP2C8, CYP2C9, CYP3A4, UGT	Yes	Yes	Yes
St. John's Wort	CYP3A4	Yes	Yes	Yes
Troglitazone	CYP2B6, CYP2C8, CYP2C9, CYP3A4	Yes	Yes	Yes

Table 2 Nuclear receptors (NR) for enzyme inducers. Enzyme inducers are now known to act as ligands to nuclear receptors, leading to gene activation and increased synthesis of the enzyme. Affinity of inducers to the receptors is now known to be responsible for the differential induction potential and can explain the observed species-differences in induction. The receptors tabulated are aryl hydrocarbon receptors (AhR), constituitively androstane receptor (CAR), pregnane X receptor (PXR), and glucocorticoid receptor (GR). The isoforms in bold type are the major isoform regulated by the corresponding receptors.

NR	P450 Isoform	Examples of Ligands (Inducers)
AhR	**CYP1A**	Aryl hydrocarbons
CAR	**CYP2B;** CYP2C; CYP3A	Phenobarbital
PXR	**CYP3A**; CYP2C	Rifampin
GR	**CYP3A5**	Dexamethasone

of the P450 gene. It is interesting to note that AhR activates its own repressor (AhR repressor), thereby forming a negative feedback loop for its regulation. The role of AhR in CYP1A induction has been demonstrated by the resistance of AhR knockout mice to TCDD-elicited CYP1A1 induction.

- Pregnane X receptor (PXR): PXR, also called SXR or PAR has been discovered by Kliewer and colleagues (Lehmann et al. 1998) to be responsible for CYP3A induction. It is now shown that this nuclear receptor is also responsible for CYP2C8 and CYP2C9 induction. Pharmaceuticals that active PXR include rifampin, phenobarbital, nifedipine, clotrimazole, mifepristone and metyrapone. Many PXR ligands also bind to the constitutive androstane receptor (CAR). Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor-α (RXR-α and transactivates the ER6 (everted repeat with a 6 base pair spacer) elements upstream of the P450 genes. It is known that PXR and RXR-α are induced by glucocorticoid receptor (GR). Dexamethasone is known to activate GR and lead to the induction of PXR/RXR and CYP3A4 induction. Cytokines such as interleukin-6 (IL-6) are known to down regulate PXR and CAR, leading to the repression of several P450 isoforms. PXR is responsible for the dramatic rodent-human difference in response to rifampin. Human PXR has a much higher affinity for rifampin than rodent PXR.
- Constitutively androstane receptor (CAR): CAR is also called a constitutively active receptor, originally characterized as a constitutive activator of retinoid acid response element (RARE). It is called "constitutive" because it transactivates RARE without being bound to a ligand. CAR is responsible for the induction of CYP2B6 and to a lesser extent, CYP3A4. Phenobarbital, a potent CYP2B6 inducer, however, is not a ligand to CAR. Unlike the well-established relationship between ligands and AhR and PXR for CYP1A and CYP3A induction, respectively, the mechanism for phenobartital activation of CYP2B is still not known. It is possible that phenobartital affects CAR via a kinase pathway, as CAR translocation and activation are inhibited by protein phosphatase and kinase inhibitors. CAR-null mice are found not to respond to the CYP2B induction effects of phenobarbital.
- Glucocorticoid receptor (GR): A well-established ligand to GR is dexamethasone. GR effects are not due to its binding to P450 genes, but rather on protein–protein interplay (cross-talk) between GR and other receptors. Dexamethasone induces

CYP3A4 in human hepatocytes due to its induction of PXR and RXR expression, and also for the same reason enhances the induction effects of PXR ligands. CYP3A5 is the only human P450 isoform induced directly by GR.

- Non-receptor related mechanisms: Enzyme induction may occur via non-receptor related mechanisms. CYP2A6, although known to be induced by phenobarbital and rifampin, may involve mRNA stabilization. CYP2E1 induction may be a result of stabilization of mRNA and protein.

PROCEDURE

Enzyme induction assays are generally performed for the evaluation of the effects of the substance tested on P450 induction. For animal studies, liver enlargement (e.g. liver weight gain) as well as increases in P450 activities after a relevant treatment period (e.g. 14 days) are used as endpoints for enzyme induction potential.

It is now known that rodents are different from humans in response to enzyme inducers. For instance, dexamethasone, a potent inducer of CYP3A2 in rats, is a poor inducer of CYP3A4 in humans. On the other hand, rifampin, a potent inducer of CYP3A4 in humans, is a poor rodent CYP3A2 inducer (Lu & Li 2001). The empirically derived species-differences can now be explained by the species-differences in the binding affinity of nuclear receptors for the inducers (Jones et al. 2000). To address human enzyme induction potential, human hepatocytes in vitro apparently represent the most appropriate preclinical experimental model – the known mechanisms of enzyme induction, including induction of gene expression, mRNA stabilization, and protein stabilization, can be adequately studied in cultured hepatocytes (Li 1997; Li et al. 1997). As of this writing, virtually all known enzyme inducers for humans in vivo (Benedetti 2000) are also found to be inducers for hepatocytes in vitro (Table 1).

The following represents the timeline and procedures for a typical enzyme induction assays with human hepatocytes (Table 3):

- Cell plating (Day 0): Freshly isolated human hepatocytes are isolated using a 2-step collagenase digestion procedure and plated onto collagen-coated plate. It is important that the cells are plated at a cell density that will lead to a confluent monolayer culture, as cell density is known to affect enzyme induction results.
- Stabilization (Days 1 and 2): The plated hepatocytes are cultured for 2–3 days to allow the P450 level to stabilize. P450 activities for inducible isoforms such as CYP1A2 and 3A4 are known to decrease with culture time at a rate of approximately 50 % per day. Medium should be changed daily during this stabilizing period. It is interesting to note that this stabilization period is critical for induction studies. Hepatocytes are less responsive to inducers if they are treated within 24 hours of plating.
- Treatment (Days 3 to 5): The culture medium is now changed to that containing the test substance at the desired concentrations. In general, the concentrations are selected from the expected plasma drug concentrations (e.g. 0.1X to 10X of plasma Cmax). It is important to ensure that the treatment doses are noncytotoxic. Cytotoxic doses will lead to cell death, and may complicate the assessment of induction potential. Cytotoxicity evaluation can be made qualitative via microscopy (evaluation of cell density and morphological changes) or quantitatively via the measurement of cellular protein and other cytotoxicity endpoints such as ATP content and enzyme leakage.
- Activity Determination (Day 6): The cells are harvested for the evaluation of enzyme activities. This can be performed in situ via the incubation of the cells with the enzyme substrates (Table 2). Activity can also be determined from the cell homogenates or microsomal fractions of the cells. The in situ method is the most efficient, and would allow one to use 24-well or 96-well plates. The microsomal method would require the use of larger cell culture plates (e.g. 100-mm diameter plates). DME substrates used in the hepatocyte enzyme induction assay are shown in Table 4.

EVALUATION

- Controls: The experiment should include a negative solvent control, and positive controls with known inducers (e.g. omeprazole (25 uM) and rifampin (25 uM) for CYP1A2 and CYP3A4, respectively). The negative control is used to determine the level of induction (see below). The positive controls are necessary to demonstrate that the experimental conditions are adequate for the evaluation of induction. A study is considered valid only if the positive controls are found to significantly induce the corresponding P450 isoforms.

Table 3 Timeline and key procedures for the human hepatocyte enzyme induction assay.

Days	Procedures	Comments
0	Isolate human hepatocytes from high quality human livers	The best procedure for hepatocyte isolation is the 2-step collagenase perfusion technique. Freshly isolated human hepatocytes are now also available from commercial vendors. However, the quality of the liver may be affected by the transportation period. One needs to ensure that the hepatocytes are near confluent and that no significant attachment occurs during the culturing period.
1, 2	Change medium daily using hepatocyte maintenance medium.	This is the stabilization period to allow the P450 activities of the hepatocytes to stabilize at a basal level.
3–5	Change medium to medium containing the chemicals to be evaluated	Selection of the appropriate concentration is important. The concentration should be high enough but still physiological relevant. Care must be taken to avoid using cytotoxic concentrations. For the best results, the hepatocyte cultures should be near 100 % confluent during the entire treatment period.
6	Remove medium and replace with medium containing DME substrates	The in situ method for enzyme activity measurement is the most efficient. Enzyme activity can also be measured from cell homogenates or microsomal preparations. Besides activity, one can also measure mRNA and protein levels.

- Data analysis: Enzyme induction results are usually expressed as relative activity, a ratio of the activity after treatment vs that of the solvent control using the following equation:
  Relative activity (%) = Activity (Treatment)/Activity (Solvent Control) × 100 %.
  Appropriate statistical methods are yet to be established for enzyme induction assays. In general, ANOVA is used for the determination of statistical significance. Biological significance such as fold induction and effective concentrations (e.g. EC50) are valuable in the interpretation of the significance of the data. One interesting approach is to compare the data to that of the positive control which also allows the normalization of data for inter-experimental comparisons.

Besides enzyme activity, enzyme induction assays can also utilize mRNA and enzyme protein level as endpoints. Gene expression studies now can be performed using branch-chained DNA and microarray techniques. Protein level quantification in general is performed using isoform-specific antibodies and Western blotting. Enzyme activity represents the most relevant endpoint for drug–drug interaction evaluations. However, an inducer may not induce enzyme activity due to its inhibitory effects on the induced enzyme.

CRITICAL ASSESSMENT OF THE METHOD

The procedures described with human hepatocytes represent the "gold standard" for the assessment of human induction potential of xenobiotics. As of now, there appears to be no evidence of known human P450 inducers which are not inducers in vitro using the assay described. The following are additional issues critical to the performance of the assay:

- Noncytotoxic doses of the test article should be used. Cytotoxicity may lead to an erroneous conclusion of no induction, as the total activity may be decreased due to the lower number of viable cells or compromised cell metabolism. All known inducers are active at noncytotoxic concentrations.
- There are inducers which are also enzyme inhibitors. Ritonavir is an example of an inducer of CYP3A4 but also a potent inhibitor of the P450 isoform. Ritonavir is not an inducer using enzyme activity as an endpoint but can be shown to be an inducer based on gene expression (quantification of CYP3A4

Table 4 Drug metabolizing enzyme (P450 isoforms (CYP), UDP-glucuronosyl transferase (UGT) and phenol sulfotransferase (PST)) substrates that can be used for the in situ measurement of activity in the human hepatocyte enzyme induction assay.

Enzyme	Substrate	Concentration (uM)	Metabolite Quantified
CYP1A1/2	Ethoxyresorufin	2	Resorufin
CYP1A2	Phenytoin	50	Acetaminophen
CYP2A6	Coumarin	100	7-OH coumarin
CYP2C9	Tolbutamide	50	4-OH tolbutamide
CYP2C19	S-mephenytoin	100	4'-OH S-mephenytoin
CYP2D6	Dextromethorphan	16	Dextrophan
CYP2E1	Chlorzoxazone	300	6-OH chlorzoxazone
CYP3A4	Testosterone	125	6βOH-testosterone
UGT/PST	7-OH coumarin	100	Glucuronide and sulfate conjugates

Table 5 Typical results for the testing of a test article for CYP1A and CYP3A induction using primary human hepatocytes. Ethoxyresorufin O-dealkylation and testosterone 6-beta-hydroxylation were used as activity endpoints for CYP1A and CYP3A, respectively. Omeprazole (25 uM) and rifampin (25 uM) were used as positive controls for the induction of CYP1A and CYP3A, respectively. Activity results are expressed as mean activity ± standard deviation. Asterisk (*) represents results that are statistically significant ($p \leq 0.05$) from solvent control values. The results show that the experimental conditions were adequate for the evaluation of CYP1A and CYP3A induction, as the positive controls provided the expected positive findings. The test article at the concentrations tested did cause induction of CYP1A and CYP3A activities.

Treatment	CYP1A2 Activity (pmol/million cells)	Percent of Solvent Control	CYP3A4 Activity (nmol/million cells)	Percent of Solvent Control
Solvent Control	15 ± 2	100 %	744 ± 76	100 %
Omeprazole	535* ± 93	355 %	767 ± 62	103 %
Rifampin	23* ± 6	151 %	2597* ± 218	349 %
TA 10 uM	16 ± 3	107 %	692 ± 53	93.0 %
TA 20 uM	15 ± 3	100 %	723 ± 67	97.2 %
TA 50 uM	16 ± 2	107 %	709 ± 85	95.3 %
TA 100 uM	16 ± 4	107 %	633 ± 57	85.1 %

mRNA). Quantification of enzyme activity as well as either total protein (e.g. using Western blotting) or mRNA (e.g. using cDNA microarray (Kier et al. 2004; Harris et al. 2004)) will allow the evaluation of these inducers.

- Primary hepatocytes are not responsive to inducers immediately after isolation. Primary hepatocytes require culturing at 37 °C for approximately 1–2 days before they are responsive to inducers. This "recovery period" is also important for hepatocytes that have been subjected to culturing at temperature lower than 37 °C (e.g. shipment from one laboratory to another).
- The induction period should be at least 48 hours, especially when enzyme activity is used as an endpoint.
- The assay requires the use of confluent or near-confluent hepatocyte cultures. It is well-known that for CYP3A4 induction, cell density is critical. Cell cultures that has low cell densities (e.g. less than 50 % confluent) will not be responsive to CYP3A4 inducers such as rifampin. Cell morphol-

ogy evaluation is therefore an important step in the performance of the assay.

MODIFICATIONS OF THE METHOD

Freshly isolated hepatocytes are universally accepted as the "gold standard" for enzyme induction assays. However, each experiment requires the availability of a liver for hepatocyte isolation. Fresh hepatocyte availability has been recently enhanced due to several commercial vendors' effort to procure livers and provide isolated hepatocytes as a product. Studies with fresh hepatocytes cannot be planned, and sometimes may be delayed due to the lack of livers. To overcome this inconvenience of the use of freshly-isolated human hepatocytes, the following approaches for enzyme induction have been developed:

- Reporter cell lines: As discussed earlier, research on the mechanism of enzyme induction has lead to the discovery that most inducers act via binding to specific receptors. Based on this knowledge, via transient or stable transfection, cell lines can be engineered to contain one of these receptors linked to a reporter gene (e.g. luciferase, so activation can be conveniently evaluated via chemilumenescence using luciferin as a substrate) (Raucy et al. 2002; Lemaire et al. 2004). These cell lines can be used for the early screening of enzyme inducers. In general, the reporter cell line would produce results similar to that of primary hepatocytes. There are some incidences of significant different results which may be due to the presence of other DME and transporters in the hepatocytes that are absent in the reporter cell line.
- Immortalized hepatocytes: A major drawback of the use of primary hepatocytes is that hepatocytes can not be expanded in culture. To overcome this problem, researchers have embarked on the immortalization of primary hepatocyte cultures (e.g. Bayad et al. 1991; Li et al. 2005). Currently, immortalized human hepatocyte cell lines are available commercially and may represent convenient screening experimental systems for enzyme induction studies. Unfortunately, at the time of this writing, there are no peer-reviewed publications on the application of human immortalized hepatocytes in induction studies. It is important to ensure that the induced isoforms in the cell lines are the mature P450 isoforms rather than the embryonic forms. For instance, the use of HepG2 cells may not be appropriate as the embryonic P450 isoforms CYP1A1 and CYP3A7 are the inducible isoforms in these cells rather than the CYP1A2 and CYP3A4 in the adult human liver.
- Cryopreserved human hepatocytes: Cryopreserved human hepatocytes are extremely useful for the evaluation of drug metabolism, but in general cannot be cultured due to their impaired cell-attachment. There are preparations of cryopreserved human hepatocytes with high plating efficiency and therefore can be cultured for induction studies. It has been shown that hepatocytes cultured after cryopreservation are responsive to CYP1A and 3A inducers, but they have a significantly lower basal (uninduced) levels of these enzymes. Cryopreserved human hepatocytes therefore represent a more convenient experimental model than freshly isolated human hepatocytes for enzyme induction studies (Roymans et al. 2005).

The above-mentioned experimental systems are more convenient than freshly isolated hepatocytes. However, as of this writing, results obtained with these alternative systems need to be confirmed with those from freshly isolated hepatocytes to assess the true induction human enzyme induction potential.

EXAMPLE

Typical results for the testing of a test article for CYP1A and CYP3A induction using primary human hepatocytes are shown in Table 5.

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Chapter II.K
Drug–Drug Interaction – Enzyme Inhibition

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INTRODUCTION

Inhibitory drug interactions received considerable attention in the 1990s because some prominent drugs (e.g. terfenadine) caused life-threatening adverse effects when prescribed with other commonly used drugs (e.g. antibiotics). At about the same time, in vitro technology was developed to study drug interaction with individual human P450 enzymes by using either enzyme specific substrates or recombinant P450 isoenzymes. Along with guidance documents issued by the US FDA and the European Agency for the Evaluation of Medicinal Products, the evaluation of in vitro drug interactions have become as an integral part of the drug development process (FDA 1997, EMEA 1997).

The following discussions and assay descriptions are related to cytochrome P450 inhibition. Most drug interaction studies are related to P450 isoenzymes. Nevertheless, other enzyme systems may contribute to significant drug interactions such as transporter (e.g. Floren 1997; Abel 2001) phase II enzymes (e.g. Dietmann 1976; Zucker 2001; Rayer 2001; Williams 2004), or cytosolic enzymes (Obach 2004). Additional assays related to pgp, phase II or cytosolic enzyme interactions are published in literature (e.g. Polli 2001; Schwab 2002; Schinkel 2003; www.bdbiosciences.com, Obach 2004).

Inhibitory drug interactions generally fall into two categories. The first involves "direct" inhibition of the metabolism of one drug by the other. Direct inhibition may exhibit Michaelis-Menten kinetic characteristics of competitive, noncompetitive, uncompetitive or mixed (competitive and noncompetitive) inhibition.

The second type of drug inhibition results from "irreversible" (or "quasi-irreversible") inhibition of cytochrome P450 and often involves metabolism-dependent inhibition or suicide inactivation of cytochrome P450 (Ortiz de Montellano 1995).

Identifying a drug as an inhibitor of a given P450 isoenzyme does not necessarily imply, that the drug will cause clinically relevant drug interactions. The inhibition potential must be considered in the following context (Madan 2002):

1. The pharmacokinetics of the inhibitory drug, particularily maximum exposure and half-life
2. The potential of co-administering the inhibitory drug together with other drugs that are substrates of the same isoenzyme (inhibition of the co-medication by the NCE must be considered separately from inhibition of the NCE metabolism by the co-medication)
3. The extent to which drug clearance is dependent on the related isoenzyme
4. The potential of saturating the capacity of the related isoenzyme
5. The clinical consequences of alteration of pharmacokinetics of the affected drug (depending on the drug's therapeutic index).

The Ki determination is inevitable for risk assessment, also to understand the mechanism of inhibition. The impact of I/Ki on the AUC ratio of a drug in presence and absence of a competitive inhibitor is given in Figure 1 (Tucker 2001).

II.K.1
"Direct" Cytochrome P450 Inhibition

PURPOSE AND RATIONALE

"Direct" inhibition occurs, when a drug inhibits P450 isoenzyme without requiring biotransforma-

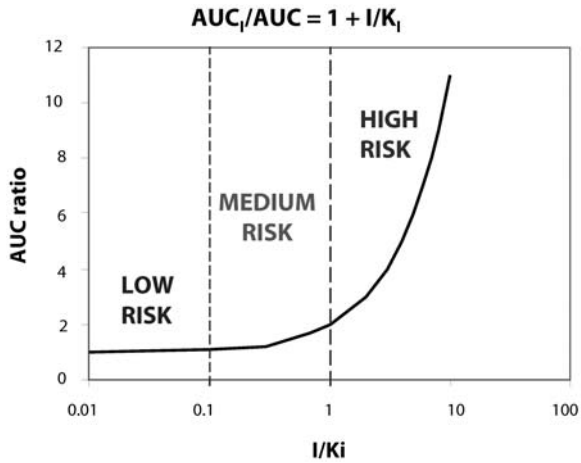

Fig. 1. Plot I/Ki versus AUCi/AUC related to potential risk for drug interaction

tion. Traditionally, direct inhibition has been divided in three categories: competive, noncompetive and uncompetitive inhibition (Madan 2002).

Competitive inhibition occurs, when substrate and inhibitor compete for binding at the same active site at the enzyme. Based on the Michaelis-Menten kinetics, Vmax is unchanged whereas Km increases. In case of noncompetive inhibition, the inhibitor and the substrate bind to different sites at the enzyme. Vmax decrease whereas the Km value is unaffected. Binding of the inhibitor only to the enzyme-substrate complex is described as uncompetitive inhibition. Both, Vmax and Km decrease. Finally, mixed (competitive–noncompetitive) inhibition occurs, either the inhibitor binds to the active or to another site on the enzyme, or the inhibitor binds to the active site but does not block the binding of the substrate.

Enzyme kinetics and the mode of inhibition are well described by transformation of the Michaelis-Menten equation. The binding affinity of the inhibitor to the enzyme is defined as the inhibition constant Ki, whereas the affinity, with which the substrate binds, is referred to the Michaelis-Menten coefficient Km. Michaelis-Menten kinetics base on three assumptions:

- The dissociation of the enzyme-inhibitor or the enzyme-substrate complex is the rate-limiting step.
- The enzyme concentration is negligible compared to the concentration of the substrate or inhibitor.
- The free concentration of inhibitor and substrate is known or well approximated by the total concentration of substrate and inhibitor.

Besides liver slices, isolated/cultured hepatocytes and purified, reconstituted P450 isoenenzymes, human liver microsomes and microsomes from cell lines transfected with cDNA encoding a given human P450 isoenzyme (recombinant P450 isoenzymes) are used for drug interaction studies, most commonly human liver microsomes and recombinant P450 isoenzymes. During the drug discovery phase, IC_{50} determinations are typically performed in a HTS format using fluorescence marker substrates and recombinant P450 isoenzymes. Within the drug development phase, detailed interaction studies (IC_{50} determination) are performed with enzyme specific marker substrates using human liver microsomes. Determination of the Ki value and the mode of inhibition are usually required for intermediate and potential inhibitors.

II.K.2 CYP Inhibition Studies Using Recombinant P450 Isoenzymes

PROCEDURE

Combinatorial chemistry and high throughput screening for pharmacological activity have identified a relatively large numbers of compounds, which have potential drug properties. Since inhibitory drug interaction has been associated with life-threatening adverse effects, an early identification for potential drug interaction of NCE is desirable. The availability of high throughput assays for cytochrome P450 inhibition facilitates the identification of those drug candidates, which have lower potential for drug–drug interactions.

Table 1 summarizes typical assay conditions for CYP inhibition studies of the most relevant P450 enzymes using recombinant P450 isoenzymes (Supersomes) which is applicable to 96 and 384 well format. Assay conditions for additional P450 isoenzymes can be found under www.gentest.com.

Usually, the NCE is pipetted together with the enzyme/substrate complex and the reaction is started with addition of the cofactor solution. Incubation times vary between 15 and 45 min at 37 °C. Afterwards, the reaction is stopped by addition a TRIS/Acetonitile solution and applied to fluorescence read out.

EVALUATION

The assays are usually performed in parallel to solvent control and a well-known inhibitor of the P450 isoenzyme investigated.

CRITICAL ASSESSMENT OF THE METHOD

For over-expressing P450 isoenzymes, several heterologous expression systems have been established, that

Table 1 Marker substrates and typical incubation conditions using recombinant P450 isoenzymes (Supersomes, Gentest, Woborn MA).

CYP	Substrate	Substrate Conc. (μM)	Enzyme (nM)	$NADP^+$ (μM)	G6P (mM)	$MgCl_2$ (mM)	G6P-DH (U/ml)	Incubation Time (min)
1A2	3-Cyano-7-ethoxycoumarin (CEC)	5	5	8.1	0.4	0.4	0.2	15
2A6	Coumarin	3	5	8.1	0.4	0.4	0.2	10
2B6	7-Ethoxy-4-trifluoro-methylcoumarin (EFC)	2.5	5	8.1	0.4	0.4	0.2	30
2C8	Dibenylfluoroscein (DBF)	1	9	8.1	0.4	0.4	0.2	30
2C9	7-Methoxy-4-trifluoro-methylcoumarin (MFC)	75	5	8.1	0.4	0.4	0.2	45
2C19	3-Cyano-7-ethoxycoumarin (CEC)	25	5	8.1	0.4	0.4	0.2	30
2D6	3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methyl-coumarin (AMMC)	1.5	7.5	8.1	0.4	0.4	0.2	30
2E1	7-Methoxy-4-trifluoro-methylcoumarin (MFC)	70	5	8.1	0.4	0.4	0.2	45
3A4	7-Benzyloxy-trifluoro-methylcoumarin (BFC)	50	5	8.1	0.4	0.4	0.2	30
	7-Benzyloxyquinoline (BQ)	40	7.5	8.1	0.4	0.4	0.2	30
	Dibenzylfluorescein (DBF)	1	1	8.1	0.4	0.4	0.2	10

Final volume: 200 μl

includes bacteria (Escherichia coli), yeast (Saccharomyces cerevisiae, Schizosaccharomyces pombe), insect cells (baculovirus), and mammalian cells (V79, CHO, HepG2, NIH 3T3, human lymphoblast cells) (Crespi 1999). High active P450 isoenzymes, overexpressed in human lymphoblast cells (Supersomes, Gentest Corp., Woborn, MA,) and in insect cells (Baculosomes, Panvera, Madison WI) have been asserted in the market and are commercially available. However, the Supersomes and Baculosomes have not been thoroughly characterized with respect to their kinetic properties and substrates/inhibitors specificities. Usually, the expression of the cytochrome b5 and/or NADPH-cytochrome P450 reductase, varies from batch to batch, which can affect the turnover number (Vmax) for a given enzyme, although the affinity (Km value) of P450 enzymes toward marker substrates is generally comparable between recombinant enzymes and human liver microsomes (McGinnity 1999). Last but not least, the catalytically inactive apoprotein contributes significantly to the total protein concentration. On the other hand, the simplicity of the test system (separate study of the P450 isoenzyme, fluorescence labeled marker substrates, which allows a rapid, compound-independent read out without any extraction procedures) allows a quick estimation of the interaction potential of NCEs in a HTS format with an excellent sensitivity, reproducibility and throughput. In addition, polymorphic P450 isoenzymes with different genotypes are available which allows detailed interaction studies. Sometimes, fluorescent excitation and emission overlaps between NCEs/metabolites, NADPH and marker substrate occurs, which might be overcome by alternative read out methods (e.g. LC/MS/MS technologies).

A major disadvantage is, that inhibitory metabolites generated from other CYPs are overseen in the assays (false-negative). False-positive are due to enzymes involved in the metabolic turnover other than the particular one studied.

MODIFICATION OF THE METHOD

Additional fluorescence labeled marker substrates with different extension/emission wavelength are on the market e.g. from Invitrogen (www.invitrogen.com) which allows some variation if the NCE/metabolite interfere with the fluorescence read out.

P450-Glo from Promega Biosciences Inc. (www.promega.com), a luminescent cytochrome p450 Assay was introduced as alternative CYP inhibition assay. The marker substrates are luciferin

derivatives (Luciferin 6′-chloroethyl-ether, Luciferin 6′-methylether, 6′-Deoxyluciferin and Luciferin 6′-benzylether), which are converted from recombinant P450 isoenzymes (Supersomes or Baculosomes) to luciferin, which in turn react with luciferase to an amount of light that is directly proportional to P450 activity. The assay promises an exquisite sensitivity with low background signals and a broad dynamic range. Since no information of NCEs towards their luciferase inhibition potential is known, an inhibition study has to be performed in parallel. The same is true for quench effect of the NCE that might alter the read out. The marker substrates are not specific for any single P450 isoenzyme, except for 6′-Deoxyluciferin (human CYP2C9). Hence, application to HLM or cell based assays are critical today. In addition, marker substrates for CYP2A6, CYP2B6, CYP2C19, CYP2D6 and CYP2E1 are currently missing. The assay is applicable to 96 and 384 well format. The throughput seems to be lower since the luciferase read out reaction needs additional 20 min incubation time.

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II.K.3 CYP Inhibition Studies Using Human Liver Microsomes

PROCEDURE

Human liver microsomes contain all P450 isoenzymes expressed in human livers, although their content and genotype, especially for polymorphic P450 isoenzymes such as CYP2D6, CYP2C9 and CYP2C19 can vary from sample to sample. To overcome the problem of variability, several individual human liver samples are pooled to get an "average" of all P450 enzymes expressed in human livers. Individual and pooled human liver microsomes are commercially available. Since all P450 isoenzymes are present in microsomes, enzyme-selective substrates must be used. Table 2 summarized recommended substrates.

Each drug (at least in five concentration which covers two orders of magnitude) is incubated with human liver microsomes in the presence of the marker substrate. Reactions are initiated with addition of NADPH

Table 2 Recommended in vitro marker substrates and inhibitors for CYPs (Tucker 2001).

CYP	Substrate preferred	acceptable	Inhibitor preferred	acceptable
1A2	Ethoxyresorufin, Phenacetin	Caffeine (low turnover), Theophylline (low turnover), Acetanilide (mostly applied in hepatocytes)	Furafylline*	α-Naphthoflavone (but also inhibits 3A4)
2A6	Coumarin			Coumarin (but high turnover)
2B6	S-Mephenytoin (N-desmethyl metabolite)	Bupropion		Sertraline (but also inhibits 2D6)
2C8	Paclitaxel		Glitazones	
2C9	S-Warfarin, Diclofenac	Tolbutamide (low turnover)	Sulfaphenazole	
2C19	Mephenytoin (4-hydroxy metabolite), Omeprazole		Tranylcypromine	Ticlopidine (but also inhibits 2D6), Nootkatone (but also inhibits 2A6)
2D6	Bufuralol, Dextromethorphan	Metoprolol, Debrisquine, Codeine	Quinidine	
2E1	Chlorzoxazone	4-Nitrophenol, Lauric acid		Fomepizole
3A4	Midazolam, Testosterone (strongly recommended to use at least two structurally unrelated substrates)	Nifedipine, Felodipine, Cyclosporine, Terfenadine, Erythromycin, Simvastatin	Ketoconazole (recent evidence that it is also a potent inhibitor of 2C8), Troleandomycin*	Cyclosporine

* mechanism based inhibitor (Donato 2003)

at 37 °C (or $NADP^+$ if a regeneration system is used). According to the Michaelis-Menten assumptions, the marker substrates should be used at concentrations below or around the corresponding Km values. The microsomal protein concentration should be as low as possible to circumvent unspecific binding. Cofactors, such as NADPH or $NADP^+$, G6P, G6P-DH and $MgCl_2$ are usually used at concentration of 5 mM, 0.5 mM, 5 mM, 0.5 U/ml and 6 mM, respectively in 50 mM Phosphate buffer pH 7.4. Organic solvent should be minimized as much as possible to circumvent inhibitory effects of the solvents (Busby 1999; Yuan 2002). Adding ice-cold acetonitrile solution following a sharp centrifugation step stops the reactions. The supernatants are applied directly to HPLC or LC/MS/MS analytics for quantification of the marker substrate and the corresponding metabolite.

Table 3 summarizes typical incubation conditions and kinetic constants of marker substrate reactions of Human P450 enzymes in a pool of human liver microsomes (Mandan 2002).

EVALUATION

The assays are usually performed in parallel to solvent control and a well-known inhibitor of the P450 isoenzyme investigated.

CRITICAL ASSESSMENT OF THE METHOD

Michaelis-Menten assumption can be violated in the case of P450 enzymes, depending on the in vitro system used. The "free" concentration of substrate or inhibitor may differ significantly from the total concentration, since microsomes usually contain a large amount of lipids and proteins that can decrease the free concentration of the drug and the marker substrate in the medium. The potency of some inhibitors is such that the free concentration of the inhibitor is in the same range as the enzyme concentration. This problem can be overcome by lowering the enzyme concentration (often limited by analytical sensitivity of the assay) or by estimating an "apparent" Ki, by correcting for the fraction of the inhibitor that is bound to the enzyme, which is calculated at the product of the fractional inhibition in the presence of a given inhibitor concentration and enzyme content (Gibbs 1999). Note, that IC_{50} values are extrinsic constants whereas Ki values are intrinsic constant. Consequently, IC_{50} values (but not Ki values) are dependent on the type of substrate, incubation conditions and are not exactly reproducible from one laboratory to another. On the other hand, IC_{50} determination are less time consuming and an external quality control can be achieved by using

Table 3 Typical incubation conditions and kinetic constants of marker substrate reactions of human P450 enzymes in a pool of human liver microsomes (Mandan 2002).

CYP	Marker reaction	Protein (mg/ml)	Incubation Time (min)	Km (μM)	V_{max} (pmol/min/mg)
1A2	Ethoxyresorufin O-dealkylation	0.1	10	0.26 ± 0.01	120 ± 2
2A6	Coumarin 7-hydroxylation	0.05	5	0.57 ± 0.02	1300 ± 12
2B6	S-Mephenytoin N-demethylation	1	30	1700 ± 40	1900 ± 30
2C8	Paclitaxel 6α-hydroxylation	0.1	10	14 ± 1	530 ± 30
2C9	Diclofenac 4'-hydroxylation	0.1	5	3.7 ± 0.2	3600 ± 59
2C19	Mephenytoin 4'-hydroxylation	1	30	35± 2	380 ± 4
2D6	Dextromethorphan O-demethylation	0.1	10	5.5 ± 0.5	360 ± 13
2E1	Chlorzoxazone 6-hydroxylation	0.1	10	27 ± 2	2500 ± 100
3A4	Testosterone 6β-hydroxylation	0.1	10	110 ± 10	9800 ± 490
4A9/11	Lauric acid 12-hydroxylation	0.1	5	7.6 ± 1.2	2200 ± 100

standard inhibitors in parallel. Nevertheless, the FDA has accepted the method of predicting the potential for drug interaction by a drug based on Ki values (together with the free plasma concentration of the drug). Referring to Table 3, Buproprione is recommended as 2B6 marker substrate since the Km value is around 89 μM (Hesse 2000), whereas 2B6 has only a low affinity for S-Mephenytoin (N-desmethyl metabolite) with a Km value of approx. 1900 μM (Ko 1998). Rae at al. (Rae 2002) and Heyn et al. (Heyn 1996) recommend Thio-TEPA or Orphenadrine as competitive inhibitor for CYP2B6. In addition, 4-Methylpyrazole and Diethyldithiocarbamate have been accepted as competitive and mechanism based inhibitor for CYP2E1 (Yamazaki 1992; Chang 1994).

MODIFICATION OF THE METHOD

Based on the result from the IC_{50} determination, determination of additional kinetic parameters such as Ki and the inhibition mode are useful (variation of the substrate concentration e.g. Km/4–4 Km with time). Transformation of the Michaelis-Menten equation are used both for calculation the Ki value as well as for graphical depiction of the type of inhibition (e.g. direct plot ([rate]/[substrate], Dixon plot [1/rate]/[inhibitor], Linewaver-Burk plot [1/rate]/[1/substrate] or Eadie-Hofstee plot [rate]/[rate/substrate]).

The most critical step in the interaction studies is a sensitive and reproducible method for quantification of the marker substrates and the corresponding metabolite. Analytical methods are usually applied to time- and resources-consuming HPLC (UV/fluorescence/radioactivity detection) or LC/MS/MS detection.

An alternative detection method was introduced by Yang (1991); Bloomer (1992; 1995); Rodrigues (1994; Rodrigues 1996; Rodrigues 1997) and Riley (1997). They used ^{14}C-Nitrosodimethylamine, [O-Methyl-^{14}C]Dextromethorphan, [O-Ethyl-^{14}C]Phenacetin and [N-Methyl-^{14}C]Erythromycin as marker substrates for CYP2E1, CYP2D6, CYP1A2 and CYP3A4, respectively with a ^{14}C-CH_2O or ^{14}C-C_2H_4O read out after a simple and rapid extraction method.

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II.K.4
CYP Inhibition Studies Using Isolated/Cultured Hepatocytes or Liver Slices

Interaction studies in isolated/cultured hepatocytes and liver slices have to be assessed critically since a couple of competing reactions occur e.g. uptake pathways or phase II metabolism of the NCE and/or marker substrate, which makes it extremely difficult to interpret the data mechanistically. Additionally, metabolic capacity of hepatocytes in culture change with time (e.g. decrease/increase of phase I and phase II enzyme activities and internalization of transporters). Usually, Michaelis-Menten kinetics does not apply directly. For reliable results, interactions studies have to be performed in hepatocytes from at least three different donors since pooled hepatocytes are not available yet. Similar problems are also discussed for interaction studies in liver slices (Ekins 1998).

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Chapter II.L
Absorption

Volker Krone

INTRODUCTION

The use of radiolabeled molecules allows a drug and its labeled metabolites to be followed throughout the body and excreta over time. The radioactivity concentration can be tracked in blood and plasma as well as in tissues. Whether the drug with its specific radioactivity administered to the body is completely captured can be proven by calculating the so-called 'mass balance'.

Whereas the radioactivity measurement alone does not allow distinguishing between drug and metabolite(s), samples obtained from the described studies should be also used for standard determination of the drug and its known metabolites, receiving information about the drug and the known metabolite kinetics directly. The gap between radioactivity concentrations and the concentrations determined by direct bioanalytical methods defines the contribution of unknown metabolites.

Using one and the same set of studies with equally withdrawn samples for determination of all radiokinetic and pharmacokinetic data possible is not only economically favorable, but allows illuminating comparisons with a minimum of assumptions and therefore high confidence to interpretation.

Radiokinetic studies deliver the key data of absorption and elimination to which all data and statements of other investigations have to fit – or in case of conflict need a plausible explanation at least.

As examples in the following chapter, the frequently used radiokinetic studies in dogs and rats are described, including placental transfer and milk transfer in rats. Investigations in other species can often be performed similarly. The final choice of species should be done in consensus with toxicology (at a later stage of development including cancerogenicity and segment II studies), regarding the metabolic patterns of in vitro cross-species comparisons and regarding the pharmacological in vivo model.

A suggestion of necessary types of radiokinetic studies and general hints for conducting those animal ADME studies can be found in Campbell DB, Jochemsen R (1994).

Choice of the Radiolabel

^{14}C is the label of choice for most drugs since it is stable biologically (when the right labeling position is chosen), the detection is comfortable[1] and in case of combustion of samples the produced $^{14}CO2$ can be nicely absorbed quantitatively. ^{3}H labeled drugs are easier to synthesize sometimes, but they are often less stable biologically or at least worse to predict in their biological stability. Because of its much higher specific activity, the ^{3}H-label is favorable in the case of high molecular weight drugs and/or very low doses. ^{35}S, ^{33}P, ^{125}J are used comparatively seldom.

The position of the label should be away from sites chemically instable or from sites of metabolic attack to ensure that the label is kept in the main metabolic fragments. Of course, this is difficult for complex molecules, especially when the metabolic attack takes part in the center of the drug molecule. Then it can become necessary to introduce a second label and to repeat the set of radiokinetic studies. A double-labeling strategy to minimize the number of radiokinetic studies has normally to be refused due to complexity of interpretation of the data.

A quality control including stability of the radiolabeled drug is mandatory. The chemical degradation and

[1] half-life of 5730y for ^{14}C, making an half-life correction unnecessary. As a weak β – radiator (up to 156 keV) the risk of handling is an acceptable compromise in the laboratory (protection area!).

also the radiolysis can be responsible for a short expiry date. The higher the purity and the content of the labeled drug is, the better the results of the radiokinetic studies can be interpreted[2].

The stability and homogeneity[3] of the radiolabeled compound should be tested also in the galenic formulation intended to be used.

DETERMINATION OF THE RADIOACTIVITY CONCENTRATION

Radioactivity measurements are carried out by the liquid scintillation counting procedure in β-spectrometers using an external standard device which permitted the counting efficiency to be determined by the channel ratio method (explained for instance by Dyer (1980)).

Samples are either measured directly after adding a commercial scintillation cocktail[4] or after dissolution and discoloration[5] or after combustion in suitable combustion machines[6].

A thorough calibration of the β-spectrometer regarding different sample matrices and sizes, the scintillator used, different dissolutions, different colorations of the samples, different amounts of CO_2 after combustion and of course different radioactivity concentrations should be ensured. Calibration and control samples can be set up by using internal standard kits (for instance ^{14}C-O Standard from PerkinElmer Wallac GmbH D-79111 Freiburg).

Blank samples (of every type of matrix) have to be measured in the batches with the study samples. Their mean value can be used as background value to estimate the limit of quantitation.

The definition of the limit of quantitation (LOQ) is handled quite differently. For example, an easy, often used approach is the definition of using the double of the mean blank value as LOQ. This definition sounds simple and a scientific theoretical justification seems not to be available. However, the thus defined LOQ is then a reasonable value when standard deviation (SD) of the blank values is low (< 10 %, according to own experience). More sophisticated is the following definition: The mean blank value plus 3 times the standard deviation is required for a limit of detection (LOD) and the mean blank value plus 10 times the standard deviation is required for LOQ (see for instance Krull (1998)). Outliers can be identified for instance by the Grubbs test (for instance explained in www.graphpad.com).

Control of Applied Dose

For small animals or dosing of a large number of animals, individual doses are determined by weighing the syringes containing the galenic formulation prior to and after each administration; stability of radioactive concentration is determined on two aliquots before and after each series of administration.

For individual dosing of larger animals/few animals the radiolabeled drug is directly weighed in separate devices regarding the individual weight of the animal. All used devices for administration are collected and washed with solvent. The collected solvents are used for back-measurement (dose loss) of radioactivity. The radioactivity found is subtracted from the originally calculated dose.

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[2] Imagine the case of a projected non-absorbable drug, for instance for topical application, and the situation of having detected 3 % absorption with a radioanalytical purity of the labeled drug of 97 %. Or imagine the case of a minor part of radioactivity with a long terminal half-life suggesting a metabolite with an accumulation potency. The radioactivity represents the sum of the original compound and/or radioactive-labeled metabolites and not to forget possible synthetic side-products which can be present in traces (depending on the purity and content of the synthetic material). Discussing traces of radioactivity, for instance traces crossing the placenta, keep in mind that these traces may be due to synthetic side-products. Thus, whenever possible try to use radiolabeled compound as clean as possible.

[3] also in case of solutions the homogenicity can be significantly impaired by surface effects

[4] for instance in case of urine samples when the quench is in the range of the calibration curve or bile samples

[5] for instance with Solvable from Packard BioScience B.V. Groningen Netherlands for dissolution and H_2O_2 for discoloration

[6] for instance with a Tri-Carb 307 combuster which can be equipped with a robot unit for automatic sample handling from Canberra-Packard/Perkin Elmer. Since carry-over effects are not negligible in case a low radioactivity sample follows a high radioactivity sample, a reasonable arrangement of samples in a sequence is essential.

II.L.1 Radiokinetics and Mass Balance in Dogs

PURPOSE AND RATIONALE

To evaluate the pattern and the rate of excretion and to investigate the time course of radioactivity concentrations in blood and plasma with the aim of getting information about the absorption process, the AUC, C_{max} and the elimination half-life of radioactivity in blood and plasma. Possibly, indices for enterohepatic

cycle or for metabolites with much different volume of distribution than the original compound may be found. An estimate of the absorption rate comparing dose normalized AUCs or the renal excretion after iv and oral (or any other) administration can be done and a major binding to formed blood elements can be noticeable by comparison of blood and plasma concentrations.

The route of administration projected to be used therapeutically should be applied. If possible – and the solubility of the drug allows – the study should also be performed after intravenous administration. The results of both routes should be compared. Since the oral administration is the most prominent route of administration, this route is described here. The method can be adapted for other routes of administration.

Normally, the radiokinetic study is performed administering a single dose per administration route. In case of extraordinary circumstances, for instance when there is a suspicion of an accumulation of any metabolite, a radiokinetic study with repeated dosing can be considered.

PROCEDURE

Three healthy male beagles receive an intravenous dose into a jugular vein or into the vena cephalica antebrachii (typically 1–5 mg/kg b.w.). After a wash out period dependent on the duration of excretion of the drug/metabolites (typically 4 weeks at minimum), the same animals are dosed orally by using a stomach tube (typical dose: 10 mg/kg b.w.). Normally, 5–10 MBq/animal is a sufficient radioactive dose for a ^{14}C-label, in case of ^{3}H about 50 MBq/animal could be used. Up to 168 h after administration, blood, urine and feces are collected. At each collection time about 3.5 mL of blood are taken for radioanalysis (2–3 aliquots) and for processing plasma for subsequent radio- and bioanalysis in plasma (also 2–3 aliquots each). The plasma is aliquoted immediately after centrifugation without warm-up. At a few preselected time points additional blood is collected for metabolite investigations (about 5 mL).

Proposal for Standard Collection Times

Blood: 0 h (before administration), 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 24 h, 48 h, 72 h, 144 h, 168 h after oral administration and 0 h (before administration), 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 8 h, 24 h, 48 h, 72 h, 144 h, 168 h after intravenous administration.

Urine: 0 h (before administration), 0–8 h, 8–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

Feces: 0 h (before administration), 0–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

Cage washing: 0–24 h, 24–48 h, 48–168 h after administration.

Radioanalysis is performed by the liquid scintillation procedure either directly after addition of a scintillator (urine, cage washing) or following combustion and addition of a scintillator (blood, plasma, feces).

Details of the Oral Administration

Material

- Bulge tube (stomach tube) for oral dosing of dogs (soft, transparent plastic tube with a diameter of about 1 cm and 60 cm of length)
- nonreturn valve
- disposable gloves
- highly absorbant paper
- glass funnel
- bottle with tap water.

Procedure

The dog is placed on an undersheet and its head is secured by an assistant. The upper and lower jaws are separated by exerting pressure on the lips with both hands. The stomach tube moistened with mains water is introduced into the open throat. Caution: do not introduce the tube in the trachea. Pour mains water into the glass funnel fitted on the stomach tube over the nonreturn valve to check that the tube is correctly positioned. If outflow difficulties occur, adjust the position of the tube by moving it slightly backwards and forwards. Then introduce the test substance to be administered. Then rinse the tube and remove.

Details of the Intravenous Administration into the Vena Cephalica Antebrachii or the Vena Saphena Parva

Material

- Curved scissors
- disposable gloves, Kleenex
- disposable syringe with formulation for administration with fixed canula
- electric hair clippers.

Procedure

An assistent fixes the dog. The vena saphena parva (hindlimb vein) or the vena cephalica antebrachii is excised from hairs. The vein is compressed using a rubber tubing tourniquet or by firmly encircling the

limb with the hand above the vein. Insert the needle of the disposable syringe containing the study substance preparation to be administered into the congested vein, release the tourniquet and check that the needle is in the correct position by aspirating a small amount of blood into the syringe. Then carefully administer the substance and remove the needle and syringe. Prevent secondary bleeding by exerting pressure on the skin injection site with a Kleenex swab.

Details of Sample Collection (Urine, Feces, Cage Washing)

Material

- Metabolism cage
- 2 l urine collection container (cooled)
- dash bottles containing warm mains water
- polyethylene bottles in various sizes
- labels, plastic feces scraper, large glass beaker or polyethylene bag, disposable gloves, Kleenex, scrubbing brush with handle, cooling device for the urine tube
- mixer.

Procedure

The urine is collected according to the preinstructed collection intervals directly from the urine collection container of the metabolism cage. The samples are weight and directly after stirring and homogenizing the urine, aliquots are taken for radioanalysis. The remainder is bottled and frozen for additional investigations planned (metabolite profile and identification and/or bioanalysis).

The feces are collected from the sitting device and the ground plate using the plastic scraper. In case of direct processing of the feces sample, the feces are collected in a weight glass beaker. In case a direct processing is not possible, the feces are collected in a weight plastic sac and are frozen.

The weights are determined and registered as raw-data. To homogenize the feces each sample is diluted with demineralised water – according to the consistence with the two to four-fold of the feces weight. The weight of the filled bottle is determined and the data stored until evaluation.

The sample with the mixture of feces and distilled water is homogenized with a mixer which should be cleaned rigorously before using it again and which is proven to be without contamination.

For preparation of samples for combustion, preweighted combustion cones were filled with an appropriate amount of homogenate (typically about 250 mg). The combustion cones and covers including the homogenate are weight again and the data stored until evaluation.

The cage washing is done after removal of feces and urine with the help of warm tap water dash bottle (about 2 L). Cleaning of the cage is done with the scrubbing brush starting with the walls, the sitting device and followed by the ground plate. The fluid is weight and after thorough mixing, aliquots are filled in scintillation cups for radioactivity measurement.

Details of Sample Collection (Blood, Plasma)

Material

- Monovets with sine use canulas (butterfly)
- tourniquet loop
- disposable gloves, Kleenex, hair clipper
- needle, needle holder, surgical suture material, scissors, protective collar for dogs, antibiotic powder.

Procedure

The site for blood collection has to be different from the site of intravenous administration. Thus, the vena cephalica antebrachii can be used for collection and the vena saphena parva for administration (or vice versa). The dog is kept by one person and the hairs at the selected venes at the forelimbs or hindlimbs are removed by gently clipping. The vein on the dog's limb is compressed by the assistant by encircling the limb with the hand or applying a rubber tourniquet loop. The puncture procedure is then performed. Blood should be withdrawn slowly by aspirating gently.

When the monovet is sufficiently filled, remove the compression and withdraw the needle. Secondary bleeding is prevented by immediately exerting compression on the puncture site with a Kleenex swab.

Alternative Route for Blood Sample Collection

A favorable, safe method for multiple blood collection is a permanent catheter in the vena jugularis. The dog is fixed in a frame with a hammock. An assistent fixed the head upwards, slightly stretched to the opposite site to which the vein puncture should be done. The animal's neck is shaved with clippers over the jugular vein and wiped with a commercial skin disinfectant solution. After applying the tourniquet loop at the base of the neck, the jugular vein is clearly prominent. The jugular vein is then fixed between two fingers and punctured towards the heart with a split needle. The catheter is located inside the split needle and, as soon as blood is visible, is

inserted into the vein. The split needle is then carefully withdrawn and opened. The base of the catheter is secured to the skin with surgical suture material. After the syringe has been inserted, aspirate until blood is visible in the syringe. Then rinse out the catheter with sterile physiological sodium chloride solution (about 2 ml). The correct position of the catheter can be checked by repeating this operation if required. Then seal the base of the catheter with a closure cap. To prevent obstruction when pronounced coagulation occurs, a drop of anticoagulant can be added to the NaCl solution.

For restless animals, the catheter can be prevented from being removed by the dog by fitting with a protective collar of the type commonly used in veterinary practice. Dusting the puncture site and fixation suture with antibiotic powder prevents inflammation.

Catheter insertion should be carried out under the most aseptic conditions possible.

Before blood sampling, the physiological saline solution has to be removed from the catheter using a syringe. This is done by withdrawing 0.5 ml from the catheter before collecting the actual sample with the prepared monovet. After blood collection, the catheter is rinsed and filled again with about 2 ml of physiological sodium chloride solution.

For generating plasma, part of the collected blood is transferred into a centrifuge tube and treated in the centrifuge with 1500 G.

Animal Maintenance

During the study period the animals are maintained on metabolism cages, one in each cage with a separating device for urine and feces, allowing to collect the excreta separately. Conventionally those cages are consisting several parts which have to be cleaned carefully before assembling (box, device for sitting, food, water, urine collection). It has shown to be favorable to control the cleanness by rinsing the cage with an alcohol-water-mixture and subsequent determination of radioactivity of the rinsing solution.

Before study start the animals should have time for acclimatization, depending on the extent of changes in the surroundings and the type and duration of transport. In case of a maintenance in a lab in the neighborhood under similar conditions as in the study, an acclimatization time of at least 1–2 days might be sufficient.

The feed (500 g commercial pelleted diet about noontime, such as ALLCO Vollkost Menü or Ssniff Spezialdiäten GmbH; D-59494 Soest, Deutschland) and tap water ad libitum is given about 20 h before administration.

Following conditions should be controled throughout the study duration:

- room temperature
- relative humidity
- lighting time.

Proposed values for these conditions:

- room temperature: 20–25 °C
- relative humidity: 30–70 %
- lighting time:natural day/night rhythm.

EVALUATION

The concentrations of radioactivity expressed as μg equivalents of drug per g of matrix (μg equiv./g) and alternatively as part of dose eliminated (% administered dose) are determined in each matrix and collection interval[7]. The temporal course of the excretion and the radioactivity concentration in blood and plasma are represented by graphs[8] and tables using either Windows Excel function, a specific pharmacokinetic software program (like WinNONLIN; www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com). The software also allows calculation of the half-lives of radioactivity elimination via urine and feces (when appropriate)[9], to generate cumulation plots or general survey plots like bar diagrams (see example).

In case that an essential part of dose is excreted via urine, an estimation of absorption is often done giving the ratio of urine excretion (oral/intravenous) as absorption rate. It should be compared with the ratio of the dose-normalized plasma AUCs (oral/intravenous).

All these data are finally drawn together for detailed discussion and evaluation. Special attention has to be taken on major differences of data following intravenous and oral administration.

An essential requirement for the quality of the study is a balance value near to 100 %. A value > 95 % and < 105 % is reasonable. Balance values of 90 % to 95 %

[7] It might be necessary to use a normalized apparent collection interval for graphical reasons in case of dissimimilar collection intervals.

[8] Instead of the collection interval, the mean time of the collection period is often used in the graphic presentations.

[9] Via determination of the rate constant which is calculated by linear regression of ln concentration on time using a least three data points which appeared to be randomly distributed about a single straight line. Half-lives were calculated as ln2/rate constant.

or 105–110 % are often accepted too, but they should indicate the investigator that something might run out of control or the process/handling could be optimized. Balances much worse than those should be explained.

In case an essential concentration is available in blood and plasma at early time points after oral administration (may be C_{max} is reached soon also), a rapid onset of absorption can be stated.

When there is no continuous decline of concentrations after intravenous administration, this might be an indication for:

- an inappropriate administration or an precipitation of the drug from the formulation at the application side or just behind the side in the blood stream. This might be the case when there is a plateau concentration or even an increase at early collection times.
- an enterohepatic circulation when a side maximum exists (time depending on absorption and elimination rate, often seen 4 to 8 h after administration)
- one or more of the generating metabolites have a much smaller volume of distribution than the original drug
- other (often more complex) explanations.

In case a concentration ratio (blood/plasma) is distinctly higher than the hematocrit value, this could indicate a binding of the drug or its metabolites to formed blood elements.

CRITICAL ASSESSMENT OF THE METHOD

Always keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled metabolites and not the drug itself. Therefore, it is worthwhile to determine also the drug and known metabolites directly by bioanalytical methods with samples withdrawn from the same study. The comparison of both, the radioactivity concentrations and the sum of concentrations determined by specific methods, allows estimation of the gap (of unknown metabolites) which often develops time-dependently: for instance at the beginning of the study radioactivity concentrations in plasma should fit to the drug concentration. Usually, the gap (as a percentage of the total radioactivity at a certain time point) between both concentrations increases with time.

The estimation of absorption via the ratio of dose-normalized plasma AUCs after oral and intravenous administration is based on the assumption that:

1. a first pass effect including an instant excretion via bile does not exist
2. the metabolic pattern is comparable independently of the route of administration[10]
3. the distribution (drug and metabolites) and the metabolism in special tissue is not effected by the bolus iv administration with high initial concentrations (see for example Chiou (1989))
4. dose-exposure linearity exists (under a more stringent view even a first order kinetic is demanded).

When these criteria are not fulfilled, then either the absorption can be underestimated (for instance when precondition 1) is not true 2) or distinctly overestimated up to a calculated "super-absorption" for more than 100 % (possible for instance when precondition 3) or 4) are not true).

Only AUCs obtained from the same "type of blood" samples should be used. For instance don't compare central arterial blood with venous peripheral blood (Chiou 1989) as long as one is not sure that both are virtually comparable.

Certainly, every radiokinetic as well as any in vivo pharmacokinetic study depends on the galenic formulation used. Aqueous solutions, solutions with organic water-miscible components, solutions with surfactants, emulsions, micro emulsions or suspensions can be responsible for distinct differences of radiokinetics (pharmacokinetics). Therefore, a conscious choice of galenic vehicle and formulation procedure is critical!

MODIFICATIONS OF THE METHOD

The radiokinetic and mass balance study can also be applied to other non-rodent species. The framework is similar to the study described above. Often monkeys, minipigs and rabbits are used. Instead of or additional to the oral administration, the method can be adopted to other routes of administration.

Similar basic procedures including evaluation are described by Webber et al. (2004); additionally, the investigation of biliary excretion and tissue distribution was integrated within the study.

Blood collection from the femoral artery is mentioned by Davis et al. (1994) after surgically preparing vascular access ports to the femoral artery. Using dogs with surgically instrumented indwelling venous access ports into the femoral vein and using a 6 min infusion instead of a intravenous bolus injection is mentioned by

[10] an example where this assumption is not fulfilled is described by Okuyama et al. (1997); consequently the authors only mention the ratio of AUCs after oral and intravenous administration and do not correlate this ratio to absorption.

Krishna et al. (2002). Occasionally, such a short term infusion is recommended instead of a bolus intravenous administration (Chiou 1989, p 283). The advantage is to avoid high blood concentrations shortly after intravenous administration which may be in conflict with the assumed linear dose-exposure relationship (validity of linear relation at border concentration?). The advantage of the intravenous bolus injection is the lower/shorter stress situation for the animals (and may be also for the experimenter) which might influence the reproducibility of the study results.

Sometimes several dose levels are investigated with the described method; normally one starts with a dose from which a pharmacological response is expected, but the dose should be lower than NOAEL. Later, during the course of development, studies at the NOAEL may follow. In the case of evaluation of altered routes of elimination at toxic doses, studies with doses higher than NOAEL may considered as an exception.

Determination of blood, plasma and feces samples not using combustion but either direct measurement (plasma) or solubilizing and de-coloration, the samples is mentioned for instance by Okuyama et al. (1997).

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EXAMPLE

AVE 3559 was developed for the treatment of obesitas. It was ^{14}C-labeled and investigated in a dog radiokinetic study to determine the time course of radioactivity concentrations in blood/plasma and to determine the pattern and rate of excretion, the residual concentrations at the end of the study 168 h after administration and the mass balance following oral and intravenous route of administration.

Three healthy male Harlan beagles weighing 15 to 16 kg received an approximate dose of 10 mg/kg ^{14}C-AVE 3559 orally by using a stomach tube (exact weights and doses see table). The identical animals received approximately 2 mg/kg ^{14}C-AVE 3559 into a jugular vein 5 weeks following the oral administration. The pharmaceutical preparation used for both the oral and intravenous route was a solution in transcutol/water (25/75 w/w). The drug concentration in the formulation was about 2 mg/g. Thus, the animals received about 75 g formulation orally and 15 g intravenously. The radiolabeled drug was blended with non-labeled drug in such a way that each dog received about 7.5 MBq radioactivity.

Blood, urine and feces were collected as described above. The remaining parts of samples were used for bioanalysis and metabolism studies.

Three aliquots from each (processed) sample were used for radioactivity determination. The aliquots from the urine samples were measured directly in the liquid scintillation counting procedure after addition of the scintillator Roth-rotiszint eco plus (Roth, Karlsruhe, Germany). The other matrix aliquots taken up on Combusto Cones (Canberra-Packard), weighed, dried at room temperature, were combusted in a Tricarb combuster (Canberra-Packard GmbH, Model 307) and the $^{14}CO_2$ formed was absorbed by Carbo-Sorb (Canberra-Packard). The subsequent radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Canberra-Packard 2500 TR) after addition of the scintillator Permafluor E+ (Canberra-Packard).

Evaluation and Discussion

As an example for the numerous generated graphs, the time course of the mean radioactivity blood and plasma concentration values (incl. standard deviation) after intravenous and oral administration is given in the summary graph (Fig. 1). Additionally, the mean amounts of dose excreted in urine and feces depending on time after administration is given in the subsequent Fig. 2.

The pharmacokinetic values were calculated by the pharmacokinetics' software WinNonlin, version 1.5. and were summarized in Table 1.

Already 15 minutes after oral administration mean radioactivity concentrations of 5.60 μg equivalents/g (range: 3.9 to 7.4 μg equivalents/g) and 8.47 μg equivalents/g (range 5.8 to 11.1 μg equivalents/g), respectively, were present in blood and plasma indicating

Table 1

Route of administration	Oral				Intravenous			
Dog No.[1]	1	2	3	MW	4	5	6	MW
Body weights (kg)	14.7	16.0	15.3	15.3	15.4	15.9	15.0	15.4
Doses (mg/kg)	10.24	10.03	10.05	10.11	2.041	1.915	1.928	1.961
				Blood values				
C_{max} (μgeqiv./g)[2]	9.5	7.3	8.4	8.4	4.5 (5.9)	4.2 (5.4)	4.3 (6.0)	4.3 (5.8)
t_{max}(h)	0.5	3	3	2.2	0.08	0.08	0.08	0.08
$t_{1/2}^{3}$(h)	8.0	9.4	7.0	8.1	7.1	7.1	7.7	7.3
$t_{1/2}z^{4}$(h)	71.4	81.5	101	84.6	66.6	66.6	79.7	71.0
AUC[5](μgequiv/g × h)	176 (187)	176 (196)	183 (211)	178 (198)	34.0 (36.4)	34.0 (36.3)	36.5 (41.0)	34.8 (37.9)
				Plasma values				
C_{max} (μgeqiv./g)[2]	13.6	10.0	11.4	11.7	6.6 (8.6)	6.8 (8.8)	5.8 (7.6)	6.4 (8.3)
t_{max}(h)	0.5	3	4	2.5	0.08	0.08	0.08	0.08
$t_{1/2}^{3}$(h)	8.3	9.7	7.1	8.4	7.0	7.9	7.6	7.5
$t_{1/2}z^{4}$(h)	68.7	78.4	102.7	83.3	61.4	92.8	67.8	74.0
AUC[5](μg equiv./g × h)	245 (262)	253 (279)	264 (305)	254 (282)	50.0 (53.1)	54.6 (62.8)	53.6 (58.7)	52.7 (58.2)
				Urine				
Total amount (%)	85.85	72.83	79.30	79.3	84.41	79.03	79.48	81.0
$t_{1/2}$(h)	22.6	21.4	15.2	19.7	16.0	14.8		
				Faeces				
Total amount (%)	13.45	22.80	15.64	17.3	10.76	18.04	16.07	15.0
$t_{1/2}$(h)	28.5	13.5	27.9	23.3	12.3	12.0	17.0	13.8
				Cage washing				
Total amount (%)	1.35	2.20	3.57	2.4	2.85	2.97	1.38	2.4
				Recovery				
Total amount (%)	100.7	97.8	98.5	99.0	98.0	100.0	96.9	98.3
				Absorption rate (%)				
cal. via urine excretion (%)	102	92	100	98				
cal. via plasma AUC(%)	97	88	94	93				

[1] dog 1 = dog 4; dog 2 = dog 5; dog 3 = dog 6
[2] dog 4–6: number in brackets give C_{max} extrapolated to t_{max} = 0
[3] half-lives calculated using noncompartmental models (200.201)
[4] terminal half-lives calculated using noncompartmental models (200.201)
[5] AUC all calculated using the trapezoidal rule (AUC inf is given in brackets)

a rapid onset of absorption. C_{max} was interindividually different (see table) and reached 0.5 to 4 hours after administration. The mean maximum concentrations amounted 8.4 μg equivalents in blood and 11.7 μg equivalents/g in plasma. The AUCs (until the last concentration point) amounted to mean values of 178 μg equiv./g × h for blood and 254 μg equiv./g × h for plasma.

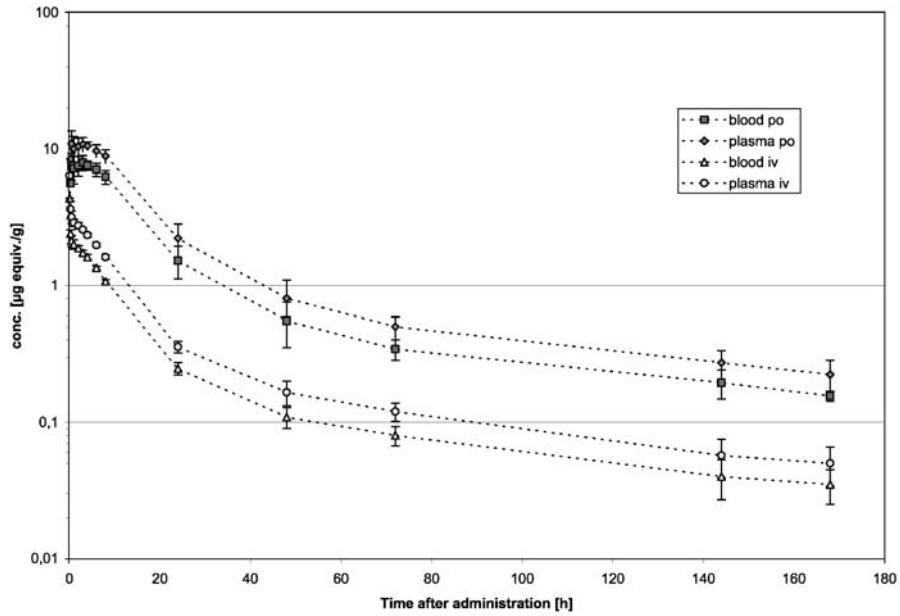

Fig. 1. ^{14}C-AVE 3559: mean concentration in blood and plasma after administration p.o. (10 mg/kg) and i.v. (2 mg/kg)

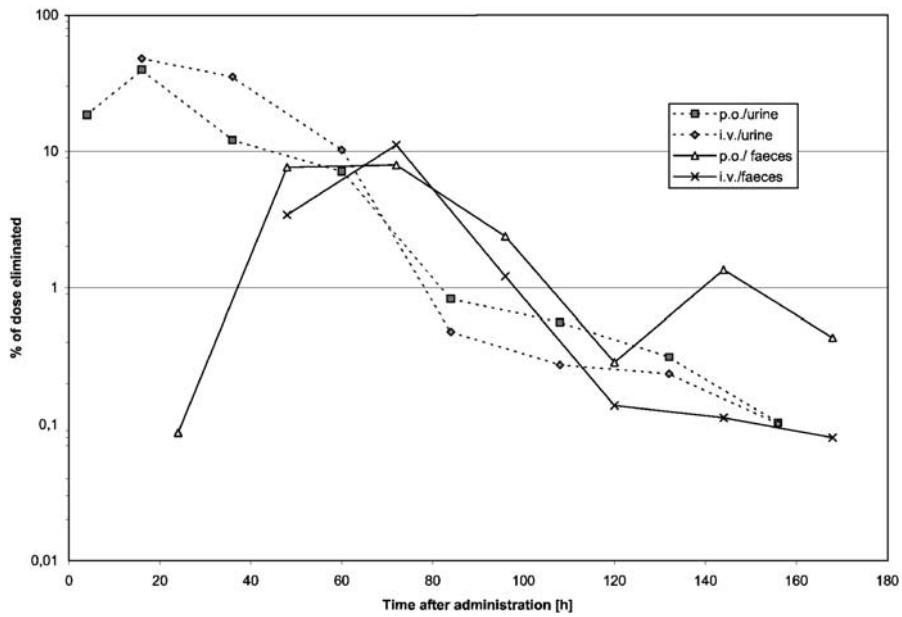

Fig. 2. ^{14}C-AVE 3559: mean % of dose eliminated via urine and feces after administration p.o. (10 mg/kg) and i.v. (2 mg/kg)

After reaching peak concentrations the radioactivity was eliminated in a first elimination phase with mean half-lives of 8.1 h from blood and 8.4 h from plasma and with mean half-lives of about 84 h from blood and plasma in a terminal elimination phase.

These half-lives correspond to the elimination processes and half-lives found after intravenous administration with mean half-lives of 7.3 h and 7.5 h in blood and plasma for the first elimination process and 71 h and 74 h for the terminal elimination phase. Mean C_{max} values 5 minutes after administration were 4.3 and 6.4 µg equivalents/g. The mean AUCs (until last concentration point) amounted 34.8 and 52.7 µg equivalents/g × h.

The higher plasma than blood concentrations at all measuring points (especially at the last measuring time points) after oral and intravenous administration

make a strong binding of a radioactive component (drug or metabolite) to blood cells in dog unlikely.

After both routes of administration the main part of the administered radioactivity was excreted renally with means of 79 % and 81 % (oral and intravenous route). In the feces an average of 17 % after oral and 15 % after intravenous administration were found.

The recovery rate after oral administration amounted to a mean of 99 %. Following intravenous administration the mean balance was 98 %.

An absorption of radioactivity between 88 % and 97 % based on plasma AUC could be calculated. Based on urinary excretion data a mean absorption rate of 98 % could be computed.

II.L.2 Mass Balance Study in Rats

PURPOSE AND RATIONALE

To evaluate the pattern and the rate of excretion, the residual concentration of the drug in the body one week after administration and the estimation of the absorption in case of a relevant renal excretion. The kind of study is obligatory for the registration as a drug when the rat is chosen as rodent model (in toxicology).

The route of administration projected to be used therapeutically should be applied also in the mass balance study. If possible and solubility allows, the study should also be performed after intravenous administration and the results of both routes should be compared. Since the oral administration is the most prominent route of administration, this route is described here. The method can be adapted for other routes of administration.

Normally, the mass balance study is performed administering a single dose per administration route. In case of extraordinary circumstances, for instance when there is a suspicion of an accumulation of any metabolite, a mass balance with repeated dosing can be considered.

The study is performed separately from the radiokinetics blood/plasma study in rats not to influence the results by collecting blood during the study course.

PROCEDURE

Animal Study Part

Out of eight healthy male Sprague Dawley rats weighing about 200 g corresponding to an age of 6–10 weeks, 4 rats receive an oral dose of the radiolabeled drug by using a stomach tube (typically 10 mg/kg body weight; 2 mg/g formulation; total radioactivity of about 1–2 MBq/animal in case of ^{14}C-label). Four other animals receive an intravenous dose into a tail vein (typically 2–5 mg/kg body weight). In this description the iv. and oral administration is described in more detail.

Material for Oral Administration

- Bulge tube (stomach tube) for oral dosing of rats
- syringes
- undersheet and disposable gloves
- drug formulation.

Procedure of Oral Administration

- The drug formulation is transferred into a syringe with the bulge tube put on the top of the syringe
- the rat is hold tight on its neck above the undersheet
- slightly turning, the bulge tube is introduced into the throat of the animal
- syringe content is administered intra-gastrally
- if appropriate a rinsing solution might follow the drug administration.

Material for Intravenous Administration into the Tail Vein

- Fixation tube for rats adjusted to the size of the rat
- electrically warmed and controled water bath (42 °C)
- Kleenex, cellulose, disposable gloves
- syringe containing the formulation (solution!) for administration
- syringe with pyrogen-free, physiological saline solution
- vein-dwell-cannula consisting of Teflon (about 0.8 mm diameter).

Procedure for IV Administration

- The rat is placed into the fixation tube. The tail of the animal is fixed outside the tube
- the tail is put into the water bath with a constant temperature between 40 and 42 °C
- this procedure causes the appearance of the tail vein without burdening the animals
- the vein is punctured with the dwell-cannula so that blood at the end of the leading-cannula appears. During extracting, the leading-cannula blood should also arrive in the dwell-cannula
- for additional control of the correct position of the dwell cannula, 0.2 to 0.3 mL of physiological saline

solution are administered. The administration has to occur easily, the aqueous saline solution has to flow into the vein immediately
- the syringe is changed cautiously by the syringe containing the drug formulation. The content is administered
- also this syringe is changed cautiously against a second syringe containing physiological aqueous saline solution. Again 0.2 to 0.3 mL are administered to transfer the formulation which rested in the cannula completely into the body.

Note: to avoid an injury of the tail vein, all the administrations should be conducted with caution.

Animal Maintenance

During the study period the animals are kept in metabolism cages, one in each cage with a separating device for urine and feces, allowing collection of the excreta separately. Conventionally those cages consist of several parts which have to be cleaned carefully before assembling (cage cylinder, device for sitting, funnel and separation device, cover, drinking bottle, tubing to the food vessel and food vessel). It has shown to be favorable to control the cleanliness by rinsing the cage with an alcohol-water-mixture and subsequent determination of radioactivity of the rinsing solution.

Before study start the animals should have time for acclimatization, depending on the extent of changes in the surroundings and the type and duration of transport. In case of a maintenance in a lab in the neighborhood under similar conditions as in the study, an acclimatization time of at least 1–2 days might be sufficient. In case of longer transport or from a different environment, acclimatization times up to 14 days can become necessary. One animal is housed per metabolism cage.

Before administration of the drug the animals should have an empty stomach but not go hungry, so that the food is withdrawn 3–4 h before the administration in the morning (for instance achieved by a timer-controled unit withdrawing the food). The feed (commercial pelleted diet, for instance Ssniff Spezialdiäten GmbH; D-59494 Soest, Deutschland) and tap water is provided ad libitum.

Following conditions should be controled throughout the study duration: Room temperature, relative humidity, lighting time

Proposed values for these conditions:

- room temperature: 20–25 °C
- relative humidity: 40–70 %
- lighting time: 6°°am–6°°pm light, 6°°pm–6°°am (dark).

Animal identification has to be ensured e.g. by permanent marking on the tail root with the animal number. Prior to the start of the study, the animals have to be assessed and have to show no clinical abnormalities.

Collection and Sample Preparation of Excreta

Material for Collection of Urine, Cage Washing and Feces

- Clear plastic bottles (100 mL) with covers, with appropriate labeling and registered tara weight (e.g. entered in LIMS)
- bottles for spraying de-ionized water
- precision balance
- Kleenex, disposable gloves
- if necessary: cooling device and light protection box
- Ultra-Turrax or mixer.

Urine Collection

Typical collection intervals are: 0–8 h, 8–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

Each collection interval the excreted urine is collected from the separation device in a labeled plastic bottle. To collect also the dried adherent parts of the urine on the surface, all contaminated devices are rinsed with de-ionized water until the clear plastic bottles are filled to about three-quarters. The weight of the filled bottle is determined and the data stored until evaluation.

Cage Washing

Typical collection intervals are: 0–24 h, 24–48 h, 48–168 h after administration.

At predetermined occasions additional washings of the cage are performed. The collection is done during the change of the bottles containing the urine. When necessary (in case of very hydrophobic drugs or metabolites) the rinsing fluid may contain organic, water miscible fluids. The cage washing samples are treated in the same way like the urine samples.

Feces Collection and Sample Preparation

Typical collection intervals are: 0–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration.

Each collection interval the excreted feces pellets are collected from the separation device in a labeled plastic bottle. The weights are determined and registered as raw-data. To homogenize the feces each sample is di-

luted with demineralized water, according to the consistency with the two to four-fold of the feces weight. The weight of the filled bottle is determined and the data stored until evaluation.

The sample with the mixture of feces and distilled water is homogenized with the Ultra-Turrax intensively. The Ultra-Turrax should be cleaned rigorously before using it again.

For preparation of samples for combustion, pre-weighted combustion cones were filled with an appropriate amount of homogenate (typically about 250 mg). The combustion cones and covers including the homogenate is weight again and the data stored until evaluation.

Preparation of the Carcass

Material

- Standard commercial small meat-mincer
- Ultra-Turrax (for example from Ika, Staufen, Germany)
- precision balance
- evaporation basins, scissors, pincer, spatula, Kleenex, disposable gloves, plastic bottles or glass bottles.

Procedure

After exsanguination/killing any organs and tissues of each rat planned to be investigated separately are removed and stored in a pre-weighed small evaporation basin. The rest of the body is ground with the meat-mincer. In order to minimize the residues in the mincer, 3 Kleenex wipes are ground in the mincer afterwards and are added to/in evaporation basin. The mincer is disassembled and residuals are removed with pincer and spatula. The residuals are added also to the evaporation basin. To homogenize the residues each sample is diluted with de-ionised water, according to the consistency with the two to four-fold of the carcass weight and mixed intensively with the Ultra-Turrax. The Ultra-Turrax should be cleaned rigorously before using it again. All weights have to be registered thoroughly. The weights of the removed organs and the blood of exsanguination also have to be collected.

For preparation of samples/aliquots for combustion, pre-weighted combustion cones (as from Schleicher & Schüll or from Canberra Packard) are filled with an appropriate amount of homogenate (typically about 250 mg) or blood/plasma of exsanguination. The combustion cones and covers including the homogenate are weight again and the data stored until radioanalytical evaluation.

Radioanalysis is performed by the liquid scintillation procedure either directly after addition of a scintillator (urine, cage washing) or following combustion and addition of a scintillator (carcass, blood, plasma, feces).

EVALUATION

The concentrations of radioactivity expressed as μg equivalents of drug per g of matrix and alternatively as part of dose eliminated (in percentage of the administered dose) are determined in the each matrix and collection interval[11]. The temporal course of the eliminated parts of excretion are represented by graphs[12] and tables using either Windows Excel function, a specific software program like Winnonlin (www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com). They also allow calculation of the half-lives of radioactivity elimination via urine and feces (when appropriate)[13], to generate cumulation plots or general survey plots like bar diagrams (see example).

These data and the radioactivity in carcass, blood and plasma of exsanguination, special organs/tissues, cage washing and the balance are finally drawn together for detailed discussion and evaluation. Special attention has to be taken on major differences of data following intravenous and oral administration.

In the case that an essential part of dose is excreted via urine, an estimation of absorption is often done giving the ratio of urine excretion (oral/intravenous) as absorption rate.

An essential requirement for the quality of the study is a balance value near to 100 %. A value > 95 % and < 105 % is reasonable. Balance values of 90 % to 95 % or 105–110 % are often accepted too, but they should indicate the investigator that something might run out of control or the process/handling could be optimized. Balances much worse than those should be explained.

CRITICAL ASSESSMENT OF THE METHOD

Always keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled

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[13] via determination of the rate constant which is calculated by linear regression of ln concentration on time using a least three data points which appeared to be randomly distributed about a single straight line. Half-lives were calculated as ln2/rate constant.

metabolites and not the drug itself. Therefore, it is worthwhile to determine also the drug and known metabolites directly by bioanalytical methods with samples withdrawn from the same study. The comparison of both the radioactivity concentrations and the sum of concentrations determined by specific methods allows the estimation of the gap which often develops time-dependently: for instance in the beginning of the study radioactivity concentrations in plasma should fit to the drug concentration. Usually, the gap (as percentage of the total radioactivity at a certain time point) between both concentrations increases with time.

The estimation of absorption based on the urine excretion ratio (oral/intravenous) should be compared with the ratio of dose-normalized plasma AUCs after iv. and oral administration from the rat radiokinetic study. In case of major differences between both methods of absorption determination, a thorough investigation/discussion of a route of administration-dependent metabolism and/or volume of distribution might help to explain the apparent discrepancy.

Both methods of absorption calculation mentioned are based on the fact that after absorption the drug/metabolites reach the central circulation. In case of oral administered drugs targeting the liver and being eliminated biliary instantly, it is obvious that both methods mentioned fail. In such a special case an absorption estimation might be possible via a biliary excretion study.

Certainly, every radiokinetic as well as any in vivo pharmacokinetic study depends on the galenic formulation used. Aqueous solutions, solutions with organic water-miscible components, solutions with surfactants, emulsions, micro emulsions or suspensions can be responsible for very distinct differences of radiokinetics (pharmacokinetics). Therefore, a conscious choice of galenic vehicle and formulation procedure is critical!

MODIFICATIONS OF THE METHOD

Instead of or additional to the oral administration the method can be adopted to subcutaneous, inhalation or dermal route (as for instance described by Dix et al. (2001), Simonsen et al. (2002), Mathews (1998)) or any other route of administration.

Sometimes several dose levels are investigated with the method described; normally one starts with a dose from which a pharmacological response is expected, but the dose should be significantly lower than NOAEL. Later during the course of development studies at the NOAEL or NOEL may follow. In case of evaluation of altered routes of elimination at toxic doses, studies with doses higher than NOAEL may be considered as an exception.

Determination of blood, plasma and feces samples not using combustion but either direct measurement (plasma) or solubilizing and de-coloration of the samples is mentioned for instance by Okuyama et al. (1997) or Mathews et al. (1998).

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EXAMPLE

HMR 1556 was a drug candidate developed for treatment of hypertension. It was ^{14}C-labeled and investigated in a rat mass balance study to determine the pattern and rate of excretion, the residual concentrations at the end of the study 168 h after administration and the mass balance following oral and intravenous route of administration.

Choice of Formulation

Because of low solubility of the compound in pure water, for the oral administration a suspension in an aqueous solution of hydroxyethylcellulose (0.5 %) was used. The solid state physical parameters (particle size, surface area, amorphicity and crystal modification) and the suspension properties (agglomeration tendency, homogenicity and dissolved part of the drug) had to be investigated. For the intravenous administration, an aqueous solution containing saline, DMSO and PEG 400 was used as formulation.

Animal and Radioanalytic/kinetic Study Part

Of 8 healthy male Sprague Dawley rats weighing between 187.4 and 197.6 g, 4 animals received an approximate dose of 10 mg ^{14}C-HMR 1556/kg body weight orally by using a stomach tube. Four other animals received a dose of approx. 5 mg/kg intravenously into a tail vein. Further details see Fig. 3.

Animal-No	1	2	3	4	5	6	7	8
Body weight (g)	195.8	195.1	190.1	197.4	197.6	191.5	187.4	194.4
Route of administration	oral				intravenous			
Dose (mg/kg)	9.535	9.694	9.949	9.543	5.794	5.591	5.658	5.444
Time of killing (h aft. adm.)	168							
Preparation	Suspension with HEC				Solution in water with DMSO/PEG 400/Saline			
Conc. in prep. (mg/g)	1.884				2.063			
Amount prep. adm. (g)	0.991	1.004	1.004	1.000	0.555	0.519	0.514	0.513
Batch	Z 29075-2				Z 29075-2			
Spec. radioact. (MBq/g)	1990.00				1990.00			
Adm. rad. (MBq/animal)	3.72	3.76	3.76	3.75	2.28	2.13	2.11	2.11
Study objectives	Mass Balance Excretion via urine and faeces Residues in carcass							

Fig. 3. HMR 1556 dosage

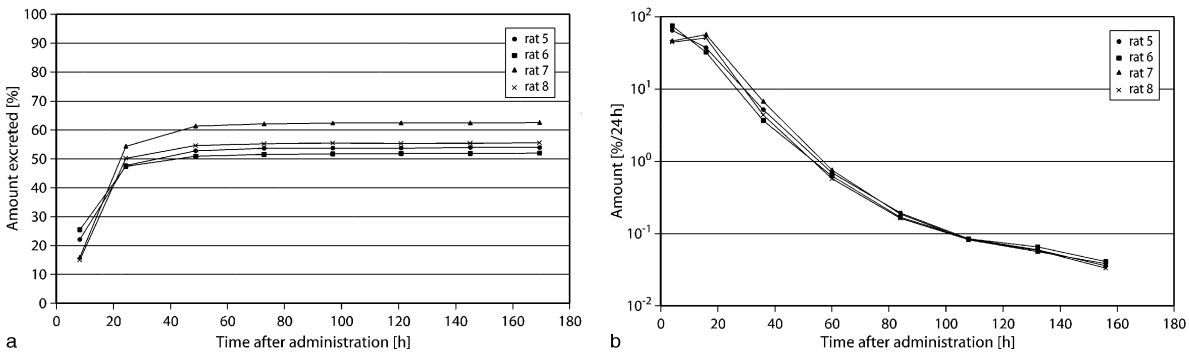

Fig. 4. ^{14}C-HMR 1556: excretion via urine following intravenous administration of approx. (a) cumulative plot (b) amount (%/24 h)

Urine samples (0–8 h, 8–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration), feces samples (0–24 h, 24–48 h, 48–72 h, 72–96 h, 96–120 h, 120–144 h, 144–168 h after administration), cage washings (0–24 h, 24–48 h, 48–168 h after administration), blood and plasma samples after exsanguination and carcass were collected and weighed as described above. Three aliquots from each (processed) sample were used. The aliquots from the urine samples were measured directly in the liquid scintillation counting procedure after addition of the scintillator Roth-rotiszint eco plus (Roth, Karlsruhe, Germany). The other matrix aliquots were combusted in a Tri-Carb307 combuster (Canberra-Packard GmbH, Model 307)

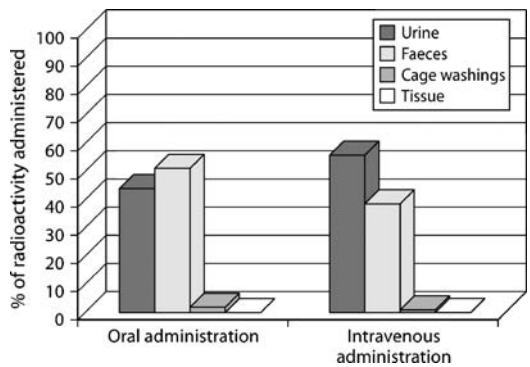

Fig. 5. ^{14}C-HMR 1556: Balance after oral administration of approx. 10 mg/kg body weight and intravenous administration of approx. 5 mg/kg body weight to male rats

Table 2 ^{14}C-HMR 1556. Excretion in urine and feces, cage washings and amount of radioactivity in blood of exsanguination and carcass after oral administration of approx. 10 mg/kg and intravenous administration of approx. 5 mg/kg body weight to male rats (% of administered radioactivity).

	oral						intravenous					
	Rat 1	**Rat 2**	**Rat 3**	**Rat 4**	**Mean**	**SD**	**Rat 5**	**Rat 6**	**Rat 7**	**Rat 8**	**Mean**	**SD**
Body weight (g)	195.8	195.1	190.1	197.4	**194.6**	3.2	197.6	191.5	187.4	194.4	**192.7**	4.3
Dose (mg/kg)	9.535	9.694	9.949	9.543	**9.68**	0.193	5.794	5.591	5.658	5.444	**5.622**	0.146
Urine												
Total amount (%)	41.81	42.95	49.31	42.01	**44.02**	3.56	53.82	51.79	62.35	55.37	**55.83**	4.58
$t_{1/2}$ (h)	9.3	11.3	12.4	15.7	**12.2**	2.7	9	8.8	9.1	8.9	**9**	0.1
Faeces												
Total amount (%)	58.3	47.75	46.88	51.8	**51.19**	5.21	38.19	43.23	33.37	39.6	**38.6**	4.08
$t_{1/2}$ (h)	7.7	9.3	11.6	12.6	**10.3**	2.2	8.7	8.7	9.4	8.3	**8.8**	0.5
Cage washings												
Total amount (%)	1	2.93	1.45	2.74	**2.03**	0.95	1.35	0.49	1.58	0.77	**1.05**	0.5
Carcass												
Total amount (%)	0.16	0.2	0.13	0.16	**0.16**	0.03	0.17	0.19	0.24	0.21	**0.2**	0.03
Recovery												
Total amount (%)	101.3	93.8	97.8	96.7	**97.4**	3.1	93.5	95.7	97.5	96	**95.7**	1.6

and the $^{14}CO_2$ formed was absorbed by Carbo-Sorb (Canberra-Packard). The subsequent radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Canberra-Packard 2500 TR) after addition of the scintillator Permafluor E+ (Canberra-Packard).

Evaluation and Discussion

As an example for the numerous generated graphs, the linear cumulation plot (% of dose amount excreted) and the semilogarithmic plot of drug amount (given as amount dose amount excreted during a 24 h interval) excreted in urine depending on time after intravenous administration is given in Figures 4a and 4b.

After the oral administration of approx. 10 mg ^{14}C-HMR 1556/kg body weight, between 42.8 % and 50.8 % (mean 46.05 %) of the administered radioactivity was excreted via the urine (including cage washings). In the feces, between 46.9 % and 58.3 % (mean 51.2 %) was found. After the intravenous administration of approx. 5 mg ^{14}C HMR 1556/kg body weight, between 52.3 % and 63.9 % (mean 56.9 %) of the administered radioactivity was excreted via the urine (including cage washings). In the feces, between 33.4 % and 43.2 % (mean 38.6 %) was found.

The calculated absorption rate based on the comparison of the urine excretion after oral and intravenous administration amounted to appox. 80 %.

The calculated half-lives for the renal elimination amounted to a mean of 12.2 h (oral dose) and 9.0 h (intravenous dose). The fecal elimination took place with mean half lives of 10.3 h and 8.8 (oral and intravenous dosing).

After both routes of administration, only very low amounts of the total radioactivity administered were to be found in the carcasses and blood of exsanguination (0.2 %).

The recovery rate after oral administration of approx. 10 mg/kg body weight amounted to a mean of 97.4 %. Following intravenous administration of approx. 5 mg/kg, the mean balance was 95.7 %.

The main results are summarized in Figure 5 and Table 2.

II.L.3
Blood/Plasma Radiokinetics in Rats

PURPOSE AND RATIONALE

Investigate the time course of radioactivity concentrations in blood and plasma with the aim to getting information about the absorption process, the AUC, C_{max} and the elimination half-life of radioactivity in blood and plasma. Possibly indices for enterohepatic cycle or for metabolites with very different volume of distribution from the original compound may be found. An estimate of the absorption rate comparing dose normalized AUCs after iv and oral (or any other) administration can be done and a major binding to formed blood elements can be noticeable by comparison of blood and plasma concentrations.

This type of study is obligatory for the registration as a drug when the rat is chosen as rodent model (in toxicology).

Since the oral administration is the most prominent route of administration, this route is described here. The method can be adapted for other routes of administration. If possible, and solubility allows, the study should also be performed after intravenous administration and the results of both routes should be compared.

Normally, the radiokinetic study is performed administering a single dose per administration route. In case of extraordinary circumstances, for instance when there is a suspicion of an accumulation of any metabolite, a radiokinetics study with repeated dosing can be considered.

The study is performed separately from the mass balance study in rats, in order not to influence the balance by collecting blood during the study course.

PROCEDURE

Animal Study Part

The radiolabeled drug is administered to 54 healthy male Sprague Dawley rats (weighing about 200 g corresponding to an age of 6–10 weeks). 27 animals receive an oral dose by stomach tube (typically 10 mg/kg bw) and 27 animals receive an intravenous dose into a tail vein (typically 1–5 mg/kg; total radioactivity of about 2 MBq/animal in case of ^{14}C-label).

The administration and the animal maintenance can/may follow the procedure and description given for oral and intravenous administration of the mass balance study.

Collection and Sample Preparation

Material

- Box for anesthesia with CO_2 or with Isofluran
- scalpel
- disposable gloves
- small evaporating basins
- commercially available aggregation inhibitor like heparin-sodium or sodium citrate
- small centrifuge tubes
- centrifuge.

At each observation time point three rats are put in the box for anesthesia separately (especially critical at early time points; a staggered approach makes sense), which is permanently flushed with narcosis gas. The cover is closed and the rat is left there until complete apnoea. Taking the rat outside the box again the reflex cornealis is proven. When the reflex is extinguished the vessels lateral below the throat are cut with the scalpel. The blood pouring out is collected in the small evaporating basins which already contain small amounts of heparin-sodium in cases where plasma preparation is intended (instead of serum).

For generating plasma, part of the collected blood is transferred into centrifuge tubes and treated in the centrifuge with 1500 G.

For typical sample collection time points in a 48 h study see example below.

Blood and plasma samples are taken up prepared for combustion (e.g. aliquots are transferred on cones for combustion), weighed, dried at room temperature, combusted and the $^{14}CO_2$ formed is absorbed by Carbo-Sorb (Canberra-Packard). The subsequent radioactivity measurements are carried out after addition of scintillator to the samples.

Remaining blood and plasma are suitable for concomitant metabolism and bioanalytical studies.

EVALUATION

The concentrations of radioactivity expressed as μg equivalents of drug per g of matrix at all collection times and in blood and plasma are determined. The temporal course of the concentrations are represented by graphs and tables using either Windows Excel functions, a specific pharmacokinetic software program like Winnonlin (www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com). They also allow calculation of the half-lives of radioactivity and the AUCs (see example). The ratio of the dose-normalized AUCs (oral/intravenous) is used as the extent of absorption.

All these data are finally drawn together for detailed discussion and evaluation. Special attention has to be taken to major differences of data following intravenous and oral administration.

In the case that an essential concentration is available in blood and plasma at early time points after oral administration (e.g. C_{max} is reached soon) a rapid onset of absorption can be stated.

When there is no continuous decline of concentrations after intravenous administration, this might be an indication for:

- an inappropriate administration or a precipitation of the drug from the formulation at the application side or just behind the side in the blood stream. This might be the case when there is a plateau concentration or even an increase at early collection times
- an enterohepatic circulation when a side maximum exists (time depending on absorption and elimination rate – often seen 4 to 8 h after administration)
- one or more of the generating metabolites show a much smaller volume of distribution than the drug itself
- other (often more complex) explanations.

In the case that a concentration ratio (blood/plasma) is distinctly higher than the haematocrit value this could indicate a binding of the drug or its metabolites to formed blood elements.

CRITICAL ASSESSMENT OF THE METHOD

Always keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled metabolites and not the drug itself. Therefore, it is worthwhile to determine also the drug and known metabolites directly by bioanalytical methods with samples withdrawn from the same study. The comparison of both the radioactivity concentrations and the sum of concentrations determined by specific methods allows the estimation of the gap which often develops time-dependently: for instance in the beginning of the study, radioactivity concentrations in plasma should fit to the drug concentration. Usually the gap (as a percentage of the total radioactivity at a certain time point) between both concentrations increases with time.

The estimation of absorption via the ratio of dose-normalized plasma AUCs after oral and intravenous administration based on the assumption, that:

1. a first pass effect including an instant elimination via bile does not exist
2. the metabolic pattern is comparable independently of the route of administration
3. the volumes of distribution (drug and metabolites) and the metabolism in special tissue is not effected by the bolus iv administration with high initial concentrations (Chiou 1989)
4. dose-exposure linearity exists (under a more stringent view even a first order kinetic is required).

When those criteria are not fulfilled, then either the absorption can be underestimated (for instance when precondition 1) is not true 2) or distinctly overestimated up to a calculated "super-absorption" for more than 100 % (possible for instance when precondition 3) or 4) are not true).

Only AUCs obtained from the same "type of blood" samples should be used. For instance don't compare central arterial blood with venous peripheral blood (Chiou (1989)).

Certainly, every radiokinetic as well as any in vivo pharmacokinetic study depends on the galenic formulation used. Aqueous solutions, solutions with organic water-miscible components, solutions with surfactants, emulsions, micro emulsions or suspensions can be responsible for very distinct differences of radiokinetics (pharmacokinetics). Therefore, a conscious choice of galenic vehicle and formulation procedure is critical!

When establishing the method, one should cautiously practise the collection of blood by the cut lateral below the throat. In any circumstances injuring the oesophagus has to be avoided, since small parts of formulation remaining in the oesophagus (for instance because of reflux or a sticky formulation) can contamine the blood of exsanguination.

MODIFICATIONS OF THE METHOD

There are several methods of collecting blood from other sites[14]. Each method has advantages and disadvantages. Whereas central blood of exsanguination enables additional bioanalytics and metabolism from the same original sample (5–7 mL of blood), the collection of tail vein blood[15] (sample sizes of about 100 to 150 µl) enables intraindividual radiokinetics to be obtained.

In case of using pre-operated animals (such as animals with jugulars and/or carotis catheter, see for instance Davis et al. (1994) or Krishna et al. (2002)), the physical suitability has to be assessed critically.

[14] for instance blood of exsanguination collected after heart puncture; jugularis puncture, jugularis or carotis cathether, retroorbital blood, blood from the vena femoralis, sublingual blood after short narcosis or blood from the tail vein.

[15] caution: the administration should not be done at the site of sample collection to avoid contamination. For instance an iv. administration into the vena femoralis after a short transient anesthesia can be recommended.

The reproducibility of these studies is much more critical and excellently prepared and recovered animals are constantly necessary. The comparison of results from central arterial blood (carotis), for instance with peripheral venous blood, is questionable (Chiou (1989)).

Sometimes it is recommended to use a short term infusion instead of a bolus intravenous administration. The advantage is to avoid high blood concentrations shortly after intravenous administration which may be in conflict with the assumed linear dose-exposure relationship (validity of linear relation at border concentration?). The advantage of the intravenous bolus injection is the lower/shorter stress situation for the animals (and may be also for the experimenter) which again has consequences for the reproducibility of the study results.

The determination of the volatile radioactivity in urine, feces and expired air can be performed within the framework of the radiokinetic study (for instance with the animals provided for the 24 h sample). The air of the metabolism cage is continuously extracted, therefore, (for instance with 3.5 L/min) and measured using a gas flow counter (Exhalameter, Raytest, Strauchenhardt, Germany). The study period is approx. 24 hours.

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EXAMPLE

HMR 1766 is a guanylat-cyclase activator and is being developed for treatment of hypertension. It was ^{14}C-labeled and investigated in a rat radiokinetic study to determine the time course of radioactivity concentrations in blood and plasma.

Animal and Radioanalytic/kinetic Study Part

Of 54 healthy male Sprague Dawley rats weighing between 165 and 216 g, 27 animals received an approximate dose of 12 mg ^{14}C-HMR 1766/kg body weight orally by using a stomach tube. 27 other animals received a dose of approx. 2 mg/kg intravenously into a tail vein. In both cases, an aqueous solution containing PEG 400 was used as formulation. As an example for which details of data are collected during administration see Figure 6 for the data for intravenous administration (analogously the data from the p.o. administration part are recorded).

Blood samples (0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h, 48 h after oral administration and 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h after intravenous administration) were collected. Parts of the blood samples were processed to obtain plasma samples. Of each sample and matrix 3 aliquots were generated, taken up on Combusto Cones (Canberra-Packard), weighed, dried at room temperature, combusted in a Tricarb combuster (Canberra-Packard GmbH, Model 307) and the $^{14}CO_2$ formed was absorbed by Carbo-Sorb (Canberra-Packard). The subsequent radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Canberra-Packard 2500 TR) after addition of the scintillator Permafluor E+ (Canberra-Packard).

Evaluation and Discussion

The time course of the mean radioactivity blood and plasma concentration values of 3 animals is given in the summary graph Figure 7.

Half-lives were calculated by the pharmacokinetics' software WinNonlin, version 1.5. In case of the intravenous administration, a 2 compartmental model was chosen; in case of oral administration, a non-compartmenal model. The AUCs were calculated using the linear trapezoidal rule. The results are summarized in Table 3.

Already 15 min after oral administration, radioactivity concentrations in blood and plasma (5.90 and 10.67 μg equivalents/g, resp.) were found indicating a fast absorption. The maximum concentrations (C_{max}) occurred 0.5 hours after administration and amounted to a mean of 9.34 μg equivalents/g (blood) and 16.95 μg equivalents/g (plasma). The elimination of radioactivity from blood and plasma was biphasic. The half-lives for the first phase amounted to 1.1 h (blood) and 0.9 h (plasma), for the second phase half-lives of 7.5 h and 6.3 h were calculated. In all animals, radioactivity concentrations were detectable up to the last measuring time 48 h after dosing and showed averages of 0.025 and 0.024 μg equivalents/g (blood and plasma, respectively).

Animal-No	113	114	115	116	117	118	128	129	130
Body weight (kg)	0.188	0.212	0.196	0.179	0.189	0.186	0.210	0.202	0.206
Route of admin.	Intravenously								
Dose (mg/kg)	1.962	1.763	2.058	2.213	2.084	2.054	1.952	2.010	1.999
Time of killing (h after adm.)	0.083			0.25			0.5		
Preparation	Solution with PEG 400								
Conc. in prep. (mg/g)	0.788						0.951		
Amount prep. adm. (g)	0.469	0.473	0.511	0.503	0.499	0.484	0.431	0.427	0.433
Batch	Z 29023-0-10						Z 29023-0-11		
Spec. radioact. (MBq/g)	1512.78						1324.47		
Radioact./animal (MBq)	0.559	0.564	0.609	0.600	0.595	0.577	0.543	0.538	0.545
Study objectives	Drug levels in blood and plasma								
Animal-No	**131**	**132**	**133**	**119**	**120**	**121**	**122**	**123**	**124**
Body weight (kg)	0.201	0.215	0.208	0.194	0.189	0.187	0.203	0.197	0.181
Route of admin.	Intravenously								
Dose (mg/kg)	2.110	1.920	2.003	1.925	2.019	1.980	2.012	1.977	2.060
Time of killing (h after adm.)	1			2			4		
Preparation	Solution with PEG 400								
Conc. in prep. (mg/g)	0.951			0.788					
Amount prep. adm. (g)	0.446	0.434	0.438	0.474	0.483	0.470	0.517	0.495	0.473
Batch	Z 29023-0-11			Z 29023-0-10					
Spec. radioact. (MBq/g)	1324.47			1512.78					
Radioact./animal (MBq)	0.562	0.547	0.552	0.565	0.576	0.560	0.616	0.590	0.564
Study objectives	Drug levels in blood and plasma								
Animal-No	**125**	**126**	**127**	**134**	**135**	**136**	**137**	**138**	**139**
Body weight (kg)	0.189	0.196	0.186	0.202	0.216	0.210	0.213	0.206	0.207
Route of admin.	Intravenously								
Dose (mg/kg)	2.053	1.984	1.941	1.883	1.818	1.789	2.009	2.017	2.049
Time of killing (h after adm.)	8			24			48		
Preparation	Solution with PEG 400								
Conc. in prep. (mg/g)	0.788			0.951					
Amount prep. adm. (g)	0.492	0.493	0.458	0.400	0.413	0.395	0.450	0.437	0.446
Batch	Z 29023-0-10			Z 29023-0-11					
Spec. radioact. (MBq/g)	1512.78			1324.47					
Radioact./animal (MBq)	0.587	0.588	0.546	0.504	0.520	0.498	0.567	0.550	0.562
Study objectives	Drug levels in blood and plasma								

Fig. 6. Data for intravenous administration

After intravenous administration, the maximum radioactivity concentrations were measured at the first measuring time 5 min after administration, exhibiting mean concentrations of 4.79 and 7.64 μg equivalents/g (blood and plasma). Following the peak values, the radioactivity decreased in a biphasic process with mean half-lives of 0.4 h for phase I and 10 h (blood and plasma, resp.) for phase II. In all animals, blood and plasma levels were observed up to 48 h after administration (0.006 and 0.007 μg equivalents/g in blood and plasma, resp.)

The extent of absorption after oral dosing was determined by using the dose corrected comparison of the mean areas under the blood (and plasma) – radioactivity concentration curves after oral and intravenous administration (AUC-values). This comparison revealed an absorption rate of more than 90 % depending on AUC values used for calculation (blood or plasma). This value was very similar to the result in a mass balance study where the absorption was calculated by a comparison of the renally excreted radioactivity following oral and intravenous administration.

Since all plasma radioactivity concentrations are distinctly higher than the corresponding blood values, there is no indication for a major binding of radioactivity to formed blood elements in this study.

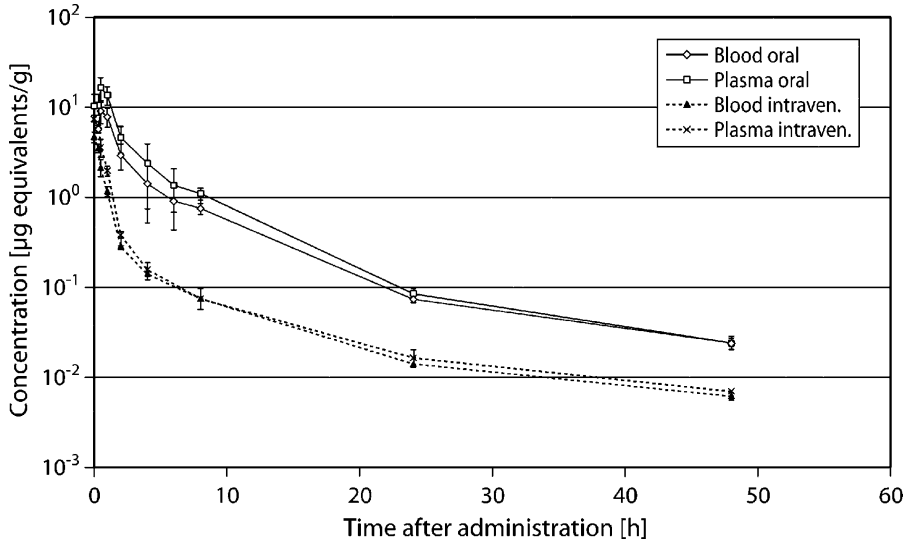

Fig. 7. ^{14}C-HMR 1766: Blood and plasma concentrations after oral administration of approx. 12 mg/kg and after intravenous administration of approx. 2 mg/kg body weight to male rats. The variability (SD) of the animal values (n = 3) is given as bars.

Table 3

Route of administration	Oral	Intravenous
Rat-Nos.	59–85	113–139
Body weights (kg)	0.165–0.189	0.179–0.216
Doses (mg/kg)[1]	10.602–13.092 (11.88)	1.763–2.213 (1.99)
Blood		
C_{max} (μg eq./g)	9.34	4.79
t_{max} (h)	0.5	0.083
$t_{1/2}$I (h)	1.1	0.4
$t_{1/2}$II (h)	7.5	9.6
AUC (μg equiv./g × h)	28.96	5.29
Plasma		
C_{max} (μg eq./g)	16.95	7.64
t_{max} (h)	0.5	0.083
$t_{1/2}$I (h)	0.9	0.4
$t_{1/2}$II (h)	6.3	10.4
AUC (μg equiv./g × h)	46.39	7.78

[1)] in brackets: mean value of 27 animals each

II.L.4 Bile Fistula Study in Rats

PURPOSE AND RATIONALE

To investigate the rate and the extent of the excretion of the drug/metabolites with the bile. If required, the investigation can be supplemented to study the enterohepatic circle. The bile fistula study becomes necessary when relevant parts of the administered radioactivity are eliminated fecally suggesting a considerable biliary excretion during the mass balance study in rats.

Since the intravenous administration ensures the maximum exposure especially in case of anesthetized or preoperated animals, this route is often favored.

PROCEDURE

Part 1 (Collecting and Investigating the Bile)

Four healthy male anesthetized Sprague Dawley (or Wistar) rats weighing about 300 g (10–12 weeks old) and provided with a permanent bile fistula receive an intravenous dose (such as 5 mg/kg; for instance into the tail vein, procedure see "mass balance study in rats" without the necessity to use the described tube for the animals).

Details of installing the permanent bile fistula:

Material

- Ketamin (for instance Hostaketfrom Intervet)
- Midazolam (for instance DormicumV from Roche)
- disposable syringes, disposable gloves, disposable cannulas
- small Braunülen (= indwelling venous cannulas)
- skin disinfectant
- heatable operating table for small animals
- surgical microscope
- electric clipper with 1/2 mm cutter head (for instance from Aesculap Favorita)
- surgical instruments: scalpel, surgical shears of various sizes, bent forceps, iridectomy scissors, surgical skin staples, stapling forceps, surgical gloves, swabs

- catheter consisting of polythene tubing ID 0.28 mm, OD 0.61 mm (for instance from Portex, Hythe, Kent, GB) or similar material (like flexible silicon catheter).

The rats are fasted for about 4 h and are anesthetized with 80 mg/kg bw Ketamin plus 5 mg/kg Midazolam administered intravenously into the tail vein via an indwelling venous catheter. The amount corresponds to a volume of about 0.3 mL. Anesthesia is monitored by checking the reflexes and is maintained by repeat dosing of ketamine/midazolam.

The abdomen of the anesthetized rat is shaved, the rat is then fixed in dorsal position on the heated operating table (thermostat set to 37 °C) and the abdomen is disinfected with a commercial skin disinfectant.

Laparatomy is carried out at the linea alba, the catheter is introduced into the common bile duct and fixed at such a distance from the hilus that the flow of pancreatic juice is not hindered. The animals are kept on heating pads to maintain body temperature throughout the experiment.

The test compound is injected into a tail vein. The study period is 8 hours as from the time of dosing. At the end of the study period, the animals are killed painlessly by an overdose of the anesthetic.

The bile removed leads to fluid depletion of the organism. The corresponding volume was thus substituted for by intravenous infusion of isotonic sodium chloride solution (about 1 mL/h).

The bile of each animals is collected separately under cooled conditions during for instance four intervals (0–2 h, 2–4 h, 4–6 h, 6–8 h) via the permanent bile fistula. The samples are weighed and aliquots thereof measured directly after the addition of a commercial scintillation cocktail and water. In addition, the amounts of radioactivity in urine (0–8 h) are determined.

The remainder of the bile is used for metabolic profiling and structure elucidation of metabolites and, if necessary, for the investigation of the enterohepatic circulation as discribed in the following.

Part 2 (Enterohepatic Circulation)

Parts of the non-diluted bile obtained are pooled in order to receive a representative mixture of metabolites on one hand (that is from different collection intervals) and on the other hand a radioactivity concentration as high as possible.

The pooled bile is intensively homogenized, the homogenicity and the exact concentration proven by radioanalysis of serveral aliquots. The amount for application (about 1 g/animal) is withdrawn. The receiver animals are prepared like the animals in the first part of the study. However, the pooled bile is administered intra-duodenally via a flexible tubing. This tube is advanced from the mouth through the stomach as far as the beginning of the duodenum. During the administration, the tube is fixed with the fingers inside the duodenum to prevent reflux of the administered bile into the stomach.

Bile samples are collected and processed as described in part 1 of the study.

EVALUATION

Part 1

With the knowledge of the amount of bile, the concentrations determined are converted into percentages of administered radioactivity. The results can be displayed graphically (versus time). In case of portions of administered radioactivity of at least three time points being distributed about a single straight line in the semilogarithmic plot, the rate constant can be calculated by linear regression and subsquently the half-lives.

The data are finally drawn together for detailed discussion and evaluation with special attention to known results from other radiokinetic studies: such as the mass balance study and the obtained fecal elimination or the quantitative whole body autoradiography.

Part 2

The part of dose found in the bile of the receiver animals dosed with a part of the bile withdrawn from the donor animals gives an estimate about the magnitude of the enterohepatic circulation.

CRITICAL ASSESSMENT OF THE METHOD

The anesthesia slows down processes of the liver. It has to be assumed that liver clearance and elimination are faster when animals are awake. Therefore, models with woken animals are sometimes favored (see modifications of the method).

A rough estimation whether anesthesia might relevantly misrepresent the situation of an awake animal should be performed comparing the bile excretion with the excretion via feces from the mass balance study (see example), the urine excretion or the radioactivity concentration in blood (so the bile fistula study should be extended to collect also body fluids such as terminal blood or the urine during the anesthesia).

The estimation of enterohepatic circulation can be valid only when biliary excretion is virtually complete in the observed time interval.

MODIFICATIONS OF THE METHOD

The intravenous administration ensures a "complete" absorption and is therefore often the favored route of dosing in this type of study. However, metabolism, distribution and excretion may change with the route of administration and might impact the results of a bile fistula study. Thus, it has to be considered which route of administration[16] has to be chosen. The comparison of the metabolic pattern in plasma or even in feces after different routes of administration may support this decision.

It may be worthwhile to dissect and measure additional tissues/organs at the end of the study, for instance to investigate whether a direct secretion into the bowels took place or whether the liver retained radioactivity.

Alternative models with woken animals during bile collection (Johnson P et al. (1978) or for instance: Tse FLS et al. (1983)) are sometimes favored excluding the influence of anesthesia. However, those models often also cannot represent the situation of a "normal" rat, since operation and catheterization may also impact the study result.

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EXAMPLE

Hoe 642 is an inhibitor of cellular Na+/H+ exchange and thus a drug with cardioprotective activity.

^{14}C-HOE 642 was intravenously injected into a tail vein (5 mg/kg; 1 MBq/animal) to 4 healthy male, anesthetized Wistar rats (approx. 320 g) with bile fistula. For administration, the test compound was used as aqueous solution with 0.9 % saline (3.5 mg/g formulation; approx. 0.5 g formulation/animal) adjusted to pH 7 with sodium hydroxide.

Bile samples were collected as described above, weighed, diluted with water, weighed again and aliqouts were measured after addition of scintillator.

The radioactivity measurements were carried out by the liquid scintillation procedure, using a β-spectrometer of type BF 5000 (Berthold, Wildbad, Germany). As scintillator Rotiszint eco plus (Roth, Karlsruhe, Germany) was used. Blank values were concurrently measured in the studies and deducted from those measured. These blank values originated from bile fluid of the animals taken before dosing.

The found amounts of radioactivity excreted with the bile are illustrated in the graph in Figure 8.

During the study period between 6.9 and 7.9 % (mean = 7.6 %) of the radioactivity administered were excreted with the bile. The major part thereof was eliminated within the first two hours after injection. The portion of radioactivity excreted with the bile decreased at later measuring intervals. In the last collection interval, i.e. 6–8 h after dosing, an average of 0.09 % of the radioactivity administered was still present in the bile.

Half-lives were calculated. They were based on a monophasic process with values between 0.7 and 1 h. A slower terminal phase was indicated in all animals except one.

A previous mass balance study with ^{14}C-HOE 642 revealed a feacal elimination of about 17 % (2 % up to 8 h, 14 % between 8–24 h and 1 % at later time points) following intravenous injection (comparable dose). Since in the bile fistula study the portion still present in the bile in the last collection period was only about 0.1 % it was assumed that biliary secretion is virtually complete. The difference to the excreted fecal elimination obtained in the mass balance study may be due to the general anesthesia of the rats in the bile study or radioactivity may reach the gastrointestinal tract by other ways (direct secretion into the intestine or the stomach, secretion by the salivary glands with subsequent swallowing of the saliva. The salivary glands are known to exhibit high radioactivity concentrations after intravouns injection from radioluminography studies with ^{14}C-HOE 642).

The presence of a lower radioactivity portion in the bile than in the feces and the virutally monophasic elim-

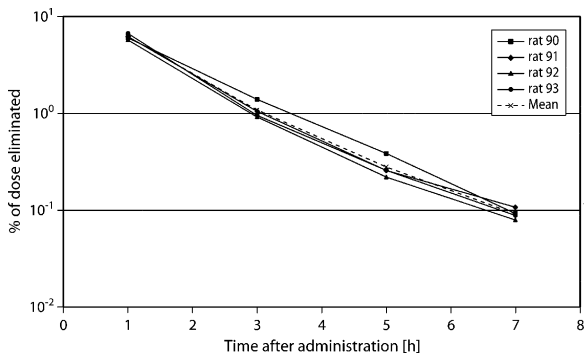

Fig. 8. ^{14}C-HOE 642: amount of dose excreted via bile after i.v. (5 mg/kg) administration to rats

[16]instead of an oral an introduodenal administration should be choosen, when using anesthetized animals

ination process makes the existence of an enterohepatic circulation improbable.

II.L.5 Diaplacental Transfer Study in Rats

PURPOSE AND RATIONALE

To obtain information about the distribution of a drug and/or its bio transformation products in dams and fetuses in dependence of time.

PROCEDURE

9 healthy female 18 days pregnant Sprague Dawley rats weighing about 300 g (approximately 12 weeks old) receive the radiolabeled compound the route of administration projected to be used therapeutically (for oral or intravenous administration to rats and animal maintenance see the procedure described in the rat mass balance study).

Material

- Box for anesthesia with CO_2 or with Isofluran
- scalpel, scissors straight and curved
- bone shears, pincers
- gloves for single use
- small evaporating basins
- plastic tubes and commercially available aggregation inhibitor like heparin-sodium
- centrifuge tubes and centrifuge
- disposable syringes and cannulas
- dash bottle with ethanol/water 1:1.

3 animals/time point are killed painlessly (CO_2 anesthesia, exsanguination as described in the "blood/plasma radiokinetic study in rats") and are each immediately dissected.

The rat is placed in a dorsal position on an undersheet and the fur is moistened with ethanol/water. The abdominal cavity is opened and the abdominal wall folded back. The uterus is exposed and the amniotic fluid is withdrawn by puncturing the amniotic sac with a syringe. The uterus is then placed on a plastic film and opened. The placentas with the fetuses are detached from the uterus. The placentas are then detached from the fetuses. After removing the fetal membranes the fetuses are removed and immediately killed under CO_2 atmosphere. The amniotic fluid is aspirated with a disposable syringe.

The organs and tissues listed below are removed. The number of fetuses is determined and documented. The fetuses per dam and their organs, respectively, are pooled. The order of removal is prescribed in the following dissection schedule.

- Blood/plasma (from exsanguination, see study "blood/plasma radiokinetics in rat")

Opening of the abdominal cavity

- liver
- extraction of uterus
- amniotic fluid
- remove/kill fetuses immediately with CO_2-gas
- fetal blood/plasma
- fetal liver
- fetal carcass
- placenta.

Examinations are performed concerning the radioactivity concentrations and portions.

Processing of Samples

Blood and plasma samples are taken on cones for combustion, weighed, dried at room temperature, combusted and the $^{14}CO_2$ formed is directed into an absorption fluid. The subsequent radioactivity measurements are carried out after addition of scintillator to the samples.

After removal, larger organs and tissues are homogenized with Ultra-Turrax appliances (for instance from Ika, Staufen, Germany) after addition of deionized water, the amount of which depended on the consistency of the tissue. Smaller tissues are finely cut. The specimens are dissolved in volumetric flasks at 60–70 °C in Solvable (Packard BioScience B.V. Groningen Netherlands) and water. Ethanol is added if required to prevent foam formation. Addition of approx. 0.2 ml Perhydrol (Riedel de Haën, Seelze, F.R.G.) is sufficient to remove discolorations. Measurements are then performed after addition of the scintillator.

EVALUATION

The radioactivity concentrations in the collected samples are determined. They are expressed in μg equivalents of drug/g and in % of administered radioactivity. The temporal course of the concentrations and the portions of radioactivity found can be represented by graph and tables. In cases where mean portions of administered radioactivity of a tissue/body fluid of all three time points are distributed about a single straight line in a semi logarithmic plot (ln concentrations on time), the rate constant can be calculated by linear regression and subsequently the half-life (ln2/rate constant).

The data are finally drawn together for detailed discussion and evaluation with special attention on the placenta border: do significant amounts of radioactiv-

ity cross the placenta and are the fetuses exposed to drug/metabolites? Does the radioactivity remain in the fetus/organs/body fluids longer than in the dams?

CRITICAL ASSESSMENT OF THE METHOD

The radioactivity represents the sum of the original compound and/or radioactive-labeled metabolites and not to forget possible synthetic side-products which can be present in traces (depending on the purity and content of the synthetic material). Discussing traces of radioactivity, for instance traces crossing the placenta, keep in mind that these traces may be due to synthetic side-products. Thus, whenever possible, try to use radiolabeled compound as clean as possible.

MODIFICATIONS OF THE METHOD

The described procedure may be seen as a minimum for investigation of the placental transfer. Of course, whenever necessary or useful, the procedure may be enlarged by additional time points and organs/tissues/body fluids (such as ovary, kidney of dams) to be investigated.

The radioactivity of the organs can also be determined after combustion alternatively. Normally the method of digestion, discoloration and direct determination of radioactivity is a little bit more sensitive, since more material can be used than in the combustion process.

Instead of the described procedure of a common quantitative distribution study, the placental transfer can be investigated nicely also by quantitative whole body autoradiography (see the section on autoradiography and for instance Endo et al. (1992) or Suwelack et al. (1985)). The quantitative whole body autoradiography was extended also for the use of pregnant rabbits (Herman 1998).

A bidirectional placental (maternal to fetal and fetal to maternal) transfer was described for instance by Thomas (1995).

Younger rats (8–10 weeks) and performing the investigation already on day 12 of gestation is mentioned for instance by Pohland (1991).

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EXAMPLE

HWA 486 (Leflunimide, Arava) is a compound against rheumatoid arthritis.

9 healthy female 18 days pregnant Sprague Dawley rats weighing between 270.0 and 317.9 g received ^{14}C-HWA 486 in an approximate dose of 20 mg per kg body weight. The compound was administered orally by a stomach tube. As formulation a suspension in starch mucilage was selected. Examinations were performed concerning the radioactivity concentrations and portions in blood, plasma, and liver of the dams as well as in amniotic fluid, placenta and in blood, plasma, liver and carcass of the fetuses.

Details of the dose and study design is given in Figure 9.

Samples were processed, the radioactivity determined and data evaluated as described above.

The radioactivity concentrations found in the different tissues/organs/body fluids are summarized in the graph (Figure 10).

Taking the weight of the investigated tissues/organs/body fluids into account and expressing the observed radioactivity as part of the dose administered (in %) the graph (Figure 11) shows the amount of drug/metabolites reaching the tissues/organs/body fluids:

At the first measuring time (6 h after dosing), the highest levels for dams were detected in the plasma (mean 58.99 μg equivalents/g), followed by the liver (43.50 μg equivalents/g) and the blood (38.16 μg equivalents/g) indicating a considerable extent of absorption. In the placenta, a mean of 16.10 μg equivalents/g were found, in the amniotic fluid 3.06 μg equivalents/g (0.30 %). Concentrations in fetal plasma (13.96 μg equivalents/g), fetal blood (11.19 μg equivalents/g), fetal carcass (7.39 μg equivalents/g) and fetal liver (6.31 μg equivalents/g) were lower than the concentration in the maternal blood. Thus a close barrier function of the placenta with complete retention of radioactivity in this organ was not present.

24 h after administration, the concentrations in all examined organs of dams had decreased as compared to the first measuring time. The concentrations in plasma (25.26 μg equivalents/g) were slightly higher than in blood (23.84 μg equivalents/g). The liver was

Animal-No	11	12	13	20	21	22	17	18	19
Body weight (kg)	0.288	0.318	0.284	0.276	0.303	0.270	0.297	0.313	0.280
Route of administration	oral								
Dose (mg/kg)	19.161	19.014	19.405	19.470	19.347	20.005	18.631	19.318	20.903
Time of killing (h after administration)	6			24			48		
Preparation	Suspension in starch mucilage								
Conc. in prep. (mg/g)	5.618			5.551			5.618		
Amount prep. adm. (g)	0.982	1.076	0.980	0.968	1.056	0.973	0.984	1.075	1.040
Batch	Z 29030-2								
Spec. radioact. (MBq/g)	849.00								
Adm. rad. (MBq/animal)	4.68	5.13	6.67	4.56	4.98	4.59	4.69	5.13	4.96
Study objectives	Determination of concentrations in maternal and fetal tissues								

Fig. 9. HWA 456 dose and study design

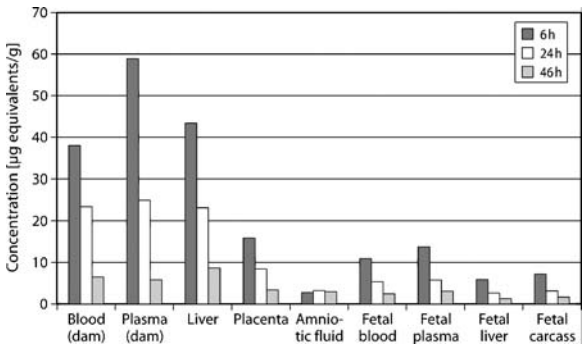

Fig. 10. ^{14}C-HWA 486: radioactivity concentrations in tissues/organs/body fluids from pregnant rats

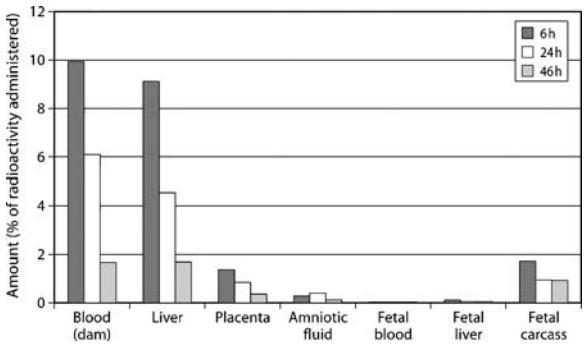

Fig. 11. ^{14}C-HWA 486: amount of dose in tissues/organs/body fluids from pregnant rats

in a similar range (23.22 µg equivalents/g) and the placenta showed 8.71 µg equivalents/g. Fetal liver, fetal blood and plasma and fetal carcass also showed lower concentrations than at the first measuring time. All concentrations were below the concentration in maternal blood.

At the last measuring time, 48 h after dosing, the concentrations in maternal organs had decreased further: The highest concentrations were detected in the liver (8.83 µg equivalents/g, corresponding to 1.7 % of radioactivity administered), followed by the blood (6.63 µg equivalents/g; 1.7 %) and the plasma (5.97 µg equivalents/g). In the placenta, 3.45 µg equivalents/g (0.4 %) were found. Concentrations in fetuses had dropped further. All measured concentrations were considerably below the concentrations in the maternal blood: Fetal blood (2.57 µg equivalents/g), fetal plasma (3.28 µg equivalents/g), fetal liver (1.36 µg equivalents/g) and fetal carcass (1.63 µg equivalents/g).

Since the concentrations showed a virtual linear decrease of radioactivity with time in the semilog scale (see Figure 12) the estimation of half-lives made sense (with the exception of the amniotic fluid). The half-lives ranged from 13 h (plasma, dam) to 20 h (plasma, fetus).

In summary, it may be stated that after oral administration of ^{14}C-HWA 486 to the dams the compound was well absorbed. The absorbed radioactivity was able to penetrate into the fetuses. The concentrations in the maternal tissues were considerably higher than in the fetus at all measuring times. The radioactivity departs

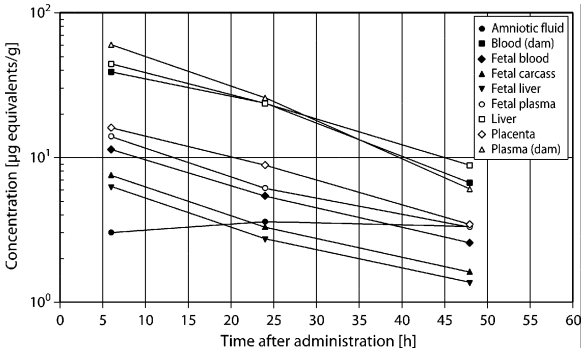

Fig. 12. ^{14}C-HWA 486: Concentration-time-profile of tissues/ organs/ body fluids from pregnant rats

from the maternal organism parallel to the fetal organism. Only in the amniotic fluid, no clear decreasing tendency could be observed.

II.L.6 Milk Transfer Study in Rats

PURPOSE AND RATIONALE

For registration of a drug, data are required on excretion with the milk as well as data about the correlation between the blood and milk level.

PROCEDURE

10 lactating healthy female Sprague-Dawley rats weighing about 300 g (approx. 13 weeks old) receive the radiolabeled compound the route of administration projected to be used therapeutically. For oral or intravenous administration to rats as well as details concerning animal maintenance see section "Mass balance study in rats".

About 5 min before the selected milking times, each dam receives about 0.03 ml oxytocin (for instance Oxytocin Injektionslsg.10IE/ml; Vetoquinol GmbH; Parkstr. 10; 88212 Ravensburg) subcutaneously, because the amounts are otherwise insufficient for analysis. At the examination times (such as given in the example), milk was first taken by manual milking, and blood then gained from the tail tip. The animals not being accustomed to the milking procedure, milk could not be obtained at each examination time.

For milking, the animals were held by the nape. A sucking pump[17] was put alternately onto the individual teats to collect the withdrawn milk into an Eppendorf tube. When the vacuum was interrupted at regular intervals with a finger, the device operated like a milking machine. The appropriate partial vacuum and rhythm had to be established for each animal. Higher milk flow was obtained by massaging and thus stimulating the teats with the fingers prior to actually beginning milking.

In cases where the milking and blood sampling are very narrow at first, it is recommended to use two alternative groups of animals.

Blood and Milk Samples

Blood and milk samples were taken on cones for combustion, weighed, dried at room temperature, combusted in a combuster and the $^{14}CO_2$ formed is directed into an absorption fluid. The subsequent radioactivity measurements are carried out after addition of scintillator to the samples. Blank values are concurrently measured in the studies and deducted from those measured. These blank values originated from identical material from untreated animals.

EVALUATION

The radioactivity concentrations of the collected samples are determined. They are expressed in µg equivalents of drug/g matrix. The temporal course of the concentrations can be represented by graph and tables. With a specific pharmacokinetic software program like Winnonlin (www.pharsight.com) or using the laboratory management system in case the functionality is given directly (such as DEBRA from LabLogic Systems Ltd. Sheffield England www.lablogic.com), the half-lives and the AUCs of the radioactivity in blood and milk can be calculated.

To calculate the portion of administered radioactivity excreted with the milk, it is necessary to know the quantitative milk secretion of each particular rat. However, this is not possible within the present study due to the stress inflicted on the animals by the unusual milking procedure which led to milk retention. Assuming values known from literature (Bornschein (1977)) 6.5 ml per day the portion excreted with the milk can be estimated.

CRITICAL ASSESSMENT OF THE METHOD

Keep in mind that the radioactivity represents the sum of the original compound and/or radiolabeled metabolites and not the drug itself.

[17] can be easily constructed connecting a water-jet pump with a micro wash bottle; an Eppendorf vessel is placed under the inlet tube; at the other end the inlet tube is fitted with a polyethylene tube and a microfunnel. The "micro wash bottle" can be assembled from a 15-mL scintillation vessel with two openings in the lid and polyethylene tubing.

MODIFICATIONS OF THE METHOD

Milking rats under anesthesia in milk transfer studies is mentioned for instance by Endo (1992), Tanayama (1974) or Saillenfait (1997).

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EXAMPLE

HWA 486 (Leflunimide, Arava) is a compound against rheumatoic arthritis. The oral route of administration was selected for this study because this was the route planned in man.

10 lactating healthy female Sprague-Dawley rats weighing between 265.0 and 351.2 g received HWA 486-[^{14}C] in an approximate dose of 16 mg per kg body weight. The compound was administered orally by a stomach tube in form of a suspension in strach mucilage. Details of the study conduct see Figure 13.

The concentrations of radioactivity in blood and milk were determined at different times up to 24 h after dosing.

Blood and Milk Samples

Blood and milk samples were taken on Combusto Cones (Canberra-Packard), weighed, dried at room temperature, combusted in a Tri-carb combuster (Canberra-Packard GmbH, Model 307, Frankfurt am Main, F.R.G.) and the $^{14}CO_2$ formed was absorbed with Carbo-Sorb (Canberra-Packard). The subsequent radioactivity measurements were carried out after addition of scintillator to the samples.

Animal-No	1	2	3	4	5	6	7	8	9	10
Body weight (kg)	0.311	0.305	0.273	0.302	0.265	0.318	0.351	0.328	0.330	0.349
Route of administration	oral									
Dose (mg/kg)	15.85	16.11	18.79	16.29	19.63	15.54	15.66	16.60	16.40	15.72
Time of blood sampling (h after adm.)	0.5, 1, 2, 3, 4, 5, 6, 8, 24									
Time of milk sampling (h after adm.)	0.5, 1, 2, 3, 4, 5, 6, 8, 24									
Preparation	Suspension in starch mucilage									
Conc. in prep. (mg/g)	4.927					4.974				
Amount prep. adm. (g)	1.000	0.996	1.039	1.000	1.056	0.995	1.106	1.095	1.088	1.104
Batch	Z 29030-2									
Spec. radioact. (MBq/g)	849.00									
Adm. rad. (MBq/animal)	4.18	4.17	4.34	4.18	4.42	4.20	4.67	4.62	4.59	4.66
Study objectives	Blood and milk levels									

Fig. 13. HWA 486 study conduct

Radioactivity Measurements

Radioactivity measurements were carried out by the liquid scintillation counting procedure in a β-spectrometer (Canberra-Packard TRI-CARB 2500 TR and T 2700), using an external standard device which permitted the counting efficiency to be determined by the channel ratio method.

The scintillator Permafluor E+ recommended by Canberra-Packard for their automatic combusters was used in the case of combusted samples. The scintillator Roth-rotiszint eco plus (Roth, Karlsruhe, Germany) was used for the samples measured directly.

Blank values were concurrently measured in the studies and deducted from those measured. These blank values originated from identical material from untreated animals.

The resulting mean concentrations (bars indicate SD) are given in Figure 14.

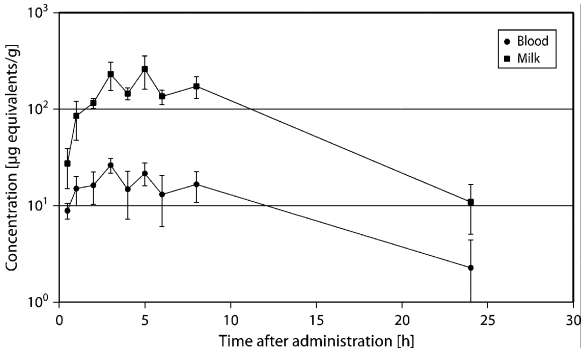

Fig. 14. ^{14}C-HWA 486: Concentration-time-profile of blood and milk from lactating rats

The pharmacokinetic values in the blood and milk given in Table 4 were calculated by the pharmacokinetics' software WinNonlin, Version 1.1. using the mean concentrations, a non-compartmental model and the linear trapezoidal rule.

Already at the first measuring time (30 min after dosing), the concentrations in milk were considerably higher than the corresponding levels in blood in all animals examined. The mean values amounted to 27.01 µg equivalents/g in milk and 8.85 µg equivalents/g in blood. This indicates a rapid penetration of the compound into the mammary gland and a rapid onset of the elimination via the milk.

Table 4

Parameters	Blood	Milk
C_{max} (µg equivalents/g)	26.3	252.9
t_{max} (h)	3	5
$t_{1/2}$(h)	6.4	4.4
Conc.$_{24\,h}$ (µg equivalents/g)	2.28	10.8
AUC (µg equiv./g × h)	282.4	2635.5
AUC_{inf} (µg equiv./g × h)	303.5	2704.2

The highest mean concentrations in the blood were observed at 3 h after dosing amounting to 26.3 µg equivalents/g. In the milk, the detected mean C_{max} concentrations were approx. 9 times higher and amounted to 252.9 µg equivalents/g. They were present at 5 h after dosing. At all measuring times, the concentrations in blood were considerably lower than in the milk, indicating an accumulation of the compound and/or its metabolites in milk.

The concentrations in the milk and in the blood decreased after reaching of the C_{max} concentrations in a similar way and speed. The calculated half-lives (from C_{max} to 24 h after dosing) amounted to 4.4 h for the elimination from the milk and 6.4 h for the elimination from the blood indicating a continuous providing of new radioactivity from the blood to the milk. There was no retention in the mammary gland longer than in the blood. A rough estimation of the amount of radioactivity administered excreted via the milk amounted to approx. 25 %.

Analogeous to the C_{max} concentrations, the calculated AUC values in the milk were approx. 9 to 10 times higher than those in the blood.

Chapter II.M
In vivo Distribution

Manfred Zimmer

II.M.1 Quantitative Whole Body Autoradiography (QWBA)

PURPOSE AND RATIONALE

Distribution studies with radiolabeled test substances in animals are an important part of the drug development process. Traditional routine methods used for these studies are quantitative tissue distribution studies (QTD) and alternatively whole-body autoradiography (WBA) with detection of the radioactivity in whole-body sections on X-ray film (John R. J. Baker 1989). WBA is a qualitative detection method with a very high local resolution which includes all organs an many small substructures.

Radioluminography (RLG) is an alternative method of radiation detection based on the phosphorus imaging technique. RLG is much more sensitive than the WBA technique, its exposure time is much shorter and it has a much wider linear measure range. Because of the latter property RLG enables a quantification of drug concentrations in whole-body sections.

Quantitative Whole-Body Autoradiography (QWBA) is based on the RLG technique and the use of standards obtained from dilution series containing known concentrations of radioactivity. Isotopes used in QWBA are mainly ^{14}C and ^{3}H. These standards were cut together with the whole-body sections to ensure an identical thickness and used for the internal calibration. The information of the calibration curve allows the determination of the concentrations in the organs and tissues of interest which can be derived from the measured area and the section thickness.

The results of distribution studies form the principle basis for the assessment of exposure and the elimination of residues in human organs and tissues. Direct determinations of exposure in man are generally limited to measurements in blood and plasma, which are easily accessible. Distribution patterns are determined in animals, usually rats, instead. Apart from the standard study design described below all kind of animals can be used up to the size of rabbits. The results obtained with QWBA provide pharmacokinetic data on the test substance and/or its metabolites, and evidence for interpretations regarding potential toxicological and pharmacological target organs.

Additionally, before the first study with radiolabeled test substance in man can be started, a risk assessment of a human radiokinetic study is mandatory. The estimation of the radiation exposure in humans given a radiolabeled dose is based on exposure data obtained typically from QWBA studies in animals.

PROCEDURE

Male pigmented and non-pigmented rats, weighing approx. 200 g, are used. At pre-selected time points (e.g. 0.25 h, 1 h, 2 h, 4 h, 8 h, 24 h, 72 h, 168 h) after administration of the radiolabeled test compound, the animals are sacrificed by CO_2-overdose, fixed on a piece of cardboard, embedded in sodiumcarboxymethylcelulosis and immediately deep-frozen using liquid nitrogen (–197 °C). In these frozen blocks a certain number of holes are drilled an filled with radioactive standards (with definite concentrations of radioactivity obtained from dilution series). These blocks are put into a cryotom (cooled microtom at a temperature of approx. –18 °C, e.g. Leica CM3600) and sagittal whole body sections are cut at different section levels (eye, brain, adrenal, kidney, thyroid; other sections as well). Before sectioning adhesive tape is placed on top of the frozen surface to enable an easy handling of the sections. The thickness of section amounts to 25 μm. The sections are dehydrated for at least 12–16 hours in the chamber of the cryotom (freeze-drying). Then the sections are cut out of the tape, fixed on a cardboard and dusted with

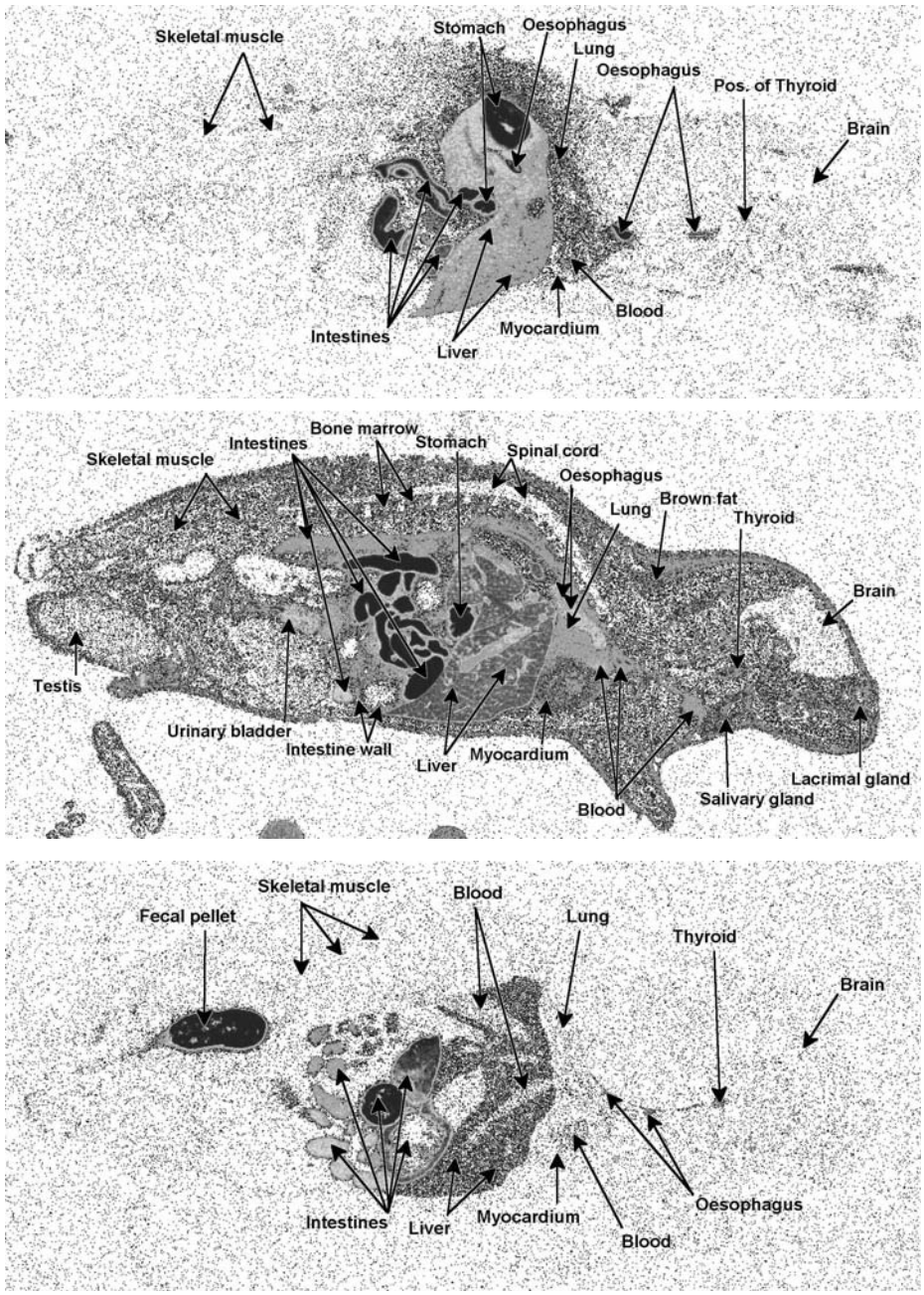

Fig. 1. Three autoradiographs at different time points (15 min, 4h, 24h) after oral administration of the radiolabelled test compound. The different radioactivity concentrations are depicted as shades of *grey*, from *dark* (high concentrations) to *white* (low concentrations).

talcum powder to prevent the sections sticking on the detection medium. After removal of redundant talcum the cardboard with the section is placed on the photo sensitive surface of an Imaging plate (IP) and both are put into a black plastic bag for exposure. The bag is sealed using the vacuum-technique (G. Kloss et.al. 1973) to enable an intimate contact with the photo-sensitive medium. Exposure time is at least 48 hours in a shielding box at room temperature. Subsequently the IPs are scanned with an image reader (e.g. BAS5000, raytest) and digitised autoradiograms are obtained.

In parallel to this investigations the biostability of the labeling position in the radiolabeled test compound is examined. For this purpose, the volatile radioactivity in the cage air after administration is monitored over 24 hours.

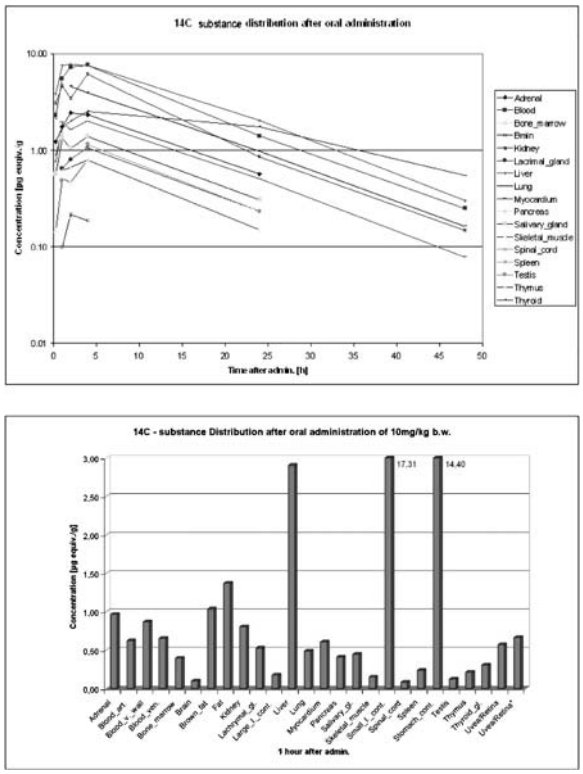

Fig. 2. Quantitative analysis of organs and tissues. The concentrations (expressed as drug equivalents/g) were calculated by dividing the measured radioactivity concentrations by the specific radioactivity of the administered compound. *Upper plot* shows the concentration-time-profile, *lower plot* shows the concentrations at the time of Cmax.

Additional to this standard procedure the following special issues should be mention where autoradiography is also used: drug penetration of blood-brain barrier, placental transfer, drug excretion via milk in dams, more detailed distribution in organs and tissues (e.g. distribution pattern in the brain: sequential coronal sectioning in anteroposterior direction; drugs being developed for treatment of e.g. osteoarthritis: extent of penetration of drug in cartilage of e.g. the knee joint), drug (e.g. herbicides) localization in plants, drug distribution at cellular and sub-cellular level, receptor microscopic autoradiography (Stumpf 2003).

EVALUATION

The digitized autoradiograms are analyzed with an appropriate software (e.g. AIDA, raytest), regions around the organs/tissues of interest can be drawn electronically. The radioactive standards, cut together with the whole-body sections are used as internal calibration. The information of the calibration curve provides the link between counts/area and radioactivity/area and allows the determination of the concentrations in the organs and tissues of interest which can be derived from the measured area and the section thickness. Considering the background counts of the IP, the specific radioactivity of the labeled test compound and the area size of the region of interest, the corrected counts can be transferred into [amount of drug equivalent/g tissue] (due to the fact that only radioactivity can be measured the value is defined as "equivalent"). The concentrations in the organs/tissues at different time points after administration are listed in tables and can be depicted as concentration-time-profiles, bar charts etc.

CRITICAL ASSESSMENT OF THE METHOD

Pros:

- Distribution of compound and/or metabolites detected
- In situ situation in the body reflected
- Different subunits of organs can be regarded separately
- Rapid, impressive, accepted method
- Accepted as basis for human mass balance studies (dosimetry calculations)

Cons:

- Radiolabeled compound required
- Total radioactivity detected, not parent compound or any metabolites
- Blood content of the organs and tissues not exactly known
- It is not possible to differentiate parent compound from conjugated, metabolites or other radiolabeled fragments of the original molecule

MODIFICATIONS OF THE METHOD

Although WBA is a relatively old method having its origin in the beginning of the 20th century this technique was undergoing some considerable modifications during the last 20 years and is now used widely in the chemical and pharmaceutical industries. The first whole body autoradiogram (WBA) of a frog which had been exposed to radium and was placed on a photographic plate was published by E.H. London (1904). The original WBA technique was invented by Swen Ullberg (1954). He did research in the field of antibiotics and wanted to investigate whether the concentrations in the different infected organs or tissues in the body were high enough for therapeutic effects. There was no sufficient method available so he developed a technique to get the appropriate information in situ. Mice were injected with radiolabeled penicillin

and snap frozen in liquid air. Whole body sections were cut on a hand driven sledge microtome in a freezer room at –15 °C. Frozen and dehydrated sections were exposed to X-ray film. Using different section levels a qualitative estimation of the distribution in organs and tissues could be obtained.

The evolution from qualitative to quantitative WBA occurred during the last 20 years.

It started from X-ray films and introduction of tritium film (increased sensitivity and removal of the antiscratch layer) and went on to the semi-quantitative analysis in the 1980s using densitometry. Finally quantification was feasible in the 1990s when digitized autoradiograms and radioluminography (RLG) were developed as a new method of radiation detection (Keiji Mori 1994; Steinke et al. 2000).

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II.M.2 Quantitative Tissue Distribution (QTD)

PURPOSE AND RATIONALE

Distribution studies with radiolabeled test substances in animals are an important part of the drug development process. Routine methods used for these studies are quantitative whole-body autoradiography (QWBA) and alternatively quantitative tissue distribution studies (QTD) with dissection of the animals and measurement of radioactivity in pre-selected organs and tissues using a liquid scintillation counter.

The results of these studies form the principal basis for the assessment of exposure and the elimination of residues in human organs and tissues, in which direct determination is of course impossible. Direct determinations of exposure in man are generally limited to measurements in blood or plasma, which is easily accessible. Distribution patterns and pharmacokinetic parameters (e.g. elimination half lives, time of maximum concentrations in organs and tissues) are determined in animals, usually rats, instead. Radiolabeled test substance is administered to ensure, as far as possible, determination of the whole of the parent compound and its metabolites. The results provide pharmacokinetic data on the distribution of the total radioactivity, information on any accumulation or specific affinities of the test substance or its metabolites, and evidence for interpretations regarding potential toxicological and pharmacological target organs (Steinke et al. 2000).

Additionally, before the first study with radiolabeled test substance in man can be started, a risk assessment of a human radiokinetic study is mandatory. The estimation of the radiation exposure in humans given a radiolabeled dose is based on exposure data obtained typically from QTD – or QWBA – studies in animals.

PROCEDURE

Male rats, weighing approx. 200 g, are used. At preselected time points (e.g. 0.25 h, 1 h, 2 h, 8 h, 24 h, 96 h; 3 animals per group) after administration of the radiolabeled test compound, the animals are sacrificed (CO_2 overdose; exsanguination) and immediately dissected. Organs and tissues are removed in the following dissection order (from expected low to high concentrations to avoid cross-contaminations): blood, plasma, eyes, brain, skin (without hair and subcutaneous fat), subcutaneous fat, skeletal muscle, bone(femur), bone marrow, thyroid, lungs, thymus, heart, testis, prostate, retroperitoneal fat, spleen, kidney, adrenal, urinary bladder, liver, pancreas, mesenterial lymph nodes, stomach contents, small intestine contents, large intestine contents, stomach wall, small intestine wall, large intestine wall.

Blood and plasma samples are taken on Combusto Cones (e.g. PerkinElmer Life Sciences), weighed, dried at room temperature, combusted in a sample Oxidizer (e.g. PerkinElmer Life Sciences, Model Oximate 80/307, Frankfurt am Main, Germany) and the 14 CO_2 formed is absorbed with Carbo-Sorb (e.g. PerkinElmer Life Sciences), The subsequent radioactivity measurements are carried out after addition of scintillator to the samples.

After removal, larger organs and tissues are homogenised with Ultra-Turrax-appliances (e.g. Ika, Staufen, Germany) after addition of deionised water, the amount of which depended on the consistency of

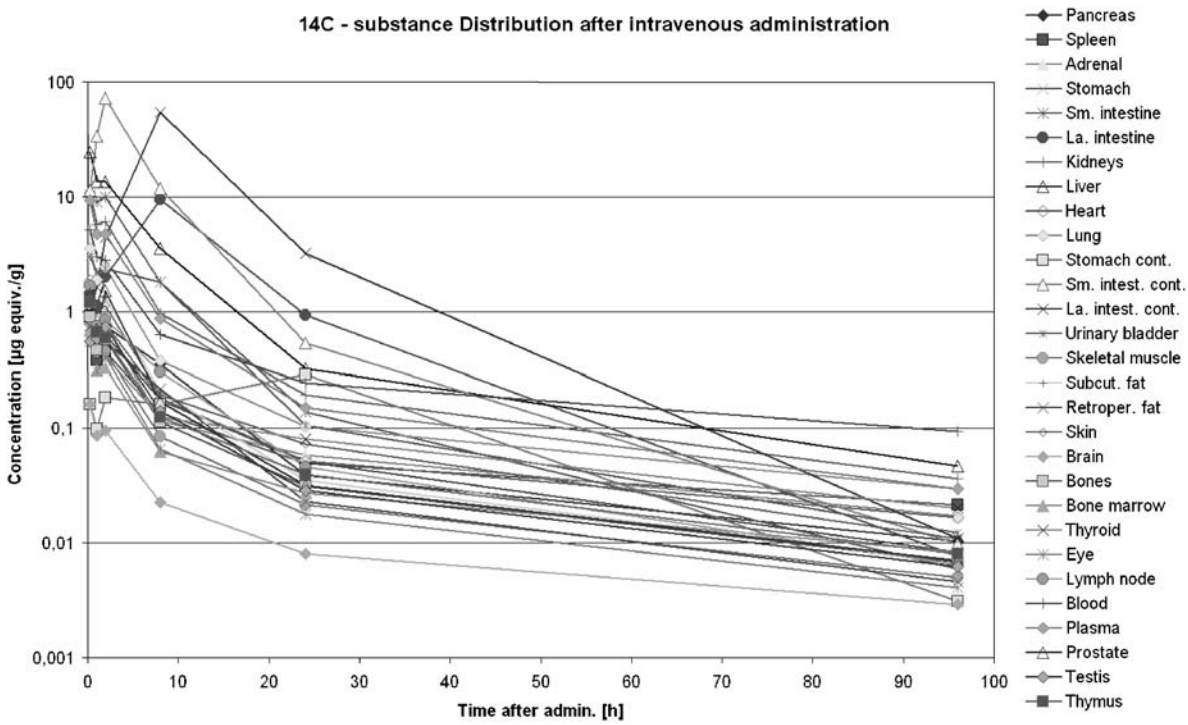

Fig. 3. The concentration-time profiles of the analyzed organs and tissues. The concentrations (expressed as drug equivalents/g) were calculated by dividing the measured radioactivity concentrations by the specific radioactivity of the administered compound.

the tissue. Smaller tissues are finely cut, The specimens are dissolved in volumetric flasks at 60–70 °C in a mixture of Digestin (e.g. Merck, Darmstadt, Germany) and water, ethanol is added if required to prevent foam formation, addition of approx, 0.3 ml Perhydrol (e.g. Riedel de Haën, Seelze, Germany) is sufficient to remove discolourations, measurements are then performed after addition of the scintillator.

Radioactivity measurements are carried out by the liquid scintillation counting procedure in β-spectrometers (e.g. Canberra-Packard 4530), using an external standard device which permitted the counting efficiency to be determined by the channel ratio method (Dyer 1980).

The scintillators Permafluor E +, recommended by PerkinElmer Life Sciences for their automatic combusters, and Rotiszint eco plus (e.g. Roth, Karlsruhe, Germany) for dissolved samples are used, blank values are concurrently measured in the studies and deducted from those measured, These blank values originates from material of untreated animals.

EVALUATION

Considering the count conditions of the analytic device, the specific radioactivity of the labeled test compound and the weights of the analyzed specimen, the corrected counts can be transferred into [amount of drug equivalent/g tissue] (due to the fact that only radioactivity can be measured the value is defined as "equivalent").

The concentrations are expressed in µg equivalents drug/g tissue and in % of administered radioactivity, i.e. they represent the sum of the original compound and/or radioactively-labeled metabolites, To obtain a unit independent of metabolisation, the test preparation concentrations in µg equivalents/g can be converted to nmol using the molecular weight:

1 µg of the free compound is equivalent to (1000/MW) nmol.

The pharmokinetic parameters in the blood, plasma, excreta and the kinetics in organs and tissues are calculated using an appropriate pharmacokinetics software (e.g. WinNonlin).

The detection limit is determined using the individual blank values for the corresponding biological material by the following formula:

*Detection limit = background (Bq)/(Initial weight (g) * specific radioactivity (Bq/µg))*

At least the double blank value (Bq) is required for evidence of a significant concentration value (µg equivalents per g or ml).

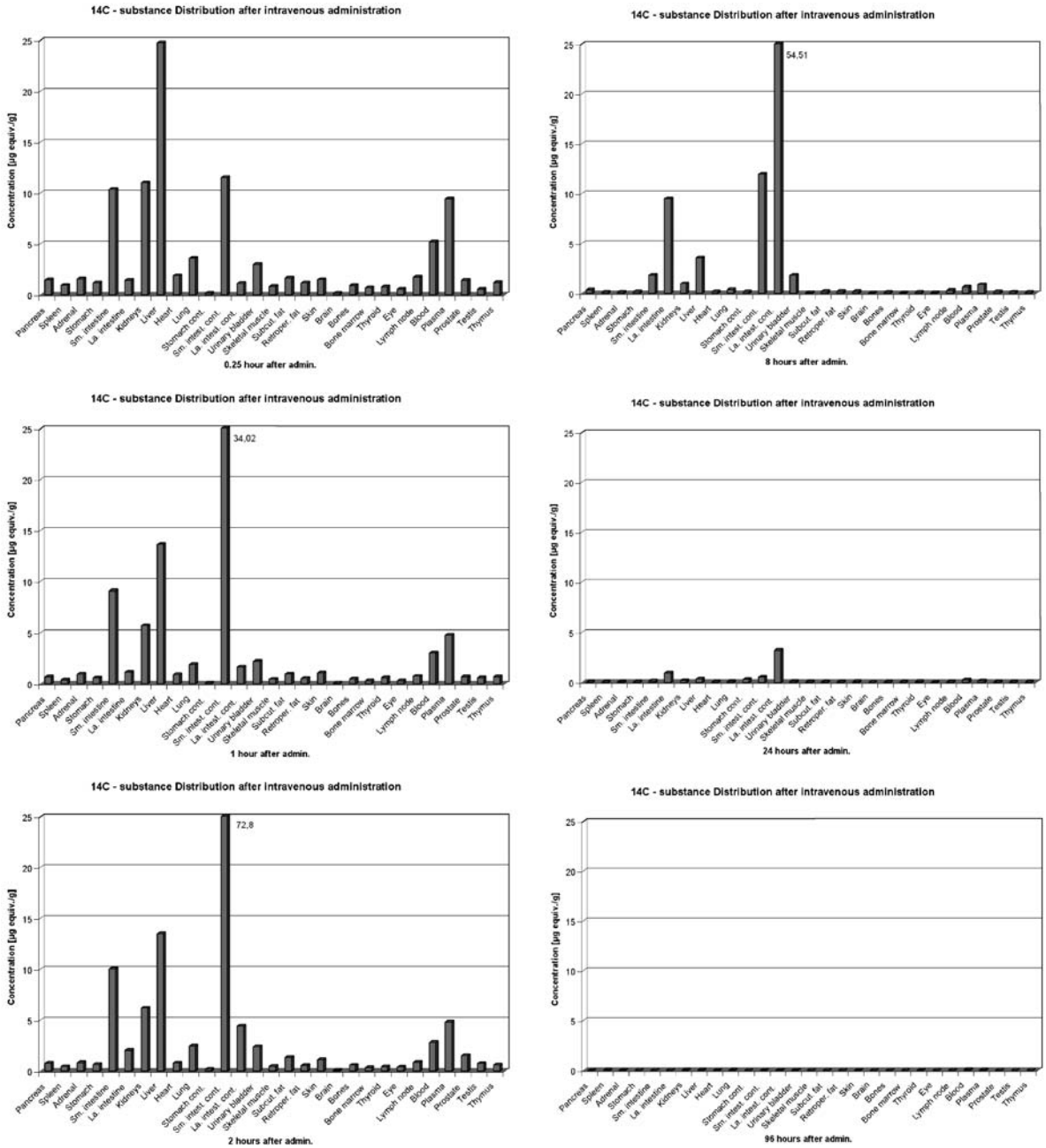

Fig. 4. The concentrations in organs and tissues at different time points, depicted as bar charts

The concentrations in the organs/tissues at different time points after administration are listed in tables and can be depicted as concentration-time-profiles, bar charts etc.

CRITICAL ASSESSMENT OF THE METHOD

QTD was the "gold standard" when the method of WBA (Ullberg 1954) was not jet able to quantify the distribution.

The results of the two methods complemented one another. Whole-body autoradiography is a qualitative detection method with a very high local resolution which includes all organs and many small substructures, whereas measurement after dissection yields quantitative concentration data for a limited, preselected number of organs.

A vast change occurred when a new technology allowed the quantification of WBA in the 1990s.

Digitized autoradiograms and Radioluminography (RLG) were developed as a new method of radiation detection (Keiji Mori 1994).

Since the organs and tissues are not destroyed during a QWBA study (in contrast to the homogenisation of the organs and tissues for LSC-measurements), substructures can be distinguished clearly (e.g. adrenal medulla and adrenal cortex often show very different concentrations of radioactivity).

To save animals and time, the quantitative tissue distribution study is more and more replaced by the quantitative whole-body autoradiography.

MODIFICATIONS OF THE METHOD

As the procedure of the animal study part of this method is well established, modifications occur mainly in the technological part by improvement of the performance of liquid scintillation counters and the appropriate software. As this method no longer belongs to the "cutting edge" skills in the drug development process, modification activities are moderate.

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Chapter II.N
Distribution – in vivo – Other Methods

Yves Archimbaud

II.N.1
Positron Emission Tomography

PURPOSE AND RATIONALE

Distribution in vivo could be studied by positron emission tomography (PET). Positron emission tomography is a sensitive and specific functional non invasive 3-D imaging method that permits rapidly and directly measurement of the total radioactivity from a drug labelled with a positron-emitting radionuclide (Gupta).

PROCEDURE

The drug should be labelled with a positron emitting isotopes such as carbon-11, oxygen-15, nitrogen-13 and fluorine-18. The radionuclides are predominantly produced by charged particle nuclear reactions using a cyclotron. Radiotracers are produced after replacement of a normal constituent in a biologically active compound of interest with a radionuclide without modifying their pharmaceutical, biological or biochemical properties.

The optimal isotopes should be selected depending of the pharmacokinetic of the drug and their half-life values (20.4, 2.03, 10 and 109.8 min for ^{11}C, ^{15}O, ^{13}N and ^{18}F, respectively).

The labelled compounds are generally administered by intravenous infusion to avoid local radiation damage. The amounts of radioactivity injected varied between 200 to 800 MBq (Bergström). Just after administration, the camera is activated to start the acquisition. When a positron-emitting radionuclide is decaying, it emits a positron, that will interact with an electron in an annihilation event whereby these particles jointly are converted to two oppositely directed gamma rays of 511 keV energy. The gamma rays readily penetrate the tissue and a fraction of them emerge out of the body to be recorded by the PET camera with rings of external nuclear detectors, which has high resolution and sensitivity. Computer processing of data generates sets of tomographic images with a spatial resolution of 0.5 to 5 mm depending of the scanner type (0.5–1 mm on animal scanners and 2–5 mm on clinical scanners) after correction for attenuation and detector efficiency. An image is acquired for a predefined time length and at a predefined time after administration. Timing of PET imaging should be well selected depending of the radionuclide and the drug. The data depict the spatial and temporal distribution of total radioactivity. Images are displayed on a computer monitor for inspection. Quantitative values on the drugs/tracers radioactivity concentrations in different organs are determined. For this, regions of interest are manually outlined in the images to represent targeted regions for analysis. Within these regions, the radioactivity concentration is determined for each image in the sequence, resulting in a time-radioactivity data set. The radioactivity is rapidly decaying according to the half-life of the radionuclide and therefore a correction is applied to compensate for this. Integral methods or compartmental analysis are used to analyse PET data. The most widely used integral method is the standardized uptake value SUV: SUV = local radioactivity concentration/(administered radioactivity/body weight). Arterial blood and tissue combined data are needed in compartmental modelling in order to define exchange parameters and assess steady-state distribution volumes of the drug in organs.

EVALUATION

Quantitative values on the drugs/tracers radioactivity concentration-time profile in different organs or sub-regions of organs are determined.

CRITICAL ASSESSMENT OF THE METHOD

PET has a number of advantages. The entire time course of the distribution can be determined quantitatively in a living animal or human with a temporal resolution of seconds to minutes. Each subject can be used as its own

control. PET is highly sensitive with the capacity to detect subnanomolar concentrations of radiotracers.

PET has a number of disadvantages: tracers with short half-lives limit the time during which they can be studied. For example, drugs labelled with ^{11}C may be too rapidly metabolised relative to the 20.4 min half-life and thus meaningful information may be limited. The logistics is challenging with the need of a cyclotron. PET can only measure radioactivity and not discern the chemical form of it, it is not possible in a PET study to be certain that the radioactivity signal is related to the original compound or including metabolites thereof. In order to make it likely that the proper conclusion is drawn, it is recommended that metabolism studies be performed in which the fraction of radioactivity constituted by intact tracer is determined in plasma and in the target organ. Additionally, the plasma profile of radioactive metabolites should be assessed in the PET study. Timing of PET imaging is not so easy to determine.

MODIFICATIONS OF THE METHOD

New materials with higher sensitivity or resolution have been designed: A 3-dimensional PET scanner using gadolinium oxyorthosilicate (GSO) crystals (Surti).

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EXAMPLE

PET has already been applied to a wide number of drugs to demonstrate activity in vivo from standard chemotherapy such as 5-fluorouracil (5-FU). The pharmacokinetics of 5-FU has been successfully investigated using radiotracers, and is the most common anti-cancer drug imaged with PET (Kissel). This is due to the ease of chemical synthesis of ^{18}F-fluorouracil (5–[^{18}F] FU). A pharmacokinetic quantitative model to quantify the intracellular 5–[^{18}F] FU concentration in liver metastases of patients with colon cancer revealed extrahepatic and intrahepatic clearances of 0.66 ± 0.33 and 0.52 ± 0.25 L/min, respectively. A pharmacodynamic relationship between tumour uptake of 5–FU and response, first seen in mice has also been demonstrated in humans by PET methodology (Moehler). Studies showed that colorectal liver metastases with a higher uptake of 5–FU at 2 h, as measured by a standard uptake value (SUV) had a negative growth rate, whereas those with a low SUV had disease progression. Therefore PET help to differentiate between responding and nonresponding metastatic sites.

II.N.2 Microdialysis

PURPOSE AND RATIONALE

Distribution in vivo could be studied by microdialysis. Microdialysis is an in vivo technique that permits measurement of unbound drug or metabolite concentrations in extracellular fluid of specific tissue location. The unbound drug concentrations have been shown to be responsible for the pharmacological effects. The basic principle is to mimic the function of a capillary blood vessel by perfusing a thin dialysis tube implanted into the tissue with a physiological liquid (Ungerstedt).

PROCEDURE

A microdialysis probe is inserted in a selected tissue or fluids (like brain, muscle, skin, liver, tumor, blood and bile (Elmquist) of an animal (mouse, rat, rabbit, dog, monkey) or a human. The probe consists of a small semipermeable hollow fiber membrane, connected to an inlet and outlet tubing with a small diameter. The probe is continuously perfused with a physiological solution such as a Krebs Ringer solution (Na^+ 148 mM, K^+ 4.0 mM, Ca^{2+} 2.3 mM and Cl^- 156 mM) (Benveniste). The perfusate should have an ionic composition comparable to the extracellular fluid surrounding the probe and should be at ambient temperature. After a 24-hours recovery, the drug is administered by a relevant route (orally, intravenously) to an animal or a human and the perfusate is infused at a flow rate of 0.1 to 5 μl/min. The drug able to pass the semipermeable membrane will diffuse over the membrane down their concentration gradient into or out of perfusate. The solution that exits the probe, the dialysate, can be collected during interval-periods. The concentrations of the drug in the dialysate reflect the concentrations in the extracellular fluid around

the semipermeable part of the probe. However, as the dialysis procedure is not performed under equilibrium conditions, the concentration in the dialysate will be less than that in the extracellular fluid. The in vivo recovery, used to describe this relationship, is determined by the use of an internal standard by retrodialysis (Larsson). The concentrations of the drug are determined by a relevant and sensitive analytical method and then are corrected for in vivo recovery.

EVALUATION

Quantitative values on the unbound drug or metabolite concentration-time profile in different tissues or sub-regions of tissues are determined.

CRITICAL ASSESSMENT OF THE METHOD

Microdialysis has a number of advantages: concentration profiles of drug could be obtained without fluid loss from freely moving individual subjects in specific sub regions of tissues.

Microdialysis has also a number of disadvantages: the probe could elicit tissue trauma after implantation, the determination of in vivo recovery is time-consuming and sensitive analytical methods are needed due to the diluting effect (De Lange).

MODIFICATIONS OF THE METHOD

New probes (Evrard), methods for analysis and for recovery are developed. The optimal conditions for composition of the perfusion solution, the flow-rate, the post-surgery interval are searched.

Microdialysis probes are now commercially available in various sizes, designs and materials. Microdialysis probes can be flexible for soft peripheral tissues and fluids or rigid for brain. Four probe geometries are available: linear, loop, side-by-side and concentric. The semipermeable membrane is generally chosen as long as possible, typically between 1 and 10 mm. The probe radius is generally chosen as small as possible, typically between 200 and 400 μm O.D. to cause minimal disturbances within the tissue. Dialysis probes are made of various materials (for example celluloses and copolymers like polyacetonitrile/sodium methallyl sulfonate and polycarbonate/ether). The molecular mass cut off (5–50 kDa), inertness and permeability to solutes of the probes could be different.

Perfusion solution used in microdialysis experiments vary widely in composition and pH. Ideally the composition, ion strength, osmotic value and pH of the perfusion solution should be as close as possible to those of the extracellular fluid of the dialyzed tissue. Perfusion fluids should be at body temperature.

Perfusion flow-rates ranges between 0.1 and 5 μl/min. The tendency is to use lower flow-rates as this may increase recovery, provided that an analytical technique is available to deal with the smaller sized samples.

Experiments should be executed between 24 to 48 hours after implantation after recovery from early tissue reactions, and before the start of long-term reactions.

Sensitive analytical methods should be used such as liquid chromatography combined with mass spectroscopy, microbore liquid chromatography and capillary electrophoreses.

Different methods to determine in vivo recovery could be used: extrapolation to zero-flow rate (Jacobson), no-net-flux (Lonnroth), dynamic-not-net flux (Olson) and retrodialysis using an internal standard (Larsson). The zero-flow rate method is based on the principle that recovery is a function of flow rate. The dialysate concentrations are plotted as a function of flow rate and by extrapolating to zero-flow rate where the dialysate is in equilibrium with the extracellular fluid, the dialysate concentration found at zero-flow rate should equal the actual in vivo concentration. The no-net-flux method is based on the principle that drug transport across the membrane is a function of perfusate concentration while maintaining the extracellular concentration of the analyte at steady state. Different concentrations are perfused through the membrane. The dynamic-no-net-flux method, an extended version of the no-net-flux method allowed the estimation of the recovery as a function of time. Several animals are continuously perfused with one perfusion concentration. The retrodialysis, method is based on the principle that an internal standard with membrane diffusion characteristics close to the analyte of interest is added to the perfusate and its relative loss by retrodialysis is calculated. The assumption is that in vivo retrodialysis of the internal standard is the same as in vivo dialysis of the analyte. An advantage of this method is that fluctuations in recovery of the probe during the experiment are taken into account by the continuous retrodialysis of the marker during the entire experiment.

Recovery of lipophilic compounds binding to components of the dialysis equipment, could be increased by including albumin or a lipid emulsion to the dialysate (Carneheim).

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EXAMPLE

Microdialysis was used to assess morphine 6-beta-D-glucuronide (M6G) and morphine brain distribution in extracellular fluid after systemic administration in rats (Stain-Texier). M6G penetrated into the brain, was distributed and trapped preferentially than morphine in the extracellular fluid and therefore was available to bind at opioid receptors, explaining how M6G induces more potent central analgesia than morphine.

Chapter II.0
Bioanalytical Assays – Toxicokinetics

Karl-Heinz Lehr

II.O.1 Design and Evaluation of Toxicokinetic Studies

PURPOSE AND RATIONALE

Toxicokinetics is defined as "the generation of pharmacokinetic data, either as an integral component in the conduct of nonclinical toxicity studies or in specially designed supportive studies, in order to assess systemic exposure. These data may be used in the interpretation of toxicology findings and their relevance to clinical safety issues" (ICH Guidance Toxicokinetics 1994).

PROCEDURE

Main Group or Satellite Animals?

Whenever possible, toxicokinetic measurements are performed in all animals of the toxicity study. This is the most representative approach and it allows that the individual PK data can be directly correlated with the toxicological findings. The second choice is the toxicokinetic measurement in representative subgroups or in satellite groups. Satellite groups are animals, which are treated and housed under conditions identical to those of the main study animals. The use of satellite animals is indicated for example in small animals, where the collection of a relatively large volume of blood may influence the toxicological findings.

Number of Animals and Timepoints?

In the ICH Guidance Toxicokinetics 1994 it states: "The number of animals to be used should be the minimum consistent with generating adequate toxicokinetic data." and "The area under the matrix level concentration-time curve (AUC) and/or the measurement of matrix concentrations at the expected peak-concentration time C_{max}, or at some other selected time $C_{(time)}$, are the most commonly used parameters." In large animals (e.g. dogs) the number of animals is usually fixed by the number of animals which are necessary for safety evaluation. The withdrawal of a sufficient number of blood samples (6 to 9) per animal is not a problem. However, in small animals like rodents it is recommended not to collect more than 10 % of the blood volume during the AUC sampling interval (Laboratory Animals 1993; Cayen 1995). According to a new guidance from Diehl et al. (2001), the volumes per day are specified according to the recovery period. Diehl et al. limit the total daily volumes of multiple sampling to 7.5 % of the circulatory blood volume at a recovery period of 1 week, 10–15 % at a recovery period of 2 weeks and 20 % at a recovery period of 3 weeks. The optimum number of time points is always a compromise between blood volume restrictions and reliable assessment of TK parameters (AUC and C_{max}). Pai et al. (1996), compared for three different compounds, the AUCs from intensive (full) (10–15 time points with 4–5 rats/time point) with sparse sampling schemes (5 time points with 2 rats/time point). Using Monte Carlo simulation, Pai et al. (1996) showed that the deviation of AUC estimation of the sparse sampling scheme from the full sampling scheme was not larger than 10 %. Thus, a sparse sampling scheme with 5 to 7 time points with 2 to 3 animals per time point is well suited for the reliable determination of systemic exposure in small animal toxicity studies.

Evaluation of Samples from Control Animals?

In Note 8 of the ICH Guidance Toxicokinetics 1994 it is stated: "It is often considered unnecessary to assay samples from control groups. Samples may be collected and then assayed if it is deemed that this may help in the interpretation of the toxicity findings, or in the valida-

tion of the assay method." In the CPMP position paper of the EMEA (2004) this topic is further discussed in the light of a survey conducted by the European Federation of Pharmaceutical Industry, which showed that controls contamination during toxicology studies often occurs. The current version of the position paper gives guidance to assay the levels of test substance in the samples from control animals in the most relevant toxicology studies. However, up to now this is a position paper and not yet a final guideline.

Analytical Methods

The analytical methods to be used in toxicokinetic studies should be specific for the entity to be measured and of an adequate accuracy and precision. The limit of quantification should be adequate for the measurement of the range of concentrations anticipated to occur in the generation of the toxicokinetic data (ICH Guidance Toxicokinetics 1994).

Toxicokinetic Evaluation

The following aspects should be considered for toxicokinetic evaluation:

- Pharmacokinetic profile of the compound (Exposure)
- Dose dependency of AUC and C_{max}
- Chances of exposure during the course of the toxicity study
- Gender differences.

Pharmacokinetic Profile of the Compound (Exposure)
For toxicokinetic purposes it is usually sufficient to describe the systemic burden in plasma or serum of the test species with the test compound and/or its metabolites. The area under the matrix level concentration-time curve (AUC) and/or the measurement of matrix concentrations at the expected peak-concentration time C_{max}, or at some other selected time[1] $C_{(time)}$, are the most commonly used parameters. According to the supplementary notes in the ICH Guidance Toxicokinetics 1994 for a profile (e.g. 4 to 8) matrix samples during a dosing interval should be taken to make an estimate of C_{max} and/or $C_{(time)}$ and area under matrix concentration time curve (AUC).

Dose Dependency of AUC and C_{max}
According to the ICH Guidance Toxicokinetics 1994, it is the one of the primary objective of toxicokinetics to describe the systemic exposure achieved in animals and its relationship to dose level.

[1] for example C(24 hr) as trough value

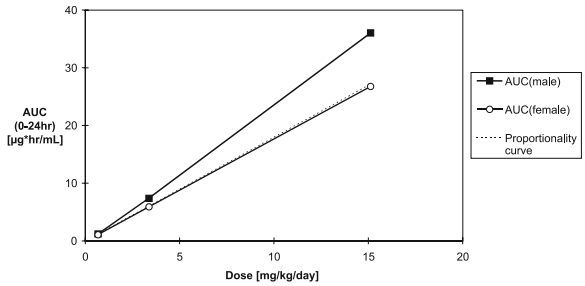

Fig. 1. Proportional dose dependency of AUC in an intravenous toxicity study in rat with the test compound A

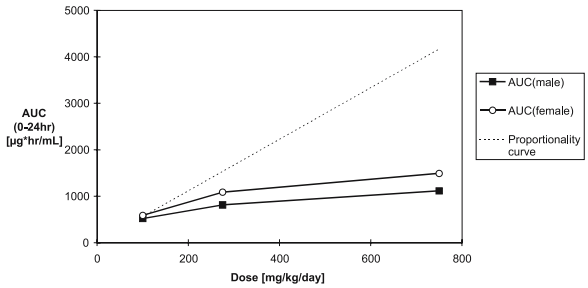

Fig. 2. Less than proportional dose dependency of AUC in an oral toxicity study in rat with the test compound B

At pharmacological and clinical doses it can be generally assumed that most of the drugs show linear pharmacokinetics. Linear pharmacokinetics is given, when exposure (AUC) is proportional to dose and principal pharmacokinetic parameters like bioavailability, elimination rate, volume of distribution and clearance are independent on dose. For toxicokinetic studies, however, nonlinear pharmacokinetics is much more frequent than linear pharmacokinetics. This in mainly due to the fact that at very high doses most systems in the body are likely to be stressed and, possibly, saturated to some degree. Thus, additional to the dose proportional increase of exposure (Figure 1) and a less than proportional increase (Figure 2) and a more than proportional increase (Figure 3) is very frequent observed. For a less than proportional increase of exposure a saturation of absorption processes or a concentration dependent change of volume of distribution should be considered as potential causes. For a more than proportional increase of exposure saturation of metabolic elimination pathways, saturation of renal of biliary excretion of parent compound or a concentration dependent change of volume of distribution should be considered as potential causes.

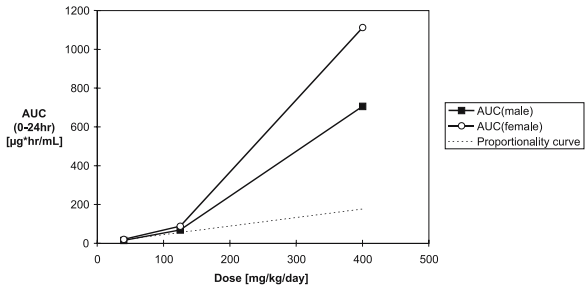

Fig. 3. More than proportional dose dependency of AUC in an oral toxicity study in dog with the test compound C

Chances of Exposure During the Course of the Toxicity Study

According to the ICH Guidance Toxicokinetics 1994, the description of the relationship of exposure to the time course of the toxicity study belongs to the primary objectives of toxicokinetics. This objective may be achieved by derivation of pharmacokinetic parameters from measurements made at appropriate time points during the course of the individual studies. In the short-term studies (1 month or shorter) Day1 and a day at the end of the toxicity study may be appropriate sampling days. In the long-term studies Day1, a day after one third of the study duration and a day at the end of the toxicity study may be appropriate sampling days. Increasing exposure may occur during the course of a study for those compounds which have a particularly long plasma half-life. Conversely, unexpectedly low exposure may occur during a study as a result of auto-induction of metabolizing enzymes. However, other facts can also play a role in changes of exposure during the course of the study. Very often rats and mice were used in an age at which they are not sexual mature and during the study the sexual maturation with their known impact on the rate and extent on metabolism takes place in the first two months. The harm of elimination pathways (e.g. nephro- or hepatoxicity) by the test compound can be another reason for changes in exposure. A more trivial reason such as aging or the change of the administered batch with impact on bioavailability should also be considered.

Gender Differences

According to the ICH Guidance Toxicokinetics 1994, it is normal to estimate exposure in animals of both genders unless some justification can be made for not so doing. For evaluation both genders should be evaluated separately. The assessment of exposure data of the two genders can be performed just by calculation of the ratio of AUC, C_{max} and elimination half-life in males and females. However, additional factors such as for example size of the investigated groups (with respect to random variation) and sexual maturity has to be considered. As a rule of thumb, it can be stated that in rodents a gender difference is quite usual when metabolism is involved as major elimination pathway, whereas in nonrodents distinct gender differences are rather seldom.

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II.0.2 Practical Examples

II.0.2.1 HPLC UV Assay

PURPOSE AND RATIONALE

Levels of drug and/or its metabolite have to be determined in plasma samples collected during the course of a toxicity study in order to evaluate the toxicokinetics of that compound. The UV absorption properties of the compound are used to determine the compound of interest. Since many of the endogenous compounds also show absorption in that wavelength range, a tailored sample work-up has to provide additional selectivity for the compound of interest. In addition, the plasma proteins have to be removed by the work-up in order to avoid blocking of the HPLC columns. Three principles are generally applied to remove the proteins from the sample: Protein precipitation, liquid/liquid extraction and liquid/solid extraction.

PROCEDURE

A typical method using liquid/liquid extraction was described by Shum et al. (1994) for the determination of an ACAT inhibitor in rat plasma. Internal standard,

1 mL water and 1 mL of n-pentane were added to 0.25 mL volume of plasma. After shaking for 15 min and centrifugation for 15 min at approx. 1200 g, the upper organic phase was transferred and evaporated. The residue was reconstituted in 200 μL of mobile phase, and 100 μL was injected onto the HPLC column for analysis. The HPLC system consisted of a Bio_Sil ODS-5S column (150 × 4 mm; 5 μm particle size) and a UV detector operating at 250 nm. The mobile phase consisted of 55 % acetonitrile, 27 % methanol, and 18 % water. The flow rate was 1.9 mL/min. The column temperature was ambient temperature.

EVALUATION

For calibration, a set of eight standards was prepared by fortifying blank plasma with concentrations ranging from 60 ng/mL to 8000 ng/mL. In addition, three sets of quality control samples are prepared of low, medium and high concentration within the range of standards. From the response of the eight standards a calibration curve is determined using the least-square linear regression using a weighting factor of 1/concentration squared. The response is generally the peak height or peak area ratio of analyte/internal standard.

MODIFICATIONS OF THE METHOD

For sample preparation, protein precipitation or solid phase extraction or any combination of the different principles can also be applied. Kim et al. (2002) described a simple toxicokinetic assay for a prodrug and its two consecutively hydrolyzed active antifungal compounds. A 0.2 mL aliquot of dog plasma was added to 0.6 mL methanol containing the internal standard compound in a 2 mL microcentrifuge tube. The tubes were vortexed at high speed and centrifuged at 4500 g for 5 min. The supernatant was transferred into a new tube and stored for a minimum of 12 h at –20 °C to complete the precipitation. The samples were again vortexed and centrifuged. 200 μL of the supernatant were injected onto the HPLC column. In order to cover all three analytes and the internal standard in a reasonable HPLC-run time, a gradient elution on an Ultrasphere ODS column was applied instead of isocratic elution.

An appropriate tool to enhance the selectivity and the feasibility of the work-up procedure using liquid/liquid extraction is the subsequent back extraction into aqueous phase. For example, Los et al. (1996) described a toxicokinetic assay for diltiazem and its two metabolites. To 0.2 mL rat plasma, internal standard solution and a pH 7.3 phosphate buffer and methyl-t-butyl ether were added. The analytes were extracted into the organic phase. The organic phase was transferred and the analytes back-extracted into 250 μL of 0.05 M phosphoric acid. The phosphoric acid was modified with 100 μL acetonitrile before injection onto HPLC.

Kawauchi et al. (2001) described a toxicokinetic assay for a thymidin phosphorylase inhibitor. This compound was water-soluble and not extracted with organic solvents. Protein precipitation failed also due to the appearance of a lot of interfering peaks. Therefore, solid-phase extraction on a strong cation exchange sorbent (= RPS) was used for sample preparation. To 0.1 ml of plasma 50 μL of internal standard solution and 0.7 mL of 0.01 M HCl were added and mixed. The mixed sample was loaded 5 onto a Bond Elut PRS column (1 cc/100 mg) set up at a Vac Elut SPS 24 (Varian) that prior to the sample loading had been conditioned with 1 ml of methanol, and then with 1 ml of water. After passing the sample, the column was washed with 1 ml of water and then with 1 ml of methanol. The eluate was collected with 2 ml of 2 % ammonia solution – methanol in a glass test tube, and dried under a stream of N gas at 37 °C. The residue was reconstituted in 0.2 ml of 10 mM acetate buffer (pH 4.3), and a 60 ml aliquot was injected onto the HPLC column. The HPLC system consisted of a Mightsil RP-18 column (150 × 4.6 mm; 5 μm particle size) and a UV detector operating at 276 nm. The mobile phase consisted of 9 % acetonitrile and 91 % 7 mM sodium 1-hexanesulfonate in 10 mM acetate buffer (pH 4.3). The flow rate was 1.0 mL/min.

CRITICAL ASSESSMENT OF THE METHODS

The described methods are typical HPLC UV assays for drug level determination for toxicokinetic purposes. However, the conditions of sample preparation, the choice of the internal standard substance, the choice of the HPLC stationary and mobile phase and the wavelength of the UV detector have to be adjusted specifically to the properties of the analytes. Particularly, the lipophilicity, the pKa value, and the pH stability of the analytes have to be considered. Regarding selectivity and sensitivity of the assay, HPLC UV assays are not the cutting edge technology. However, in most cases the sensitivity is adequate, since doses, and concomitantly the drug levels, are usually quite high in toxicity studies. A very important aspect for assays used in toxicokinetics is huge range of drug concentration, which can be expected due to very different doses. Therefore, an optimal assay should have a large dynamic range, in order to avoid

time consuming and error prone dilution procedures. The assay in plasma described by Shum et al. (1994) was validated from 0.06 to 8 μg/mL (dynamic range of 133), the assay described by Kim et al. (2002) was validated from 0.05 to 50 μg/mL (dynamic range of 1000), the assay described by Los et al. (1996) was validated from 0.01 to 5 μg/mL (dynamic range of 400) and the assay described by Kawauchi et al. (2001) was validated from 0.05 to 40 μg/mL (dynamic range of 800).

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II.0.2.2 Fluorescence Assay for Analytes with Native Fluorescence

PURPOSE AND RATIONALE

Levels of drug and/or its metabolite have to be determined in plasma samples collected during the course of a toxicity study in order to evaluate the toxicokinetics of that compound. The native fluorescence of the compound is used, to determine the compound with a relatively high sensitivity and selectivity.

PROCEDURE

A typical method was described by Kim et al. (1999). for the determination of a dopamine D receptor antagonist in rat plasma. 0.2 mL volume of plasma was diluted with 1.5 mL of 0.25 M potassium phosphate buffer, pH 8, followed by the addition of 20 μL of the internal standard (1000 ng/mL in methanol), which was a methyl analog of the analyte. The diluted sample was slowly applied on a 1 mL disposable ethyl cartridge which had been successively prewashed with 2 mL of methanol, 1 mL of acetonitrile, 1 mL of water and 1 mL of 0.25 M phosphate buffer, pH 8, using a vacuum manifold. The loaded cartridge was washed with 2 mL of water, then dried by airflow through under vacuum for 5 min. The analyte was eluted from the cartridge with 2 × 0.6 mL of methyl-t-butyl ether (saturated with water and containing 8 % triethylamine). The eluate was evaporated to dryness under nitrogen. The residue was dissolved in 0.4 mL of mobile phase and a 90 μL aliquot was injected onto the HPLC column. The HPLC system consisted of a YMC basic column (150 × 4.6 mm; 5 μm particle size) and a fluorescence detector. The detector was set at an excitation wavelength of 260 nm and an emission wavelength of 400 nm. The mobile phase consisted of 35 % acetonitrile and 65 % 0.05 M ammonium acetate. The flow rate was 1 mL/min. The column temperature was ambient temperature.

EVALUATION

For calibration, a set of five standards is prepared by fortifying blank plasma with concentrations ranging from 5 ng/mL to 100 ng/mL. In addition, three sets of quality control samples are prepared of low, medium and high concentration within the range of standards. From the response of the five standards a calibration curve is determined using the least-square linear regression with or without weighting factor. The response is generally the peak height or peak area ratio of analyte/internal standard.

MODIFICATIONS OF THE METHOD

For sample preparation, protein precipitation or liquid/liquid extraction can also be applied instead of solid phase extraction. Gluth et al. (1988) described for a toxicokinetic assay for Sotalol a threefold combination of these principles. A protein precipitation using 5 M perchloric acid was followed by a liquid/liquid extraction into a mixture of n-pentanol-chloroform 1/3 at pH 9. Thereafter, the organic phase was transferred to another glass tube and the analyte back extracted into 0.05 M sulfuric acid.

For the case that two analytes with quite different native fluorescence and different concentration ranges have to be determined, Chollet et al. (1998) describe an elegant solution. They developed a gradient elution program which generated an elution order suitable for an automated wavelength change in respect to reliable peak integration. The carboxylate and lactone form of Irinotecan and its metabolite were detected at λ_{ex} = 362 nm/λ_{em} = 425 nm and λ_{ex} = 375 nm/λ_{em} = 560 nm, respectively.

CRITICAL ASSESSMENT OF THE METHODS

The described method is a typical HPLC fluorescence assay for drug level determination for toxicokinetic purposes. However, the conditions of sample extraction, the choice of the internal standard substance, the choice of the HPLC stationary and mobile phase and the combination of excitation and emission wavelength has to be adjusted specifically to the properties of the analytes. Particularly, the lipophilicity, the pKa value and the pH stability of the analytes have to be considered.

Regarding selectivity and sensitivity of the assay, HPLC fluorescence assays are clearly preferable to UV assays, however a prerequisite is the native fluorescence of the analytes. A very important aspect for assays used in toxicokinetics is a huge range of drug concentration, which can be expected due to very different doses. Therefore, an optimal assay should have a large dynamic range, in order to avoid time consuming and error prone dilution procedures. The assay in plasma described by Kim et al. (1999) was validated from 5 to 100 ng/mL (dynamic range of 20), the assay in plasma described by Gluth et al. (1988) was validated from 50 to 1500 ng/mL (dynamic range of 30) and the assay in urine was validated from 2 to 100 μg/mL (dynamic range of 50).

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II.O.2.3 HPLC MS/MS Assay

PURPOSE AND RATIONALE

Levels of drug and/or its metabolite have to be determined in plasma samples collected during the course of a toxicity study in order to evaluate the toxicokinetics of that compound. One of the most selective and sensitive assay method is the LC-MS/MS technique. Due to the high intrinsic selectivity of the detection method there is no necessity for a selective or compound specific work-up procedure. However, proteins and matrix interferences for the ionization process have to be removed.

PROCEDURE

A typical semi-automated procedure using solid phase extraction was described (Bakhtiar et al. 2002) for the determination of the anti-leukemia drug Gleevec and its metabolite in monkey plasma. A 0.25 mL volume of plasma was placed in a Packard Multi-Probe I Model 204 automated sample handling system (Packard Instrument company, Meriden, CT). A 96-well plate 3 M Empore C_8-S.D. containing 20 mg of sorbent (Varian Assosiates Inc., Harbor City, CA) was used for the SPE procedure. The SPE procedure involved the following automated steps. Sorbent conditioning using 200 μL of methanol and 200 μL of water, addition of a stable isotope labeled internal standard solution, sample loading to the 96-well plate, mix, wash using 200 μL of 5 % methanol in water, elution using 200 μl of 1 % 1N HCl in methanol. 10 μL sample volume was injected onto the HPLC column for analysis. The HPLC stationary phase was a Waters Symmetry Shield^{TM}-RP_8 column (50 × 4.6 mm; 3.5 μm particle size). The mobile phase consisted of 72 % methanol, and 28 % water containing 0.05 % (by weight) ammonium acetate. The flow rate was 1.0 mL/min. The column temperature was ambient temperature. A Sciex API 3000 triple quadrupol mass spectrometer with atmospheric pressure chemical ionization (APCI) in positive ion mode was used for detection. The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode. The peak areas corresponding to the m/z 494.5 → 394.0 reaction for Gleevec and m/z 480.4 → 394.0 reaction for its metabolite were measured relative to that of the m/z 502.4 → 394.0 reaction of the stable isotope labeled internal standard.

EVALUATION

For calibration, a set of six and five standards was prepared by fortifying blank plasma with concentrations ranging from 1 to 1000 ng/mL and 2 to 1000 ng/mL for Gleevec and its metabolite, respectively. In addition, sets of quality control samples were prepared within the range of standards. From the response of the standards a calibration curve was determined using the least-square linear regression using a weighting factor of 1/concentration squared. The response was the peak area ratio of analyte/internal standard.

MODIFICATIONS OF THE METHOD

For sample preparation, protein precipitation or solid phase extraction or liquid/liquid extraction or any

combination of the different principles can also be applied. Zimmer et al. (2000) described a toxicokinetic assay for the enantiomers of an Alzheimer drug in whole blood using liquid/liquid extraction for sample preparation. A 0.5 mL aliquot of whole blood was dripped into 0.5 mL of 0.74 M orthophosphoric acid. To this, acidified blood sample internal standard and 3 mL of dichloromethane was added and shaken for 10 min. After centrifugation, the lower organic phase was transferred and evaporated. The dry residue was reconstituted in 150 μL of sample solvent for expected low concentration levels (5 to 1000 ng/mL) and in 2 mL for expected high concentration levels (0.2 to 40 μg/mL).

For HPLC MS/MS assays the use of stable isotope labeled internal standards is by far the best method to overcome any potential matrix effects and random variation in the MS/MS detector. If for any reason this stable isotope internal standard is not available, an analog compound with a mass different from the analyte can also be used. The chromatographic retention time of the internal standard, however, should be as close as possible to the retention time of the analyte. This ensures, that time dependent random variation in the ionization chamber, or whereever else in the MS/MS detector, are compensated by the internal standard. In a toxicokinetic assay described by Chi et al. (2003), for example, an internal standard was used which showed the same retention time as the analyte.

CRITICAL ASSESSMENT OF THE METHODS

HPLC MS/MS has been increasingly used to perform bioanalytical determination with maximum selectivity, sensitivity, and throughput. Therefore, the application of HPLC MS/MS is currently considered to be the method of choice for toxicokinetic studies. One important aspect for assays used in toxicokinetics is a huge range of drug concentration, which can be expected due to very different doses. Therefore, an optimal assay should have a large dynamic range, in order to avoid time consuming and error prone dilution procedures. The assay in plasma described by Bakhtiar et al. (2002) was validated from 1 to 1000 ng/mL (dynamic range of 1000) and the assay described by Chi et al. (2003) was validated from 0.005 to 2.5 μM (dynamic range of 500).

A critical aspect, however, is the so-called matrix effect in biological samples. Mass spectrometric detection can be adversely affected by ion suppression (or enhancement) caused by other unknown components within the sample matrix. These matrix effects can lead to quantitatively false results. J Krause has described in this book (chapter II.P.1) how to identify and how to overcome matrix effects.

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II.0.2.4 Immunoassay

PURPOSE AND RATIONALE

Levels of drug and/or its metabolite have to be determined in plasma samples collected during the course of a toxicity study in order to evaluate the toxicokinetics of that compound. For high molecular compounds and/or very low concentration of the analyte immunoassays may be the method of choice to determine the compound in toxicokinetic samples.

PROCEDURE

A typical method was described by Damle et al. (1998) for the determination of BMS-191352 in rat and dog plasma. BMS-191352 is a 67 kDa immunotoxin linked to a monoclonal antibody for the treatment of human carcinomas. The concentration of BMS-191352 was determined by a double antibody sandwich ELISA method. Polystyrene plates (96-well) were coated overnight at 4 °C with 100 μL of the monoclonal capture (coating) antibody [4 μg/mL in phosphate buffered saline (PBS)]. The coating solution was removed and the plates were blocked for 1–2 h with 200 μL of blocking buffer [PBS containing 0.05 % Tween-20 and 3 % BSA]. The plates were rinsed 5 times with wash buffer [PBS containing 0.05 % Tween-20] and incubated for 2 hr with 100 μL of standards, quality control samples or study samples; the final amount of plasma on-plate was 10 %. After rinsing the plates 5 times with wash buffer, 100 μL of biotinylated monoclonal detection antibody [80 g/mL in PBS containing 0.05 % Tween-20 and 0.25 % BSA] was added, and the plates were incubated for 1 h. The plates were then

rinsed 5 times with wash buffer, and incubated for 30 min with 100 μL of diluted Streptavidin/horseradish peroxidase conjugate (1:15 000). Excess conjugate was removed by washing 5 times with wash buffer and the color reaction was initiated by adding 100 μL of tetramethylbenzidine. The color reaction was stopped after 10 min by addition of 100 μL of 1M H_3PO_4. The optical density (OD) was measured within 1 h at 450 nm by a Tecan 340 ATTC reader.

EVALUATION

For calibration, a seven-point standard curve ranging from 2–32 ng/mL in rat or dog EDTA plasma was used. The four-parameter logistic model was used to describe the relationship between the OD readings and nominal concentration (CONC) of the analyte (DeLean et al. 1978):

$$OD = MAX + [(MIN - MAX)/(1 + (CONC/EC_{50})^B)],$$

where, MIN and MAX are the minimum and maximum OD readings, EC_{50} is an estimate of the concentration that yields 50 % of the maximum OD, and B is the slope coefficient.

MODIFICATIONS OF THE METHOD

Instead of the sandwich ELISA-system, which was applied by Damle et al. (1998), Hildebrand et al. (1996) and Audebert et al. (1994), the competitive RIA-system was applied by Wring et al. (1996) and Basu et al. (1996) for toxicokinetic assays. The competitive RIA-system needs only one antibody but this may lead to a loss of selectivity.

CRITICAL ASSESSMENT OF THE METHODS

The described methods are typical immunoassays for drug level determination for toxicokinetic purposes. However, for each analyte specific monoclonal or polyclonal antibodies have to be generated in animals, which takes a relatively long time period. Having these antibodies available, the work up and the assay conditions have to be adjusted for the combination of antibody and analyte (antigen). The use of immunoassays for drug level determination in toxicokinetics is in many cases mandatory for high molecular weight drugs like proteins or therapeutic antibodies. For low molecular weight drugs, however, chromatographic methods show advantages due to their higher selectivity and shorter assay development times. The high sensitivity of the immunoassays can nowadays be reached in many cases by MS/MS-methods too.

A very important aspect for assays used in toxicokinetics is the huge range of drug concentration which can be expected due to very different doses. Therefore, an optimal assay should have a large dynamic range, in order to avoid time consuming and error prone dilution procedures. The sandwich assay in plasma described by Damle et al. (1998) used a standard curve from 2 to 32 ng/mL (dynamic range of 16), whereas the radioimmunoassay described by Wring et al. (1996) was operating in a calibration range from 0.1 to 26 ng/mL (dynamic range of 260).

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Chapter II.P
Bioanalytical Assays – Liquid Chromatography Coupled to Tandem Mass Spectrometry

Joern Krause

PURPOSE AND RATIONALE

Powerful analytical techniques are one key requirement for successful drug research and drug development. The concentration of drugs, pro-drugs and metabolites has to be determined in very diverse matrices such as plasma (blood), urine, faeces and also in different organ tissues (depending on the nature of drug and on the targeted organs).

Gas chromatography in combination with different detection techniques, such as mass spectrometry, FID and others, has been successfully used for this purpose (Jennings 1987) as well as liquid chromatography in combination with UV or fluorescence detection and others (Dorschel 1989, Chu 1999, Jin 2004, Abu-Qare 2001).

However, these techniques have certain limitations. One prerequisite for a successful GC analysis is often the need for one or several chemical derivatization steps, since most analytes are not volatile enough for GC separation. Liquid chromatography in combination

with e.g. UV-, fluorescence- or radioactivity-detectors is usually sensitive and does not require any sample derivatization. The only common drawback in this method is the lack of analyte specificity and in some cases also sensitivity.

PROCEDURE

Ionization

A big step in resolving this issue was achieved by the introduction of Electrospray (ESI)- and Atmospheric Pressure Chemical Ionization (APCI)-mass spectrometry (and recently also atmospheric pressure photo ionization – APPI; Raffaelli 2003) as a detection system for liquid chromatography. These techniques, established in the late 1980s (Lim 2002, Dole 1968, Whitehouse 1985, Cole 1997, Gaskell 1997, Robb 2000) have very rapidly become the method of choice for drug quantification throughout the drug research community and in the pharmaceutical industry (Tiller 2003, Hopfgartner 2003).

The basic principle is a soft ionization (no or almost no fragmentation of the analyte molecules is usually happening during the ionization step) of samples out of the liquid phase (ionization occurs in the liquid phase in case of ESI or in the gas phase after solvent evaporation in case of APCI). Samples are separated and purified using liquid chromatography. The eluent of the LC is transferred into the mass analyzer via a capillary. Usually no sample derivatization is needed. As a result of the ionization process, analytes will form predominantly protonated or deprotonated molecular ions ($[M+H]^+$ or $[M–H]^-$) but also adducts such as $[M+Na]^+$, $[M+NH4]^+$ and other adducts. In addition to that and depending on the size of the molecules, multiple charging might also occur (Lim 2002, Cole 1997, Gaskell 1997).

Mass Analyzers

A large variety of mass analyzers has been tested and is in use for all kinds of bioanalytical assays. The type of detector predominantly used throughout the pharmaceutical community for drug quantification is the triple-quadrupol mass spectrometer. This type of mass analyzer has several key advantages for the analysis of biological samples in combination with liquid chromatography. Triple quadrupol mass analyzers allow for the selection of one or several analytes, which can be filtered in the first quadrupol, allowing only ions of a certain mass to charge ratio to pass this first quadrupol (first mass filter). In a second step, the filtered ions can be fragmented in a collision cell by collisions with background gas molecules. The fragmentation pathway is a characteristic property of a chemical compound or chemical compound class, which allows the use of fragment formation as a fingerprint for a specific compound or compound class. In a third step, the fragments formed during this process can be filtered again in order to let only one specific fragment ion reach the detector of the mass analyzer. In this way, a substantial reduction of background is achieved in combination with a very high selectivity against interference of endogenous compounds in the analyte matrix. Multiple compounds can be analyzed in one run since the instruments are able to switch within milliseconds from one ion/fragment to the other and back. As a result, a separate chromatogram is yielded for each and every analyte, which can be integrated and processed (Venn 2000, Willoughby 1998).

Besides the triple quadrupol instruments, other types of mass spectrometers might be used as well. Examples for these types of instruments are ion traps, time of flight mass spectrometers and also single quadrupol mass analyzers. Due to the characteristic and specific advantages and disadvantages of different instrument types, the overall assay performance (e.g. sensitivity, dynamic range and selectivity) may vary quite a bit from one instrument type to the other.

Internal Standards

In order to compensate for variations during sample analysis (e.g. thermal instabilities, variability in flow rate and also electronic instability in the mass analyzer), samples are usually analyzed together with an internal standard, which is always added to the sample in the same amount. All measured peak areas or peak heights can be normalized on the signal of the internal standard, which helps to eliminate fluctuations during the individual measurement.

Two different types of internal standards are used. The first and usually ideal choice is a stable isotope of the analyte itself. In most cases, ^{13}C or ^{2}H isotopes are used for this purpose. It is important to note that the number of atoms replaced by the stable isotope should be large enough in order not to overlap with the natural isotope distribution of the analyte. The replacement of 6 ^{12}C or 6 ^{1}H atoms by ^{13}C or ^{2}H is usually a good choice for most pharmaceutical analytes ($m/z < 500$). Such isotopes are ideal as internal standards since they will have identical properties in terms of the ionization efficiency and sensitivity, but also in terms of the sample preparation procedure (solubility, extraction rate and so forth). They will have an identical retention time and will therefore correct any fluctuations on the chro-

matography to the best possible extend (Venn 2000). Unfortunately, the synthesis of labelled compounds can be difficult, time consuming and expensive. In case that no stable isotope of the analyte is available, another compound has to be chosen as standard. For this purpose, one should look for a compound with similar structure, preferably from the same compound class, since such a molecule will have the best chance for similar physical and chemical properties like ionization efficiency, retention time and so forth. The difference in retention time should be small in order to correct for fluctuations in the liquid chromatography–tandem mass spectroscopy (LC-MS/MS system). However, in case of multiple analytes this might not be achievable. Therefore it will be necessary for some analytical problems to use more than one internal standard.

LC Conditions

In drug analysis, LC-MS usually means reversed phase liquid chromatography coupled to mass spectrometry. Although normal phase LC can be used as well (especially in combination with atmospheric pressure chemical ionization – APCI), predominantly reversed phase LC is used in drug research and drug analysis due to the typical physical and chemical properties of the analytes (e.g. polarity, size).

Gradients of aqueous and organic mobile phases are typically used for LC-MS/MS analysis of drug compounds and metabolites. The most common aqueous solvents are water with 0.1 % formic acid or 0.1 % acetic acid (v/v) or volatile buffers like 5 mM ammonium-acetate or ammonium-formate. Often adjusted to a certain pH value with the corresponding acid or base (the pH of the eluents will have to be optimized with respect to the polarity of the analytes, since ionic species will have very low or no retention on the reversed pahse LC-columns). Other volatile buffers can be used as well. Phosphate buffers should be avoided, since they will cause suppression of the ionization and thus lead to very bad analytical performance (Venn 2000). Reagents like triethyl-amine should also be avoided as mobile phase or as part of mobile phases. They induce ion suppression as well. In terms of the organic solvents, methanol and acetonitrile are very widely used and they are very well suitable for LC-MS. Other solvents can be used as well, as long as they are compatible with the materials used in the LC-MS system.

The gradients used are typically as short as possible (often less than 5 minutes) in order to realize a short analysis time and high sample throughput. A chromatographic separation of all components is usually not required since the analyzer is mass selective and very specific. The LC-method is mainly necessary for sample clean up, which in most cases means the separation of matrix related compounds from the analyte molecules. In terms of the flow rates, a very wide range can be used. Depending on the instrument used and on the source design, flow rates can typically vary between 10 or 20 μL/min up to 2000 μL/min or more. LC-columns should be selected with respect to the flow rate that is going to be used (inner diameter, particle size and length of the column). A very broad variety of packing materials are in use. However, C18-reversed phase columns are probably the basic standard columns, which are in use (Venn 2000, Willoughby 1998).

Sample Preparation

Sample clean up and sample preparation is a crucial step for a successful analysis. Three major approaches are used on a routine basis in many assays, which have been reported in the literature (O'Connor 2002, Venn 2000).

a) protein precipitation/dilution
b) solid phase extraction (SPE)
c) liquid/liquid extraction

Protein Precipitation

Protein precipitation is a very simple method of sample preparation. The sample (typically a plasma or urine sample) is spiked with internal standard solution and in case of the calibration standard or quality control samples also with analyte solution. In case of unknowns, pure solvent is added instead of the analyte solution. Following this step, samples are diluted with an organic solvent (in most cases acetonitrile or methanol), which leads to protein precipitation. Samples are typically centrifuged after this step and the resulting supernatant is either analysed directly by LC-MS/MS, or a dilution step is implemented prior to sample analysis (Beck 2004, de Jonge 2004, Viberg 2004, Crommentuin 2004, Stovkis 2004b, Hou 2004). In many cases, this kind of sample preparation proofed to be sufficient. However, more advanced sample clean up might be necessary, depending on the matrix and analytes that need to be handled.

Solid Phase Extraction

Another simple and effective method for sample preparation is the solid phase extraction (SPE). In a typical approach, samples will be mixed with aqueous internal standard solution and with a small amount of acid (typically 0.2 % formic acid). The resulting sample will be loaded on to the SPE extraction column (columns need to be conditioned before use, typically by flushing with

methanol and water). After loading the sample on to the column, the loaded column is washed with water. Finally the sample is washed off using an organic solvent combination such as $CHCl_3$/methanol (e.g. 2:1 v/v with 0.1 % formic acid). The resulting sample solution is lyophilized in order to yield the solvent free sample. The dry sample is reconstituted with mobile phase and is now ready for LC-MS/MS analysis. In many cases, an online approach is used as well, where the sample is eluted from the SPE cartridge directly onto the analytical LC-column (Venn 2000, Sottani 2004, Pichini 2004, Ding 1999).

Liquid/liquid Extraction

A general recipe for sample preparation by liquid–liquid extraction is not available, since the necessary procedures (solvents, pH etc.) are depending on the chemical nature of the analyte that needs to be extracted (e.g. pKa; it could be an acidic or basic compound or might be neutral) and of course also on the properties of the matrix that is present. However, when a basic compound needs to be extracted out of plasma samples, the following steps might be appropriate in many cases. Typically in a first step, the internal standard is added to the unknowns. In case of the calibration standards and quality control samples, the blank matrix samples should be spiked with the analyte as well. This will guarantee that all extraction steps following this step will be applied on the standard and analyte. In case of a basic analyte, the sample pH should be basic. This can be achieved by adding e.g. 0.05 % NH_3-solution. In case of acidic compounds, the use of formic acid or acetic acid is recommended. Following the addition of the acid or base, the samples can be extracted with CH_2Cl_2 (or another organic solvent). After shaking and centrifugation, the aqueous phase should be removed and the remaining organic phase (which should contain the analyte) could be evaporated in order to yield the purified dry sample. The sample will then be reconstituted by adding a suitable solvent (e.g. starting mobile phase for the LC) (Stovkis 2004a, Bonato 2003, Baker 2004, Xia 1999, Laurito 2004). In any case, a recovery experiment should be performed in order to assess the efficacy of the extraction procedure.

Recovery can be assessed by comparing the results for an extracted sample of known concentration with an unextracted sample, containing the theoretical concentration (assuming 100 % recovery) in the mobile phase. In cases where fat or fatty tissues need to be analyzed, a washing step for the samples (e.g. with pentane) might be implemented as well in order to remove as much of the fat as possible (Getie 2004). In these cases, one needs to make sure that the analyte is not too lipophilic. Otherwise it might be extracted as well. Methods for the determination of compound levels in different tissues are also often needed. Liquid–liquid extraction is used in these cases very often (Getie 2004, Boner 2003, Barratè 2004, Bogialli 2003, Hows 2004, Ito 2004). Recovery considerations are of special importance in these cases in order to get an idea on the completeness of the compound extraction procedure.

11 Steps of Method Development

1. Compound: Obtain information on the test article: solubility, purity, polarity and stability in order to avoid analytical problems due to compound precipitation or compound decomposition. One should also estimate, which LOQ level will be required for the assay and it should be estimated which calibration range is desirable (the calibration range should reflect the expected sample concentration range)
2. Tune compound on your mass spectrometer: Optimize the intensity of the precursor ion as well as the selected product ion. If necessary, try positive and negative ionization as well as different ionization sources such as Electrospray, APCI or APPI. Usually, the most intense fragment ion is selected as the product ion mass. Make sure, that the selected product ion mass is not too close to the mass to charge ratio (m/z) of the precursor ion (e.g. loss of water, –18, is not characteristic and might question the selectivity of the method). Fragments with very low mass to charge ratio are also less characteristic and might sacrifice specificity
3. Solvent selection: select start solvents for method development. A mixture of 0.1 % formic acid/acetonitrile is usually a good starting point
4. Optimize Chromatography (column, solvents, flow rate, gradient) using a solution of the analyte in mobile phase
5. The response for the selected transition of analyte(s) and internal standard(s) should be optimized by repeated flow injections of a dilute solution in the mobile phase (resulting in a weak signal of may be 10:1 signal to noise) of analyte and standard. All instrument parameters (gas flows, temperature, source position etc.) should be optimized for maximum response according to the specific instrument type that is used
6. Sensitivity in different ionization modes and with different ion sources should be tested as well in order to choose the best setup for the method
7. Sample preparation: Depending on the analyte (SPE, Liquid/liquid, protein precipitation, dilution)

8. Run first matrix samples in order to identify LLOQ, dynamic range, analyte recovery and confirm suitability of the chromatographic setup
9. Tests on sample stability, carry over, specificity, matrix interference, sample stability (freeze/thaw stability and so forth)
10. Run Validation samples (batch to batch reproducibility, within batch reproducibility)
11. Validation Report.

EVALUATION

Validation

LC-MS/MS methods are usually subjected to a validation procedure before they are used for routine analysis. In case of GLP studies or clinical studies, a validation is considered to be mandatory. During the validation procedure, the assay is evaluated with respect to the overall performance. Parameters tested are the limit of quantification (5:1 signal/noise ratio), within batch and inter batch reproducibility (accuracy and precision), recovery, specificity and long-term sample stability in matrix (Shah 2000, EEC Guidance on validation 1994, EEC Guidance on validation 1996, FDA Guidance for Industry 2001).

Sample Analysis (Routine Application of the Assay):

A series of unknown samples is usually measured together with two sets of calibration standard samples (covering the concentration range for the assay, usually two sets of six or more calibration standards) and two or more sets of quality control samples. The calibration standard samples will be used to establish the calibration for the unknowns. Quality control samples (usually at least 5 % of number of unknowns) are matrix samples of known concentration, which are equally distributed over the analytical run (usually two sets of three different concentration levels; 2–3 times the LOQ, mid concentration range and close to the upper limit of quantification). They establish a set of control samples in order to verify the assay performance within the run. Typically, the calibration standards and quality control samples should be within +/–15 % of the nominal value. However, in typical assays, it is considered to be acceptable, if 75 % of the standards are within the +/–15 % criteria. Outliers will not be used for the calculation of the calibration curve. Not all standards at one concentration should be excluded. A similar criteria is applied for the quality control samples: 2 out of 3 of the quality control samples should be within +/–15 % of their nominal value.

CRITICAL ASSESSMENT OF THE METHOD

Matrix Effects

Although there is usually no need for any chemical derivatization, caution has to be applied when LC-MS/MS data are reviewed. The ionization of analytes might be affected and altered by endogenous compounds, which can be present in the matrix and which might coelute together with the analyte or internal standard. This can lead to ion suppression (predominantly observed with ESI ionization) as well as ion enhancement, which more often is observed when APCI-ionization is used. Matrix effects can lead to false results.

Matrix effects are sometimes not obvious to recognize, which is one of the major pitfalls when using LC-MS/MS. The most practical experiment is probably a recovery experiment. A spiked matrix sample is analyzed and the result is compared to a spiked solvent sample. If no matrix effect is present, the same concentration should be found in both samples.

In case that matrix effects are present (or in case that the absence of matrix effects should be shown), samples can be diluted and reanalyzed. Matrix effects are usually concentration dependant. Lowering the sample concentration in many cases helps to minimize matrix effects. If this does not help, other measures have to be taken in order to eliminate matrix suppression. Such measures could be the use of a different sample preparation/sample clean up procedure, change of LC-column or the LC-conditions (gradient, solvents). One important source of matrix effects might be the drug formulation as well. Especially when plasma samples originating from intravenous administration are analyzed, effects of the vehicle (e.g. PEG-400 or Transcutol, Solutol and others), which can be present in the samples of the first time points in substantial amounts, should be considered. These compounds can falsify the analytical results. A reanalysis of the samples in dilution should be considered in order to reveal a potential matrix effect (Dams 2003, Pascoe 2001, Annesley 2003, Hopfgartner 2003, Schuhmacher 2003, King 2000, Liang 2003).

One way to compensate for matrix effects is also the use of stable isotopes as internal standards. Since the standard will coelute with the analyte, the signal suppression or enhancement should have the same effect on analyte and standard, which will usually compensate quite well for the matrix effect.

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II.P.1
Quantification of D-24851 (Anticancer Drug) in Human Plasma and Urine by Liquid–Liquid Extraction and High-Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, Using a Deuterated Internal Standard

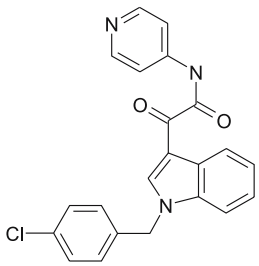
PURPOSE AND RATIONALE

This assay is based on work by E. Stovkis et al. (2004a).

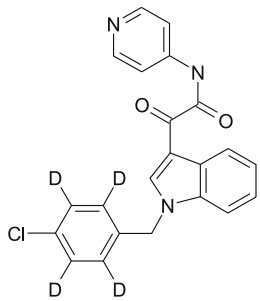
The described assay is used for the quantitative determination of the anti-cancer drug D-24851 (formula) in human plasma and urine in a concentration range of 1–1000 ng/mL (plasma) and 0.25 to 0.50 ng/mL (urine). The calibration range for the urine method is very limited in this assay. The reason for this is the limited solubility of the drug in an aqueous and protein free environment such as urine. Therefore, linearity of the assay was only found up to a maximum concentration of 50 ng/mL.

However, the concentration range could be extended as long as the extended calibration range would also be subjected to a validation procedure similar as described

for the calibration range shown in this assay. In principle, the calibration range used in a method should be selected with respect to the concentration of samples that can be expected and of course also with respect to such important physical properties like solubility.



D-24851



D-24851-D_4

PROCEDURE

Reagents

D-24851 and the internal standards D-24851-D_4 (formula) were obtained from Baxter Oncology (Frankfurt/Main, Germany). Methanol and hexane were used in HPLC supragradient grade quality from Biosleve Ltd. (Amsterdam, Netherlands). Dimethyl sulfoxide (DMSO), ammonium acetate, formic acid (pro analysis quality) and diethyl ether (uvasol quality) were purchased from Merck KgaA (Darmstadt, Germany).

Chemicals, Preparation of Stock Solutions and Working Solutions

Two independent solutions of the test subject were prepared in DMSO (1 mg/mL). One of these solutions was used for the preparation of the Calibration standard samples. The other one was used for making up the quality control samples.

The solution for the calibration standards was further diluted with methanol in order to obtain calibration standards with concentrations ranging from 0.05 to 100 μg/mL (plasma) or 0.3 to 100 μg/mL in urine. These concentrated stock solutions were diluted with the respective matrix in order to obtain the calibration standard samples (8 calibration levels, i.e. 1, 3, 5, 10, 50, 100, 500 and 1000 ng/mL) in case of the plasma samples. For the urine standards, solutions of 0.25 to 50 ng/mL were prepared (8 calibration levels).

The other stock solution was diluted with methanol in order to yield workings solutions with concentrations in the range of 0.3 to 100 μg/mL. Those are the working solutions for the preparation of the quality control samples at three levels (3, 100 and 800 ng/mL in plasma and 0.6, 25 and 40 ng/mL in urine).

All dilutions of the analyte were carried out in a way that the resulting solutions never contained more than 2 % of methanol in order to maintain the integrity of the biological matrix.

A stock solution of the internal standard was prepared as well by dissolving the standard in DMSO to a concentration of 1 mg/mL. The DMSO solution was diluted to 1 μg/mL with methanol for the plasma assay and a solution of 0.1 μg/mL was made up for the assay in urine.

Sample Preparation

- In a first step, 25 μL of the internal standard working solution were added to a 250 μL aliquot of a plasma or urine sample
- Add 1.25 mL of the extraction solvent (hexane/diethylether, 1:1, v/v)
- The resulting samples were mixed for 5 minutes
- Centrifugation of samples for 5 min at 23 100 g
- Freeze the aqueous layer (on the bottom) in an ethanol/dry ice mixture
- Decantation of the organic phase (containing the sample) into a clean tube
- Remove the organic solvent by evaporation, using a gentle stream of nitrogen
- The resulting dry sample was reconstituted in 100 μL of mobile phase (vigorous mixing for 10 min)
- Centrifugation in order to precipitate any undissolved material (5 min, 23 100 g)
- Analysis of a 10 μL aliquot of the resulting clear supernatant phase, using the LC-MS/MS method.

LC-MS/MS System Used and Analytical Conditions

A Perkin Elmer Series 200 pump and a Perkin Elmer ISS200 autosmapler (Perkin Elmer, Norwalk, CT, USA) have been used as the HLPC system, connected to an API 365 triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada). The mass spectrometer was equipped with an Electrospray ionization source.

LC-Column: Inertsil ODS-3, 50X2.0 mm, 5 μM particle size (Chrompack, Middelborough, Netherlands). An in-line solvent filter frit (5 μM from Upchurch scientific was also implemented for column protection.

Mobile Phase:

Solvent A: methanol

Solvent B: 0.004 % formic acid with 5 mM ammonium acetate

Flow Rate: 200 μL/min

Gradient:

An isocratic LC-method was used in this assay:

Solvent composition: 80:20 (solvent A/B). The run time was 5 minutes.

Injection volume: 10 µL

Mass Spectrometric conditions:

Ion source: Electrospray, positive mode.

Source Temperature: 350 °C

Mass transitions (optimized precursor and product ion mass to charge ratio) in positive ion mode:

D-24851: 390 → 268 m/z

D-24851-D: 394 → 272 m/z

The instrument parameters have been optimized for the analysis of D-24851 and the internal standard.

Total analysis time: 5 minutes.

EVALUATION

Linearity: A calibration curve was obtained using the eight calibration standards that were described above. A $1/x^2$ weighted least squares linear regression using the area ratios of analyte/internal standard against the nominal concentration was performed. A regression line was obtained from these data, which was used for back calculation of the concentration for unknowns and quality controls.

CRITICAL ASSESSMENT OF THE METHOD

Precision and Accuracy

The precision and accuracy of the method was assessed by analyzing batches on three occasions (days). Each run included a duplicate set of calibration standards (CS) and a set of quality control samples (5 QC levels in 5 replicates). The following QC levels have been used: 1, 3, 100, 800 2500 ng/mL in plasma and 0.25, 0.6, 25.2, 40 and 128 ng/mL in urine. The highest QC level was above the upper limit of quantification (ULQ). These samples were analyzed after 5-fold dilution in human plasma/urine prior to analysis in order to evaluate the possibility of diluting samples with concentrations above ULQ.

Statistical Results in Plasma

The calculated intra-assay accuracies ranged from 92.8–108.6 % for all concentrations with an overall intra-batch precision lower than 7.3 %. Similar values were obtained for the inter-batch accuracy (92.9–108.7) and precision (1.3–7.3 %).

Statistical Results in Urine

The calculated intra-assay accuracies ranged from 85.9–114.1 % for all concentrations with an overall intra-batch precision lower than 10.5 %. Similar values were obtained for the inter-batch accuracy (91.0–107.3) and precision (3.1–13.2 %).

Specificity, Selectivity and Stability

The selectivity and specificity of the method has been evaluated by the analysis of double blank, blank and LLOQ samples (originating form six independent batches). No coeluting (interfering) peaks have been found.

Samples were stable over three freeze thaw cycles an also when left at ambient temperature for 24 h.

In addition to that, the stability of D-24851 was also assessed in the dry extract (after drying the organic solvent phase) and the compound proofed to be stable for at least three days (in case of plasma sample preparation at 2–8 °C). The dry extract of urine samples was stable for at least 7 days under the same conditions.

Ion Suppression/Recovery

This assay was tested with respect to potential ion suppression effects (matrix effect) as well as with respect to the extraction recovery of the sample preparation procedure.

In order to evaluate a potential matrix effect, blank plasma was extracted and the dry extracts were dissolved in a working solution containing analyte and internal standard. The response for these samples is compared to the results obtained for the pure working solution. The response for the pure working solution is considered to be 100 %. An ion suppression of 16.1 ± 7.66 % was found for the plasma samples and a suppression of 6.89 ± 5.5 % was found for the urine samples.

The extraction recovery was investigated by the comparison of prepared QC samples with extracted blank samples that were reconstituted with the nominal amount of drug and internal standard in working solution.

An extraction recovery of 95.7 ± 9.7 % was found for the plasma samples and a value of 86.5 ± 8.5 resulted for the urine samples.

All of these tests were performed in triplicate at three QC levels.

MODIFICATIONS OF THE METHOD

A similar method using a structure analogue internal standard (D-24843, which is the identical molecules as D-24851 but lacking the chlorine atom), has been developed as well. However, the overall performance of the assay in terms of accuracy and precision was clearly inferior compared to the presented method using the labeled internal standard.

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II.P.2 Quantification of Docetaxel (Taxotere) in Mouse Plasma by Protein Precipitation and High-Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, Using Paclitaxel as Internal Standard

PURPOSE AND RATIONALE

This assay is based on work by W. Hou et al. (2004).

The described assay is used for the quantitative determination of the anti-cancer drug docetaxel in mouse plasma in a concentration range of 25–2500 nM (0.02–2 μg/mL).

PROCEDURE

Reagents

Docetaxel and paclitaxel were obtained from Hande Tech Development Co. (Houston TX, USA) with a purity of 99 %. Methanol and acetonitrile were used in HPLC grade quality from Fisher Scientific (Houston TX, USA). Organic solvents have been filtered using 0.2 μM nylon membrane filters from Whatman (Maidstone UK) with a diameter of 47 mm. Milli-Q deionised water was used in this study. All water based mobile phases were filtered using 0.22 μM GP Express plus membranes from Millipore (Bedford, USA). Formic acid (95 % or higher) from Sigma (St. Louis USA) was used.

Chemicals, Preparation of Stock Solutions and Working Solutions

Stock solutions of the drug and the internal standard were prepared in methanol in a concentration of 2 mM (docetaxel) and 1 mM in case of the internal standard, paclitaxel. A separate stock solution was made up for the preparation of the calibration standards and quality control samples in order to uncover possible errors during the preparation of stock solutions.

These solutions were used for the preparation of the working solutions of docetaxel and paglitaxel. Solutions with a concentration of 250 nM, 1.25, 5, 12.5 and 25 μM docetaxel were prepared as calibration standard working solutions by dilution of the stock solution with a mixture of methanol/water 1:1. For the quality control samples, dilutions with concentration of 500, 4000 and 20 000 nM were prepared in the same way.

A solution with the concentration of 1.5 μM paclitaxel in methanol-water 1:1 was prepared by dilution of the internal standard stock solution in order to obtain an internal standard working solution.

Sample Preparation

Standard and Quality Control Samples

Standard and quality control samples have been prepared in mouse plasma (anticoagulant: heparin) obtained from Biomeda, Foster City, USA.

For all 5 standard solutions and for the quality control solutions, 10 μL of the respective working solution were mixed with 90 μL of blank mouse plasma. The resulting samples were mixed for about 1 minute. As a result of this dilution step, calibration standard solutions with concentrations of 0.25, 1.25, 5, 12.5 and 25 μM and quality control samples with a concentration of 50, 400, 2000 nM were obtained. The spiked samples were used immediately.

Sample Preparation for Standards, Quality Controls and Unknown Matrix Samples

- 40 μL of plasma-sample were pipetted into a 1.5 mL micro-centrifuge tube
- 10 μL of the internal standard working solution were added
- the tubes were closed and mixed for 1 min
- For protein precipitation, 100 μL of a mixture of methanol/acetonitrile (1:1; v/v) were added
- The resulting mixture was mixed again for 30 s in order to allow for protein precipitation
- Precipitated samples were centrifuged for 10 min at 10 000 g
- The supernatant was transferred into Autosampler vials
- 10 μL thereof were injected into the LC-MS/MS system.

LC-MS/MS System Used and Analytical Conditions

LC-10 Advp and controller SCL-10 Avp (Shimadzu Scientific Instruments, USA) connected to a Micromass Quattro Micro mass spectrometer (Micromass, UK). The mass spectrometer was equipped with an Electrospray ionization source.

LC-Column: Waters XTerra MS C18 3.5 μM, 2.1 mm × 50 mm

Mobile Phase:

Solvent A: 0.1 % formic acid in water

Solvent B: 0.1 % formic acid in acetonitrile

Flow Rate: 250 μL/min

Gradient

A linear gradient was used for this assay. The starting solvent was 20 % solvent A and 80 % solvent B. Within 5 minutes, the organic content was increased to 100 % solvent B. Following each injection, the column was equilibrated for 5 min with 20 % solvent B.

Mass Spectrometric conditions:

Ion source: Electrospray, positive mode.

Mass transitions (optimized precursor and product ion mass to charge ratio) in positive ion mode:

Docetaxel: 808.2→ 527.1 m/z

Paclitaxel: 854.2 → 285.9 m/z

The instrument parameters have been optimized for the analysis of docetaxel and paglitaxel.

Analysis time: 10 minutes

EVALUATION

Linearity: A calibration curve was obtained using the five calibration standards that were described above. A 1/x weighted least squares linear regression using the area ratios of analyte/internal standard against the nominal concentration was performed. A regression line was obtained from these data, which was used for back calculation of the concentration for unknowns and quality controls. A very good linearity was found for this method over the concentration range from 25–2500 nM, with a correlation coefficient of $r^2 > 0.997$.

LOD (limit of detection): 8 nM (S/N = 3)

LOQ (limit of quantification): 25 nM

CRITICAL ASSESSMENT OF THE METHOD

Precision and Accuracy

The precision and accuracy of the method was assessed by analyzing batches on three occasions (days). Each run included a duplicate set of calibration standards (CS) and a set of quality control samples (QC). The following number of QC samples has been analyzed in the three batches:

Batch 1: 10 × QC1 (low QC), 5 × QC2 (medium QC) and 5 times QC3.

Batch 2: 5 × QC1 (low QC), 10 × QC2 (medium QC) and 5 times QC3.

Batch 3: 5 × QC1 (low QC), 5 × QC2 (medium QC) and 10 times QC3.

Statistical Results

Intra-batch precision (CV): 9.5 % for the low LQC (low quality control), 5.5 % for the MQC (mid quality control) and 3.9 % in case of the high quality control (HQC).

Similar values were obtained for the inter-batch precision: 9.7 % (LQC), 4.9 % (MQC) and 6.3 for the HQC.

The calculated accuracies ranged from 86.2–122.8 % for the low quality controls and from 88–106.8 for the MQC and finally from 93.6 to 116.5 % for the high concentration quality control samples (HQC).

Typically, 5 sets of all three QC's (low, mid and high) are analyzed on three occasions for establishing intra batch and within batch statistics (Shah 2001). In general, the accuracy for the mean result of the QC samples is expected to be within +/–15 % of the nominal value. However, the exact way of assessing the statistical performance of a method can vary depending on the type of analysis that the assay is going to be used for (e.g. GLP or non-GLP analysis).

Recovery

A recovery experiment is very useful not only in order to gain information on the sample preparation procedure, but also in order to reveal potential matrix effects on the analysis of the plasma samples (Schuhmacher 2003).

For this purpose, the results obtained for the prepared matrix samples have been compared to the data, which were obtained by direct analysis of the standard solutions.

The following recovery values have been found: 100 % (CV 8.5 %, n = 3) for the 50 nM samples. 97.0 % (CV 1.2 %, n = 3) were obtained for the 400 nM samples and 92.2 % resulted for the 2000 nM samples.

MODIFICATIONS OF THE METHOD

A method using isocratic LC-conditions has been developed earlier (Baker 2004).

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II.P.3
Simultaneous Quantification of Flunitrazepam (Rohypnol) and its Major Metabolites in Human Plasma by Solid Phase Extraction and High-Performance Liquid Chromatography Atmospheric Pressure Chemical Ionization Tandem Mass Spectrometry, Using Deuterium Labeled Flunitrazepam as Internal Standard

PURPOSE AND RATIONALE

This assay is based on a work by M. Kollroser et al. (2002).

The described assay is used for the simultaneous quantitative determination of the psychotropic drug flunitrazepam and its major metabolites (7-aminoflunitrazepam and N-desmethyl-flunitrazepam) in human plasma.

Flunitrazepam and 7-amino-flunitrazepam were analyzed with a limit of quantification (LOQ) of 0.001 μg/mL in plasma. In case of N-desmethyl-flunitrazepam, a LOQ of 0.005 μg/mL was achieved.

The described assay covers a dynamic range from 0.001 to 0.1 μg/mL for the drug and for the 7-amino metabolite. In case of the N-desmethyl metabolite, a dynamic range from 0.005 to 0.1 μg/mL was obtained.

PROCEDURE

Reagents

Flunitrazepam and both metabolites were obtained from Promochem (Herts, UK).

The following solvents/reagents were used:

Concentrated formic acid and acetonitrile, both obtained from Promochem (Wesel, Germany) in HPLC grade quality. Isoproanol, ammonia (33 %), methylene chloride and ortho-phosphoric acid (85 %) were supplied by Merck (Darmstadt, Germany) in analytical grade quality.

Milli-Q Plus deionised water was used in this study.

Preparation of Stock Solutions and Working Solutions:
Separate stock solutions of the drug, both metabolites and the internal standard were prepared in methanol by dissolving 1 mg of each compound in 1 mL of solvent. Two separate stock solutions were made up for the preparation of the calibration standards and quality control samples, in order to uncover possible errors during the preparation of stock solutions.

The resulting solutions for the drug and both metabolites were combined and they were diluted down using methanol in order to obtain calibration standard working solutions with concentrations of 0.1, 1 an 10 mg/L (for each analyte).

These three working solutions are used in order to spike drug free human plasma samples (1.5 mL of plasma) to a final concentration of 1, 2, 5, 10, 20, 30, 40, 50 and 100 μg/L of each compound.

The internal standard stock solution was diluted with methanol to a working concentration 1 mg/L.

Sample Preparation for Standards, Quality Controls and Unknown Matrix Samples

- 1 mL of plasma-sample (study sample, quality control or calibration standard) were pipetted into a 1.5 mL micro centrifuge tube
- Add 20 μL of ortho-phosphoric acid
- Add 40 μL of the internal standard working solution (1 mg/L)
- the tubes were closed and mixed for about 1 min
- Following this step, solid phase extraction (SPE) was performed on all samples, using Oasis MCX cartridges (1cc/30 mg from Waters), a vacuum manifold device and a vacuum source (pump)
- All SPE cartridges were conditioned prior to loading the samples by rinsing with 1 mL of methanol and subsequently with 1 mL of water
- Each samples was loaded on a SPE cartridge and was allowed to pass through the cartridge bed at a constant flow rate of approximately 1 mL/min
- Each cartridge was washed using 1 mM of 0.1 N hydrochloric acid and 1 mL of methanol
- Elution of the analytes is achieved with 1 mL of methylene chloride: isopropanol: ammonia (78:20:2)
- The eluent is collected into a glass tube and the samples were evaporated to dryness, aided by a nitrogen stream at 35 °C
- The dried samples were reconstituted in 100 μL of the mobile phase (0.1 % formic acid/acetonitrile, 75:25, v/v) and were transferred into Autosampler vials
- 20 μL of the resulting samples were injected into the LC-MS/MS system.

LC-MS/MS System Used and Analytical Conditions
An LC system from TSP, USA was used. The system consisted of an vacuum degasser, a quaternary pump (P4000) and an Autosampler (AS3000), connected to a LCQ^{DUO} ion trap mass spectrometer (Finnigan MAT, USA), supplied with an APCI source (Brewer 1998).

LC-Column: Symmetry C18, 5 μM, 3.0 × 150 mm (Waters, USA) with a C18 guard column 5 μm, 3.9 × 20 mm (Sentri, from Waters, USA). Column temperature: ambient air temperature.

Mobile Phase:
Solvent A: Acetonitrile
Solvent B: 0.1 % formic
Flow Rate: 600 μL/min
Gradient:
The following gradient was used for all analyses:

Table 1

time [min]	% mobile phas A
0	25
3	25
4	50
8	50
9	25
12	25

Mass Spectrometric conditions:

The instrument parameters have been optimized for the analysis of the analytes as well as for the internal standard.

Ion source: APCI, positive mode

Mass transitions (optimized precursor and product ion mass to charge ratio) in positive ion mode:

Table 2

funitrazepam:	314 → 268 m/z
7-aminoflunitrazepam:	284 → 264 m/z
N-desmethyl-flunitrazepam:	300 → 254 m/z
Flunitrazepam-d_7:	321 → 275 m/z

The instrument parameters (settings for the mass spectrometer) have been optimized in order to achieve the best possible response for the analysis of flunitrazepam, 7-aminoflunitrazepam, N-desmethyl-flunitrazepam and fluhnitrazepam-d_7.

Analysis time: 12 minutes.

EVALUATION

Linearity: A calibration curve was obtained for each analyte using the nine calibration standards that were described above. A least squares linear regression using the area ratios of analyte/internal standard against the nominal concentration was performed for each analyte. A regression line was obtained from these data, which was used for back calculation of the concentration for unknowns and quality controls. A very good linearity was found for this method over the entire concentration range used. The correlation coefficient r^2 was > 0.997 for all three analytes (0.998 for flunitrazepam, 0.997 for N-desmethylflunitrazepam and 0.997 for 7-aminoflunitrazepam.

LOD (limit of detection; S/N = 7):
Flunitrazepam: 0.25 ng/μL
7-aminoflunitrazepam: 0.5 ng/μL
N-desmethyl-flunitrazepam: 2.0 ng/μL
LOQ (limit of quantification):
Flunitrazepam: 1.0 ng/μL
7-aminoflunitrazepam: 1.0 ng/μL
N-desmethyl-flunitrazepam: 5.0 ng/μL

CRITICAL ASSESSMENT OF THE METHOD

Precision and Accuracy

The precision and accuracy of the method (inter and intra batch statistics) was assessed by analyzing batches on five different occasions (days). Each run included a duplicate set of calibration standards (CS) and 5 sets of quality control samples (QC levels of 8, 15 and 50 μg/L).

Statistical Results

Intra-batch precision (CV): ≤ 7.1 % for all three QC levels and for all three analytes.

Similar values were obtained for the inter-batch precision (CV): ≤ 9.2.

The calculated intra- and inter-assay accuracies ranged from 101–107 %, calculated over all analytes and all three QC levels.

Recovery/Matrix-Effects

A recovery experiment is very useful not only in order to gain information on the sample preparation procedure, but also in order to reveal potential matrix effects on the analysis of the plasma samples.

The extraction recovery has been assessed for this assay as well. A very good recovery of > 98 % was found for all analytes, and for all concentration levels of the quality control samples (5, 15 and 50 μg/L). The recovery experiment was done by comparing analyte peak areas that were obtained from plasma samples spiked before the extraction, with equal concentration levels spiked post-extraction.

The possibility of ion suppression or ion enhancement effects (Schuhmacher, 2003) was assessed as well in order to characterize the presented assay. This was done by spiking blank plasma samples (n = 5) post-extraction to a concentration of 15 μg/L of each analyte. The results for these samples were compared with pure eluent samples, spiked to the same concentration. An average signal suppression of less than 1.5 % was found (1.2 % in case of flunitrazepam, 1.3 % for N-desmethylflunitrazepam and 1.5 % for 7-aminoflunitrazepam).

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II.P.4 Simultaneous Quantification of Cyclophosphamide and its Metabolites in Human Urine by Sample Dilution and High-Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, Using a Deuterium Labeled Internal Standard

PURPOSE AND RATIONALE

This assay is based on work by D. Kasel et al. (2004).

The described assay is used for the simultaneous quantitative determination of the antineoplastic drug cyclophosphamide (CP) and three of its major metabolites (N-dechloroethylcyclophosphamide, Carboxyphosphamide and 4-ketophosphamide, abbreviated as DCL-CP, CarboxyCP and 4KetoCP) in human urine.

CP and 4-KetoCP were analyzed with a limit of quantification (LOQ) of 5 ng/mL in urine. In case of DCL-CP, a LOQ of 1 ng/mL was achieved and for CarboxyCP, the LOQ was found to be 30 ng/mL.

A narrow dynamic range was used for this assay which was adapted to the expected concentration levels of the drug and its metabolites in urine (24 h collection period) following the dose levels which were administered in a poly-chemotherapy scenario.

The described assay covers a dynamic range from 3 to 175 μg/mL of CP, 0.5 to 27 μg/mL of 4KetoCP and 0.17 to 9 μg/mL for CarboxyCP and DCL-CP. However, the same assay could be used with a wider calibration range following a validation, which supports the accuracy and precision of the assay over a wider range. In principle, the calibration range should always be selected with respect to the concentration levels which are expected for a certain application (Shah 2000; CPMP/ICH/381/95 1994; CPMP/ICH/281/95 1996, U.S: Department of Health 2001).

PROCEDURE

Reagents

CP hydrochloride, D4-CP, DCL-CP, Carboxy-CP and 4KetoCP were obtained from Asta Medica AG (Frankfurt am Main, Germany).

The following solvents/reagents were used:

Concentrated formic acid was obtained from Merck kGaA (Darmstadt, Germany) in p.a. quality. Acetonitrile and methanol were both ordered from Roth (Karlsruhe, Germany) in HPLC gradient grade quality.

Deionised water, purified using a Milli-Q water purification system was used in this study.

Preparation of Stock Solutions and Working Solutions

Separate stock solutions of the drug, all three metabolites and the internal standard were prepared in water/methanol (70:30, v/v) to a concentration of 8 mg/mL in case of CP and D_4-CP and 0.5 mg/mL in case of the three metabolites.

For this specific application, the stock solutions of all 4 analytes were combined in the ratio of 20:1:1:3 (CP : DCL-CP : CarboxyCP : 4KetoCP) in order to obtain calibration curves with different calibration ranges.

Two separate stock solutions were made up for the preparation of the calibration standards and quality control samples, in order to uncover possible errors during the preparation of stock solutions.

The resulting combined stock solution is diluted down to the desired concentration levels for calibration samples and quality controls with blank urine (drug free urine).

The final calibration standard and quality control sample solutions were stored at – 24 °C.

A working solution of the internal standard (D4-CP) is prepared by dilution of the 8 mg/mL stock solution to a final concentration of 100 ng/mL in water.

Sample preparation for calibration standards, quality control samples and unknown urine samples:

- All frozen urine samples should be allowed to thaw at room temperature
- Samples were mixed well and have been centrifuged at 4 °C for 10 minutes (2000 g)
- 300 μL of the internal standard working solution (100 ng/mL) were added to a 100 μL aliquot of each sample
- 9.6 mL of methanol were added
- All samples were thoroughly mixed for 30 s and centrifuged again at 4 °C for 10 minutes (5000 g)
- 1.5 mL of the clear supernatant liquid phase were transferred into a glass vial

Table 3 Concentration levels of calibration standards

	CP [μg/mL]	4KetoCP [μg/mL]	CarboxyCP [μg/mL]	DCL-CP [μg/mL]
CS1	193.800	28.815	9.677	9.627
CS2	98.000	14.570	4.893	4.868
CS3	35.530	5.282	1.774	1.764
CS4	18.220	2.709	0.910	0.905
CS5	6.502	0.967	0.325	0.323
CS6	3.372	0.501	0.168	0.168
CS7	1.226	0.182	0.061	0.069

Table 4 Concentration levels of quality controls

	CP [μg/mL]	4KetoCP [μg/mL]	CarboxyCP [μg/mL]	DCL-CP [μg/mL]
QC1	174.700	26.859	8.997	8.995
QC2	24.530	3.772	1.264	1.263
QC3	3.845	0.591	0.198	0.198
QC4	3.237	0.498	0.167	0.167

- 10 µL of the resulting samples were injected into the LC-MS/MS system.

LC-MS/MS System Used and Analytical Conditions

An LC system from Thermo Finnigan, USA was used. The system consisted of a quaternary pump (Surveyor series) and an Autosampler (Surveyor series, equipped with a temperature controlled tray and a column oven), connected to a TSQ-Quantum triple quadrupole mass spectrometer, supplied with an electrospray ion source (Thermo Finnigan, San Jose, CA, USA).

LC-Column: Beta Basic C8, 5 µM, 3.0 × 100 mm (Waters, USA) with a C8 pre-column (10 × 3.0 mm). Column temperature: kept at 35 °C.

Mobile Phase:

Solvent A: 0.1 % formic acid in water (v/v)

Solvent B: Methanol

Flow Rate: 300 µL/min

Gradient:

The following gradient was used for all analyses:

Table 5

time [min]	% mobile phas b
0	15
1	15
5	90
7.5	90
8.5	15
11.5	15

Mass Spectrometric conditions:

The instrument parameters have been optimized for the analysis of the analytes as well as for the internal standard.

Ion source: Electrospray (ESI), positive mode

Mass transitions (optimized precursor and product ion mass to charge ratio) in positive ion mode:

Table 6

Cylophosphamide	261 → 140 m/z
D_4-CP	265 → 145 m/z
4KetoCP	275 → 221 m/z
CarboxyCP	293 → 221 m/z
DCL-CP	199 → 171 m/z

The instrument parameters (settings for the mass spectrometer) have been optimized in order to achieve the best possible response for the analysis of CP and its metabolites as well as for the analysis of the deuterated standard, D_4-CP.

Analysis time: 11.5 minutes

A diverter valve has been used. The first 4.5 min LC-effluent of each sample have been directed to the waste.

Retention time of the analytes:

Table 7

Cylophosphamide	7.2 min
D_4-CP	7.2 min
4KetoCP	6.4 min
CarboxyCP	6.6 min
DCL-CP	5.3 min

EVALUATION

Linearity: A calibration curve was obtained for each analyte using the seven calibration standards that were described above. A least squares linear regression (weighted $1/X^2$) using the area ratios of analyte/internal standard against the nominal concentration was performed for CP, CarboxyCP and DCL-CP. A regression line was obtained from these data, which was used for back calculation of the concentration for unknowns and quality controls. In case of 4 KetoCP a quadratic regression had to be used (weighted $1/X^2$ as well).

CP and 4-KetoCP were analyzed with a limit of quantification (LOQ) of 5 ng/mL in urine. In case of DCL-CP, a LOQ of 1 ng/mL was achieved and for CarboxyCP, the LOQ was found to be 30 ng/mL.

CRITICAL ASSESSMENT OF THE METHOD

Precision and Accuracy

The precision and accuracy of the method (inter and intra batch statistics) was assessed by analyzing batches on five different occasions (days). Each run included a duplicate set of calibration standards (CS) and 5 sets of four quality control samples.

Statistical Results

The overall accuracy for this assay was within +/– 6.7 % for CP, 7.0 % for DCL-CP, 5.7 % for 4KetoCP and 13.4 % for CarboxyCP. Intra-day precision was within 2.1 % for CP, and less then 8.3 % for all three metabolites. An inter-day precision within 3.5 % was found for CP, and the inter-day precision of the metabolites ranged from 2.4 to 9.4 %.

Recovery/Matrix-effects/Stability

A recovery experiment is very useful not only in order to gain information on the sample preparation procedure, but also in order to reveal potential matrix effects on the analysis of the plasma samples.

The recovery has been assessed for this assay as well. Samples were prepared in 0.1 % formic acid in the same concentration as in the urine quality control samples.

Very good recovery values in the range of 92.0 to 114.1 % of the nominal value (measured in triplicate and averaged) were found for all analytes and for all concentration levels of the quality control samples.

A recovery experiment performed in this way usually exhibits the sum of two possible effects at the same time. The first effect is a potential loss of sample during any sample preparation step or during sample extraction. The second effect is given by potential signal enhancement or signal suppression during analysis of the samples, caused by interfering endogenous constituents of the matrix. In case of this method, were no extraction was carried out, any loss of sample in the recovery experiment can most likely be considered as a matrix effect. In case of this method, no significant matrix effect was observed.

MODIFICATIONS OF THE METHOD

In order to obtain calibration curves for other ratios of parent drug and metabolites, the ratio can be modified as well. In many cases, all analytes are used in equimolar ratios in order to obtain the same calibration range for all of the analytes. However, the assay calibration procedure has to cover the whole calibration range that is going to be used for each and every analyte.

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II.P.5
Quantification of Hyperforin in Mice Brain by Liquid–Liquid Extraction and High-Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, Using External Calibration (No Internal Standard)

PURPOSE AND RATIONALE

This assay is based on work by Keller et al. (2003).

The described assay is used for the quantitative determination of the anti-depressant drug Hyperforin in mouse brain in a concentration range of 0.25–10 ng/mL (2.5–100 ng/g brain tissue).

Note: This assay was developed without the use of an internal standard. This is somewhat unusual since almost all assays nowadays use an internal standard, in many cases even an isotopically labelled standard. However, the use of an internal standard is not mendatory and is not specifically required by the relevant guidelines (Shah 2000; EEC Guidelines 1994 and 1996; FDA Guidelines 2001). As long as the assay can be validated according to the relevant guidelines,

it is acceptable, although an internal standard usually minimizes the risk of false measurement associated with variations during sample preparation and analysis.

PROCEDURE

Reagents

Hyperforin (containing traces of adhyperforin) was obtained form Dr. W. Schwabe Pharmaceuticals (Karlsruhe, Germany). Methanol and acetonitrile were purchased from Caledon (Georgetown, ON, Canada) in HPLC-grade quality. Purified water from a Milli-Q water purification system (Millipore, Bedford, MA) was used. All other solvents and reagents (Tris buffer, ascorbic acid, formic acid and ammonium formate) were obtained from Sigma-Aldrich (Missisauga, ON, Canada).

Chemicals, Preparation of Stock Solutions and Working Solutions

Stock solutions of the drug (hyperforin sodium salt) were prepared in methanol/water (70:30 v/v) to a final concentration of 100 ng/mL. Dilutions of the stock solutions (as spike solutions) were prepared by dilution of the stock solution with methanol/water (70:30, v/v) to the following calibration standard concentrations: 0.25, 0.5, 0.75, 2.5, 5, 7.5 and 10 ng/mL. A second stock solution of hyperforin should be diluted in order to obtain quality control solutions. The following concentration levels of the drug should be made up: 0.4, 4 and 8 ng/mL.

Note: A separate stock solution was made up for the preparation of the calibration standards and quality control samples in order to uncover possible errors during the preparation of stock solutions.

Sample Preparation

Collection and Treatment of Brain Samples

Brains were obtained form animals, which were sacrificed 3 h post oral dosing of the drug. Brains of untreated (control) animals were used in order to obtain the blank brain homogenates for calibration standard and quality control samples (control animals received the pure vehicle, 0.2 % w/v agarose gel).

- Brain stem and cerebellum were collected from each animal.
- Wash brain carefully with ice cold Tris buffer
- Each brain was weighed separately
- 1 mL of 5 mM Tris-HCl buffer (pH 7.4) per 100 mg of brain were added to the brain samples
- Samples were homogenized
- Brain homogenates were stored at –20 °C until further use.

Sample Preparation for Standards, Quality Controls and Unknown Matrix Samples

- Standard and quality control samples have been prepared by spiking mouse brain homogenates with the calibration standard or quality control spike solution.
- 50 μL of the corresponding spike solution (calibration standard or quality control solution) were added to a 450 μL aliquot of blank brain homogenate. In case of the unknown samples, 500 μL of the brain homogenate were used
- In a next step, 50 μL of an ascorbic acid solution (200 mg/mL) were added and the resulting mixture was mixed for 1 minute
- Following this step, 750 L of ethyl acetate were added in order to extract hyperforin form the homogenate
- The resulting biphasic mixture was mixed for 2 min, followed by 5 min centrifugation at 13499 pm
- An aliquot (always the same volume) of the organic layer was transferred to a clean vial and was evaporated to dryness using the aid of a gentle nitrogen stream (30 °C)
- The dry residue was reconstituted using 200 L of methanol/water (70:30, v/v)
- In a last step, the resuting samples were mixed again for 1 min followed by sonication and centrifugation for 1 min
- 20 L of the sample obtained in this way were injected into the LC-MS/MS system.

LC-MS/MS System Used and Analytical Conditions

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-system was used, connected to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems MDS Sciex, Concord, ON, Canada). The mass spectrometer was equipped with an Electrospray ionization source (negative ion mode).

LC-Column: Merck (Darmstadt, Germany) Chromolith Performance Rod 100 mm, i.d. 4.6 mm

Mobile Phase:

Solvent A: water, containing 3.5 mM formic acid and 2 mM ammonium formate

Solvent B: acetonitrile

Flow Rate: 3 mL/min.

Gradient

An isocratic LC-method was used in this assay:

Solvent composition: 12:88 (solvent A/B). The run time was 2 minutes.

Injection volume: 20 μL

Mass Spectrometric conditions:

Ion source: Electrospray, negative mode

Mass transitions (optimized precursor and product ion mass to charge ratio) in negative ion mode:

Hyperforin: 535 → 383 m/z

The instrument parameters have been optimized for the analysis of hyperforin. Both quadrupoles (Q1 and Q3) were operated in unit resolution.

Analysis time: 2 minutes.

EVALUATION

Linearity: A calibration curve was obtained using the five calibration standards that were described above (concentration range from 0.25 to 10 ng/mL). A least squares linear regression using the area of the analyte against the nominal concentration was performed. A regression line was obtained from these data, which was used for back calculation of the concentration for unknowns and quality controls. A very good linearity was found for this method over the concentration range from 0.25–10 ng/mL, with a correlation coefficient of $r^2 > 0.998$.

LLOQ (lower limit of quantification): 250 pg/mL

CRITICAL ASSESSMENT OF THE METHOD

Precision and Accuracy

The precision and accuracy of the method (inter and intra batch variability) was assessed by analyzing batches on 3 different occasions (days). Each run included 3 sets of three quality control samples.

Statistical Results

Intra-day precision ranged from 4.6 to 10.6 % for hyperforin. The corresponding accuracies ranged from 4.3 to 8.4 %. The following results were found for the inter-day precision: 6.7 to 12.2 %. The inter-day accuracy was in the range from 2.0 to 5.0 %.

Recovery/Matrix-Effects/Stability

A recovery experiment is very useful in order to gain information on the sample preparation procedure. One very important question is the recovery rate for different concentration levels of the drug.

The recovery has been assessed for this assay in the following way: 5 replicates of all three QC levels have been prepared according to the sample preparation recipe, which is given in this assay. The resulting data are compared to blank brain extracts (again 5 replicates), which were spiked with the analyte to the expected concentration level after the extraction procedure. In this way, the completeness of the extraction process can be determined.

An average recovery of 73.5 % has been found (75.3 % for the 0.4 ng/mL concentration, 71.4 % for the 4 ng/mL and 73.8 % for the high QC sample). Of course, it is desirable to achieve the best possible extraction rate, in the ideal case 100 %. However, the most important criteria is the variability of the recovery. In this case, the recovery is very similar (precision of the recovery is 2.7 %) over the whole calibration range.

Sample stability was also assessed. No stability issue was found when spiked brain homogenates were stored at room temperature (4 h), or in the processed samples over 48 h. The stability of hyperforin was also tested over three freeze-thaw cycles, which again did not reveal any significant degradation of the drug.

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II.P.6
Quantification of Simvastatin and Simvastatin Acid in Human Plasma by Direct Injection High-Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, Using Lovastatin and Lovastatin Acid as Internal Standards

PURPOSE AND RATIONALE

This assay is based on work by Jemal et al. (2000).

The described assay is used for the quantitative high throughput determination of the cholesterol-lowering drug simvastatin in human plasma. Simvastatin is orally administered as a lactone prodrug, which hydrolyzes in vivo to the active drug (simvastatin acid). A calibration range from 0.5 to 200 ng/mL plasma is covered by the assay (for both pro-drug and drug). This assay utilizes on-line sample cleanup using a switching valve and a combination of an extraction column with an analytical column. No sample pre-treatment such as protein precipitation or liquid-liquid extraction is required (Jemal 1998 and 1999).

PROCEDURE

Reagents

Simvastatin and lovastatin (internal standard) were obtained from U.S.P. Both hydrolyzed compounds (simvastatin acid and lovastatin acid) were obtained from Bristol-Myers Squibb Pharmaceutical Research Institute. Acetonitrile (in HPLC grade quality) as well as formic acid (88 %) were ordered from VWR Scientific (Piscataway NJ USA). In addition to that, glacial acetic acid and sodium acetate trihydrate (both ACS grade) were purchased from Baker JT (Phillipsburg NJ USA). Purified water from a Milli-Q water purification system (Millipore Bedford MA USA) was used. Blank plasma was obtained form Bioreclamation (Meadow E, NY USA).

Chemicals, Preparation of Stock Solutions and Working Solutions

A 0.1 M sodium acetate buffer (pH 4.2) was prepared by dissolving 13.6 g of the salt in 1000 mL of purified water. The pH of 4.2 was adjusted by addition of glacial acetic acid.

A 3.0 mM formic acid solution was made up by dissolving 128 µL of the 88 % formic acid in 1000 mL of water. This is the mobile phase A.

The 0.3 mg/mL stock solutions of the pro-drug (simvastatin) was prepared in acetonitrile. An analogue solution in water/acetonitrile (90:10, v/v) was prepared with simvastatin acid.

The stock solutions of the internal standards lovastatin and lovastatin acid were prepared in the same way (0.3 mg/mL). Combined working solutions of the analytes (5.0 µg/mL) were prepared by dilution with acetonitrile. Two completely separate sets of stock solutions and dilutions were made up for the preparation of working solutions for calibration standards and quality control samples.

The highest plasma standard (200 ng/mL) was made up by adding a calculated amount of the 5.0 µg/mL working solution to blank plasma. All other standards (100, 50, 25, 10, 5, 1 and 0.5 ng/mL) were prepared by diluting the 200 ng/mL sample with blank plasma.

Quality control samples were made up containing three different ratios of simvastatin / simvastatin acid: One group contained both compounds in a ratio of 1:1 (1.5, 80, 160 and 500 ng/mL of each compound). The second series contained both drugs in a ratio 9:1 and 1:9 (72.0/8.0 ng/mL or vice versa). A third group contained only one of the analytes at a concentration of 80.0 ng/mL.

An internal standard working solution was made up by dilution of the stock solutions with sodium acetate buffer (0.1 M, pH 4.2) to a concentration of 200 ng/mL.

Sample Preparation for Standards, Quality Controls and Unknown Matrix Samples

- 25 µL of the internal standard working solution were added to 100 µL of each plasma sample, calibration standard or quality control sample resulting in an internal standard concentration of 50 ng/mL.
- The resulting mixture was mixed again for 30 s
- Samples were centrifuged for 5 min in order to remove any particles from the samples
- 10 µL of the supernatant were injected into the LC-MS/MS system.

LC-MS/MS System Used and Analytical Conditions

A Shimadzu liquid chromatography system was used (Shimadzu Scientific Instruments, USA). The system consisted of a Shimadzu SCL-10A VP system controller with four LC-10AD VP pumps. A Perkin-Elmer series 200 autosampler, equipped with a cool-tray (Perkin Elmer, Norwalk, CT, USA) was used for sample injection. Moreover, a six-port switching-valve was implemented in order to switch between an extraction column and the analytical column. Samples were injected onto the extraction column (Oasis HLB column). After a short extraction/equilibration time of 0.3 min, the samples were eluted from the extraction column onto the analytical column (Symmetry C18) (Figure 1).

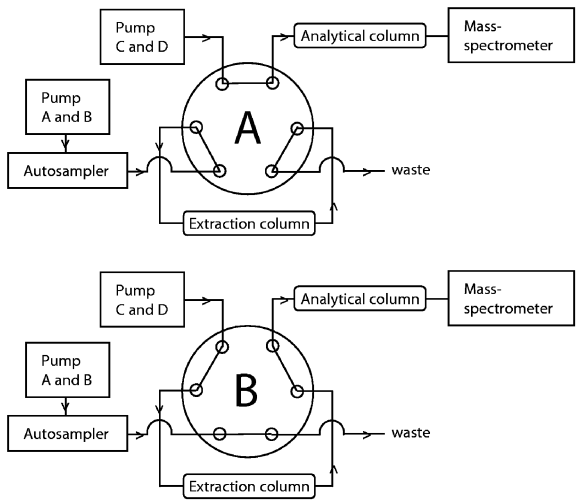

Fig. 1.

For mass spectrometric detection, a TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT, USA) equipped with an Electrospray ionization source was.

LC-Columns:

Extraction column: Oasis HLB column

(1 × 50 mm, 30 μm from Waters, Milford, MA, USA)

Analytical column: Symmetry C18

(3.9 × 50 mm, 5 μm from Finnigan, San Jose, CA, USA)

Mobile Phase:

Sample cleanup solvent system (used for sample loading onto the extraction column and for flushing/conditioning of the extraction column):

Solvent A: 90 % 3.0 mM formic acid in water

Solvent B: 10 % 3.0 mM formic acid in acetonitrile

Flow Rate: 4000 μL/min

Duration: 0.3 min (valve position A)

The elution of the compounds from the analytical column (using solvent system C and D) was combined with a parallel washing and conditioning cycle for the extraction column:

Table 8

time [min]	flow [mL/min] pump A	pump B
0.0	3.6	0.4
0.3	3.6	0.4
0.4	0.005	0.095
1.0	0.0	4.0
1.5	3.6	0.4
2.0	3.6	0.4
2.01	0.09	0.01
2.5	0.09	0.01

The solvent flow is reduced in steps 3–4 and 7–8 in order to save solvent.

Sample analysis solvent system (used for eluting samples from the extraction column and subsequent mass spectrometric analysis):

Isocratic run

Solvent C: 25 % 3.0 mM formic acid in water

Solvent D: 75 % 3.0 mM formic acid in acetonitrile

Flow Rate: 1000 μL/min (a solvent split was used, allowing only 500 μL/min of the LC-eluent to enter the mass spectrometer)

Duration: 2.5 min (valve position B).

Ion source: Electrospray, positive mode.

Mass transitions (optimized precursor and product ion mass to charge ratio) in positive ion mode:

Table 9

Simvastatin	419 → 285 m/z
Simvastatin acid	437 → 303 m/z
Lovastatin (int. std.)	405 → 199 m/z
Lovastatin acid (int. std.)	423 → 285 m/z

The instrument parameters have been optimized for the analysis of all four compounds.

Analysis time: 2.5 minutes.

EVALUATION

Linearity: A calibration curve was obtained using the eight calibration standards that were described above. A 1/x weighted least squares linear regression using the area ratios of analyte/internal standard against the nominal concentration was performed. A regression line was obtained from these data, which was used for back calculation of the concentration for unknowns and quality controls.

LOQ (limit of quantification): 0.5 ng/mL

CRITICAL ASSESSMENT OF THE METHOD

The performance of the presented bioanalytical assay method was assessed following the guideline for biological method validation presented by Shah et al. (Shah 2001).

In addition to the tests recommended by Shah et al., the accuracy of the lowest level of quantification has been evaluated for this assay by analyzing six different spiked LOQ samples (from different plasma batches) against a standard curve. The mean CV found for simvastatin in this test is 7.2 %. A value of 18 % was obtained for simvastatin acid.

Precision and Accuracy

The precision and accuracy of the method (inter and intra batch variability) was assessed by analyzing batches on 3 different occasions (days). Each run included 5 sets of eight quality control samples (4 QC samples containing equal amounts of both drugs, 2 QC samples containing the drugs in a ratio of 1:9 and 9:1 and two QC samples containing only one of the drugs), as described above.

Statistical Results

The intra-day precision ranged from 4.0 to 6.6 % for simvastatin (4.5 to 9.7 % for simvastatin acid). The corresponding accuracies ranged from –9.3 to 0.4 % for simvastatin and from –8.4 to 2.0 % for simvastatin acid. The following results were found for the inter-day precision: 0 to 6.8 % for simvastatin and 0 to 3.8 % fro simvastatin acid. These values also include the results obtained for the 1:9 and 9:1 ratios in the quality control samples. As a conclusion, the presence of different amounts of simvastatin does not influence the results for simvastatin acid and vice versa.

Recovery/Matrix-Effects/Stability

A recovery experiment is very useful in order to gain information on the sample preparation procedure and

on possible interfering compounds during sample analysis. One very important question is the recovery rate for different concentration levels of the drug.

The recovery has been assessed for this assay in the following way: The response for spiked QC plasma samples (1.5, 80 and 160 ng/mL) was compared to the response obtained for a spiked mixture of acetonitrile/0.1 M sodium acetate solution (1:1, v/v). The recovery of the internal standards was assessed in the same way (50 ng/mL). Recoveries for simvastatin and simvastatin acid were found to be $\geq$ 75 % and $\geq$ 38 % at all concentration levels tested. For the internal standards, recoveries of 52 % (lovastatin) and 57 % (lovastatin acid) were determined.

Note

The recovery experiment carried out for this assay resembles somehow the sum of two potential effects: The loss of compound during the sample preparation process (in this case on-line extraction) and a potential matrix effect during sample analysis. However, since an online sample clean up procedure is used here, these two potential effects cannot be separated in the usual way (One experiment would be the comparison of a processed spiked plasma sample with a blank plasma sample spiked post sample processing in order to determine the recovery of the sample preparation step. In a second experiment, again a processed blank plasma sample would be spiked post processing and the result would be compared with the response of a spiked solvent sample in order to reveal a potential matrix effect during analysis).

Sample stability was also assessed. Both compounds (simvastatin and simvastatin acid) were stable for at least 2 months at –70 °C and also for at least three freeze-thaw cycles. Processed samples also proofed stability over 24 hours at 4 °C. Some degradation of simvastatin was found within 24 hours at room temperature (between 23 and 39 %). For simvastatin acid on the other hand, the found concentration increased within 24 h at room temperature (+ 23 to + 29 %), indicating that a fraction of simvastatin was hydrolyzed within 24 h at room temperature.

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Chapter II.Q
Bioanalytical Assays – Gas Chromatography

Dietmar Schmidt

INTRODUCTION

The quantitative determination of analytes in biological matrices such as blood, urine, etc. is called bioanalysis. Regulatory authorities and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidance requires that the concentration-time profiles of drugs and/or metabolites in man or in animals are studied so their respective pharmacokinetics can be calculated and used as a basis for the evaluation of preclinical (especially toxicological) and clinical studies.

Gas chromatography (GC) is a chromatographic technique that is used to separate volatile organic compounds. A gas chromatograph consists of a mobile (gas) phase, an injection port, a separation column containing the stationary phase, and a detector. The organic compounds are separated due to differences in their partitioning behavior between the mobile gas phase and the stationary phase in the column. Mobile phases are generally inert gases such as hydrogen, helium, argon/methane, or nitrogen. The upper part of the injection port is sealed with a rubber septum through which a syringe needle is inserted to inject the sample. The injection port is maintained at a higher temperature than the boiling point of the least volatile component in the sample mixture. Since the partitioning behavior is dependent on temperature, the separation column is positioned in a programmable, thermostat-controled oven. Starting at a low oven temperature and increasing the temperature over time to elute the high-boiling point components accomplish separation of analytes showing a wide range of boiling points.

Gas chromatography columns are of two different designs: Either packed or capillary. Packed columns are typically glass or stainless steel coils (typically 1–5 m total length and about 5 mm inner diameter) that are filled with the stationary phase. Capillary columns are thin fused-silica (purified silicate glass) capillaries (typically 5–50 m in length and 250 μm inner diameter) that have the stationary phase coated on the inner surface. Capillary columns provide much higher separation efficiency (a typical 25 m wall-coated open tubular GC column gives about 50,000 theoretical plates[1]) than packed columns (an average packed column of 5 m has only 5000 theoretical plates), but are more easily overloaded by too much sample.

After the components of a mixture are separated using gas chromatography, they must be detected as they exit the GC column. There exist a number of detectors, which can be used in gas chromatography. Different detectors give different types of selectivity: *Non-selective* detectors like the thermal-conductivity

[1]The number of theoretical plates is the number of discrete distillations that would have to be performed to obtain an equivalent separation. This number is commonly used as a measure of separation efficiency and is a valuable number to use when comparing the performance of various chromatographic columns.

(TCD) detector or the flame-ionization (FID) detector, which are the two most common detectors in gas chromatography, respond to all (organic) compounds except the carrier gas. On the contrary, a *selective detector* responds to a range of compounds with a common physical or chemical property. Representatives of the latter group of detectors are the nitrogen-phosphorus detector (NPD), the electron capture detector (ECD), the mass selective detector (MSD) and – last, but not least – the tandem mass spectrometer (MS/MS).

However, gas chromatography is not usually the method of choice in the bioanalytical field because it requires the vaporization of the analytes, and – unfortunately – most analytes in bioanalysis are not very volatile. This low volatility may result from the sheer size of the molecules and thus from large dispersion forces holding the molecules together (Knapp 1979). For smaller molecules (as for the many drug candidates) the observed low volatility may result from strong intermolecular attraction between polar groups (e.g. N–H, O–H and S–H groups that can undergo hydrogen bonding).

It is beyond the scope of this article to describe the theory of GC, for a detailed description the reader is referred to one of the many textbooks devoted to this topic, but the textbook *Analytical Gas Chromatography* (Jennings 1987) with its detailed discussion of capillary GC is strongly recommended.

As mentioned earlier, given the limitations of GC, alternative methods such as high pressure liquid chromatography (HPLC) with ultra-violet (UV) or with fluorescence (FL) detection and – particularly in the past few years – high pressure liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) have evolved as the major bioanalytical techniques for the bioanalysis of analytes in biological matrices. Nevertheless, due to the inherent advantages of modern fused silica capillary columns in terms of resolution, inertness, and detection limits, in combination with different detectors like the flame-ionization detector (FID), the nitrogen-phosphorus detector (NPD), the electron capture detector (ECD), the mass selective detector (MSD) or even the tandem mass spectrometer (MS/MS), GC continues to play a role in modern bioanalytics (Venn 2000). An example of the implementation of each of the above-mentioned GC detectors will be discussed in this article but necessarily not in the referred order.

To overcome the already mentioned low volatility of a number of analytes, one approach has been to generate more volatile derivatives before the actual bioanalysis. Although derivatization cannot increase the volatility of large molecules, for smaller molecules, masking of polar groups by derivatization can yield dramatic increases in volatility. Polar N–H, O–H and S–H groups that can undergo hydrogen bonding contribute significantly to intermolecular attraction, and thus low volatility. Replacement of hydrogen in these groups by alkylation, acylation, or silylation significantly increases volatility, especially in compounds with multiple polar groups. The monosaccharides are a prime example of a group of relatively low molecular weight compounds that exhibit low volatility even up to temperatures at which they begin to decompose. Replacement of the active hydrogens with trimethylsilyl groups yields volatile products that readily undergo GC analysis (Knapp 1979). Carboxylic acids are another molecule class, which can easily be derivatized using diazomethane (CH_2N_2) to yield the respective methyl-esters, which often are volatile enough to allow the use of GC. In 1984, Hajdù et al. introduced a specific GC-assay, which allowed the rapid analysis of ramipril, an angiotensin converting enzyme (ACE) inhibitor, in human urine. To our knowledge, this was the first time, that an ACE inhibitor bioanalysis was performed directly using GC and not by either radioimmunoassay (RIA) (Hitchens et al. 1981 and Ribeiro et al. 1996) or a discontinuous enzyme assay (Horiuchi et al.; Tocco et al., both 1982).

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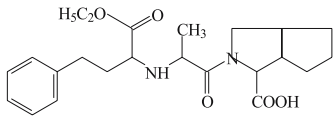
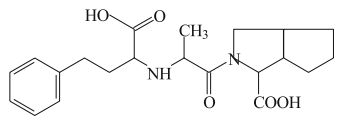
II.Q.1
Urinary Analysis of Ramipril Using Gas Chromatography with Nitrogen-Phosphorus-Detection (GC-NPD)

PURPOSE AND RATIONALE

This assay is used for the quantitative determination of the angeotensin converting enzyme (ACE) inhibitor ramipril, (2-[N-[(S)-1-Ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(1S,3S,5S)-2-azabicyclo[3.3.0]octane-3-carboxylic acid), and its pharmacologically active metabolite, ramiprilat, in human urine using capillary gas chromatography with nitrogen specific detection (NPD).



Ramipril



Ramiprilat

Note: The nitrogen-phosphorus detector responds to nitrogen-phosphorus compounds about 100 000 times more strongly than normal hydrocarbons. Due to this high degree of selectivity, the NPD is commonly used to detect pesticides, herbicides, and drugs.

The NPD is similar in design to the FID (flame ionization detector), except that the hydrogen flow rate is reduced to about 3 mL/min, and an electrically heated thermionic bead (NPD bead) is positioned near the column orifice. Nitrogen or phosphorus containing molecules exiting the column collide with the hot bead and undergo a catalytic surface chemistry reaction. The resulting ions are attracted to a collector electrode, amplified, and output to the data system. The NPD is 10–100 times more sensitive than FID.

Reagents

Ramipril, ramiprilat, and the internal standard were supplied by Hoechst AG[2] (Frankfurt, Germany). Analytical grade reagents were used at all times, methanol and chloroform were redistilled before use. Vac Elut and Bond Elut C18 and Si extraction columns are proprietary products manufactured by Varian Incorporated (Harbor City, CA; USA) and obtained from ICT (Frankfurt/Main, Germany). Dimethyldichlorosilane (DMCS) and trifluoroacetic anhydride (TFAA) were purchased from Macherey-Nagel & Co. (Düren, FR Germany) and used without further purification. N-Methyl-N-nitroso-p-toluene-sulfonamide (Diazald), for diazomethane generation, was supplied by Aldrich (Milwaukee, WI, USA). As internal standard 2-[N-[(S)-1-Ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-spiro-[5,6]-2-azadecane-3-carboxylic acid was used.

- Buffer solution pH = 1.0: 3.73 g KCl and 134 mL 0.1 mol/L HCl were filled up to 1 L with redistilled water
- Water pH = 3.3: Redistilled water was adjusted to pH = 3.3 with buffer solution 1.0
- Elution mixture: Redistilled chloroform and methanol were mixed in the ratio 2:1 (v/v)
- n-Hexane/toluene: n-Hexane and toluene were mixed in the ratio 1:3 (v/v)
- Diazomethane: Ethereal diazomethane solution was prepared by the diazomethane generator kit.

Assay

The need to isolate the analytes from the biological matrix in a form suitable for GC bioanalysis constitutes the most labor-intensive step. The isolation procedure is largely independent of the chromatographic technique, i.e. is not specific to the employed GC-method. The only GC-specific requirement is that the final extract is obtained in a relatively volatile solvent and, because of injection volume constraints, in the smallest practical volume (about 50 μL). For the extraction of ramipril and of ramiprilat from urine, disposable C18 columns were used. After derivatization with diazomethane (Albro et al. 1969), the sample was purified by the use of a Si column, to eliminate a number of endogenous impurities from urine, which are not removed during purification over the C18 column. After a second derivatization step with trifluoroacetic anhydride (Walle et al. 1972), both compounds were analyzed in form of their methyl ester-, trifluoroacetyl derivatives by capillary gas chromatography using a nitrogen specific detector.

PROCEDURE

1. To prevent adsorption of the compounds at the glass surface, the centrifuge tubes are treated with 2 mL n-

[2]Successor company: Aventis Pharma Deutschland GmbH

hexane/DMCS (5 %) for at least 10 min prior to use, rinsed with methanol and subsequently dried.
2. To 1 mL urine in a centrifuge tube, 2 μg internal standard dissolved in 50 μL redistilled water are added and then adjusted to pH = 1.4–1.5 with 0.1 mol/L HCl
3. the sample thus prepared is applied onto a C18 column – which has been preconditioned with methanol (6 mL) followed by redistilled water (6 mL) and 0.1 mol/L HCl (3 mL) –, and slowly sucked through under vacuum (using the Vac Elut System at about 20 kPa pressure drop)
4. subsequently, the column is washed with 4 × 3 mL redistilled water at the same reduced pressure
5. the pressure drop is increased to 50–60 kPa and the column is sucked dry for 10 min
6. the column is placed into a centrifuge tube and the sample is eluted with 2 × 0.75 mL elution mixture
7. the column is dried by centrifuging at 4000 rpm for 1 min, whereby the entire eluate is collected in the tube
8. the eluate is methylated by adding 0.5 mL ethereal diazomethane solution at room temperature for about 10 min and subsequently evaporated to dryness at 40 °C under N_2 gas
9. the residue is taken up in 100 μL methanol followed by adding 1 mL n-hexane/toluene
10. the sample is applied onto a Si column – which has been preconditioned with methanol (5 mL) followed by chloroform (5 mL) and n-hexane (5 mL) –, and slowly sucked through under vacuum (20 kPa pressure drop)
11. the pressure drop is increased to 50–60 kPa and the column is sucked dry
12. the column is placed into a conically tapered centrifuge tube and the sample is eluted with 2 × 2 mL chloroform, and
13. the eluate is evaporated to dryness at 60 °C under N_2 gas
14. the residue is taken up in 250 μL ethyl acetate, treated with 1 mL n-hexane/TFAA (5 %), and
15. for derivatisation, the stoppered tube is placed into a heating block of 60 °C for about 30 min
16. subsequently the sample is evaporated to dryness at 60 °C under N_2 gas and taken up in 50 μL toluene, 2 μL of which are injected.

GC-System

Hewlett Packard 5880 gas chromatograph with nitrogen specific detector (NPD), (Hewlett–Packard, Palo Alto, CA, USA).

Column: Crosslinked fused silica capillary column, Hewlett–Packard OV 101, 12 m × 0.21 mm inner diameter (ID);
Injection: Split mode;
Gases: Carrier gas (helium); inlet pressure 10 p.s.i.g.
Split ratio: 1:20 mL/min;
Septum rinsing (helium): 3 mL/min;
Detector make-up (helium): 20 mL/min;
Detector (hydrogen): 3 mL/min;
Detector (air): 60 mL/min;
Temperatures: Injection port 250 °C;
Detector 300 °C;
Oven program:
Initial temperature: 160 °C
Heating rate: 16 °C/min;
Final temperature 260 °C (4 min);
Detection: NPD detector;
Sample size: 2 μL.
Analysis time: about 12 min per sample.

EVALUATION

Ramipril and ramiprilat were admixed with human urine in concentrations of 0.050 μg–10.000 μg/mL. The urines were divided into 5 aliquots and analysed.

Precision: The standard deviation (SD) is used as a measure of precision; the respective data are as follows:

For values less than 1.000 μg/mL the precision was 2.4 % of the measured value for ramipril, and constant 0.003 μg/mL for ramiprilat.

From 1000 μg/mL upward, the precision can be formally divided into an absolute and a relative error term:

Ramipril: 0.01 ± 2.1 % of the measured value;

Ramiprilat : 0.033 ± 1.8 % of the measured value.

Accuracy: The bias served as measure. The largest deviation was for ramipril: 8.0 % relatively;

for ramiprilat: 13.2 % relatively.

Linearity: In the measuring range given, the results obtained by this method were linear.

Limit of Detection: About 0.020 μg/mL was found as limit of detection. This corresponds to a signal-to-noise ratio of 3:1.

Specificity: The method is specific and permits simultaneous determination of both compounds. Even a 10-fold excess of one compound does not impair the results.

CRITICAL ASSESSMENT OF THE METHOD

The NPD can detect compounds like drugs that contain nitrogen or phosphorus in the ppb range. However, the NPD requires regular maintenance; reduced sensitivity

often indicated the depletion of the active element on the thermoionic bead.

The NPD is a destructive detector that can be used in series only after non-destructive detectors (e.g. ECD). The NPD is sensitive to water that affects the condition of the thermoionic bead. The active element of the bead eventually will become depleted (especially when using halogenated solvents like dichloromethane, chloroform etc.) and requires replacement.

MODIFICATION OF THE METHOD

Due to the discovery of two further urinary metabolites of ramipril (the respective diketopiperazine derivatives of ramipril and ramiprilat), the above-described method was slightly modified (Schmidt et al. 1985). Instead of the rather time consuming second extraction step by means of a disposable Si column, the sample is cleaned by a liquid/liquid extraction step. After methylation of the compounds with diazomethane, the eluate is evaporated to dryness at 40 °C under N_2 gas. Subsequently the residue is dissolved in n-pentane/diethyl ether (3:2. v/v) and washed with 5 % hydrogen carbonate solution. After separation of the upper organic layer, this is evaporated to dryness at 40 °C under N_2 gas and then treated with 1 mL n-hexane/TFAA (5 %) as described before (Hajdù et al. 1984). This method allows the selective determination of ramipril and its three metabolites in human urine; the limit of quantification amounted to 0.020 μg/mL for each of all four analytes. Using this assay, thousands of urine samples originating from phase I–III clinical studies were analysed.

In the following assay, the application of capillary gas chromatography for the determination of another ACE inhibitor, but this time in human plasma and with mass selective detection, is described.

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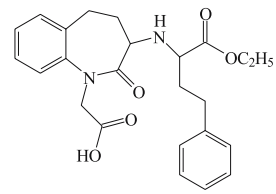
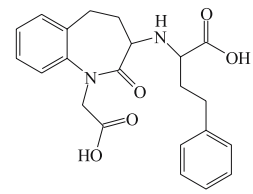
II.Q.2
Plasma Analysis of Benazepril Using Gas Chromatography with Mass-Selective Detection (GC-MSD)

PURPOSE AND RATIONALE

This assay is used for the quantitative determination of benazepril and its active metabolite, benazeprilat, in human plasma by capillary gas chromatography-mass selective detection (Pommier et al. 2003). Benazepril hydrochloride, 3-{[1-ethoxycarbonyl-3-phenyl-(1S)-propyl]amino}-2,3,4,5-tetrahydro-2-oxo-1-(3S)-benzazepine-1-acetic acid hydrochloride, is a prodrug-type angiotensin-converting enzyme (ACE) inhibitor which, on absorption, is hydrolysed to a pharmacologically active metabolite, the dicarboxylic acid (benazeprilat).



Benazepril



Benazeprilat

Note: Mass spectrometers use the difference in mass-to-charge ratio (m/z) of ionized atoms or molecules to separate them from each other. Mass spectrometry (or mass selective detection) is therefore useful for quantitation of atoms or molecules and also for determining chemical and structural information about molecules. Molecules have distinctive fragmentation patterns that provide structural information to identify structural components.

The general operation of a mass spectrometer is:

Create gas-phase ions

Separate the ions in space or time based on their mass-to-charge ratio

Measure the quantity of ions of each mass-to-charge ratio.

Reagents

Benazepril, benazeprilat, and the corresponding deuterium-labelled internal standards (I.S.) were supplied by Novartis (Basle, Switzerland). All the chemicals were of analytical grade. Hexane and toluene (Pestipur SDS) were obtained from Solvants Documentation Synthèse (Pépin, France). Methyl *tert.*-butyl ether and 2 mol/L trimethylsilyldiazomethane solution in hexane were purchased from Fluka (Saint-Quentin Fallavier, France). Hydrochloric acid (0.1 mol/L), 0.5 mol/L sulphuric acid, and sodium carbonate were from Merck (Darmstadt, Germany). 3M Empore C18-SD 96-well disk plates containing 12 mg C18 were obtained from Varian (Les Ulis, France).

Assay

An analytical method for the determination of benazepril and its active metabolite, benazeprilat, in human plasma by capillary GC with mass-selective detection was developed and validated according to international regulatory requirements (Guidance for Industry 2001; Shah et al. 2000). After addition of the internal standards, the compounds were extracted from plasma by solid-phase extraction using automated 96-well plate technology. Unlike earlier published gas chromatography-mass spectrometry (GC-MS) methods (Kaiser et al. 1987; Sioufi et al. 1988), the present method halved the volume of plasma (0.5 mL instead of 1 mL formerly required) without diminishing the lower limit of quantification (LLOQ), and reduced considerably the volume of solvent for conditioning, washing and eluting. After elution, the compounds were converted into their methyl ester derivatives by means of a safe and stable diazomethane derivative (Blau et al. 1993; Rimmer et al. 1996), trimethylsilyldiazomethane solution. The methyl ester derivatives were determined by mass-selective detection at m/z 365 for benazepril and benazeprilat and m/z 370 for D_5-benazepril and D_5-benazeprilat.

PROCEDURE

1. To 0.5 mL plasma in a polypropylene tube, successively 100 μL of the internal standard solution (150 ng) and 100 μL of 0.1 mol/L hydrochloric acid are added.
2. All tubes are then placed on the platform of the Packard MultiProbe II and a 96-well disk plate is placed on top of a vacuum manifold.
3. The 96-well disk plate is conditioned automatically with 100 μL of methanol, and 300 and 80 μL of 0.1 mol/L HCl.
4. Then, 500 μL of the prepared samples are loaded onto the 96-well disk plate.
5. The loaded samples are washed with 100 μL of water and then the analytes and the internal standards are eluted twice with 500 μL of methanol.
6. The eluates are transferred into extraction tubes and 300 μL of trimethylsilyldiazomethane solution are added.
7. The reaction runs at room temperature in a dry bath for 30 min and the reaction mixture is evaporated to dryness under N_2 gas at 40 °C.
8. To the dry derivatized plasma sample are added 0.5 mL of 0.5 mol/L sulphuric acid and 1 mL of hexane.
9. The mixture is shaken mechanically for 5 min at 240 r.p.m. and centrifuged at 1600 g for 2 min.
10. The upper organic phase is discarded and the aqueous phase is alkalised with 1 mL of 2 mol/L sodium carbonate and shaken with 2 mL methyl *tert.*-butyl ether for 5 min at 240 r.p.m.
11. After centrifugation at 1600 g for 5 min, the organic phase is separated and evaporated to dryness under nitrogen at 40 °C.
12. The residue is dissolved in 100 μL toluene, 2 μL of which are injected onto the gas chromatograph.

Robotic System

For sample preparation, a Packkard MultiProbe II liquid handling robotic system was used (Packard Instruments, Meride, CT, USA) using the WinPrep software.

GC-System

Hewlett–Packard 5890 Series II gas chromatograph equipped with a capillary inlet system and an HP 7673 automatic sampler, was used (Hewlett–Packard, Palo Alto, CA, USA).

Column: Fused silica capillary column, Resteck Rtx-1, 15 m × 0.25 mm inner diameter (ID); (Restek, Evry, France).

Injection: Splitless mode, 30 sec splitless period
Gases: Carrier gas (helium); inlet pressure 8 p.s.i.g.
Split flow: 1:50 mL/min;
Septum rinsing (helium): 3 mL/min;
Temperatures: Injection port 280 °C;
Detector 300 °C
Oven program:
Initial temperature: 190 °C (0.5 min)
Heating rate: 30 °C/min;
Final temperature 290 °C

Detection: Hewlett–Packard 5970B mass-selective detector (MSD); the MSD was maintained at 280 °C.

The detector was turned on from 3.5 to 5 min after injection. The selected ions monitored for the methyl ester derivatives were m/z 365 for benazepril and benazeprilat and m/z 370 for D_5-benazepril and D_5-benazeprilat. These fragments are obtained by cleavage of the carboxyethyl [M–73] or carboxymethyl group [M–59] in the side chain.

Sample size: 2 μL

Analysis time: about 6 min per sample.

EVALUATION

Daily calibration standards were prepared at six different concentrations, in duplicate, in the range of 2.5–1000 ng/mL for benazepril and benazeprilat. Calibration curves ($y = ax + b$) were presented by plots of the peak area ratios (y) of the methyl ester derivative of benazepril or benazeprilat to the methyl ester derivative of the I.S. versus the concentration (x) of the calibration standards, and were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model. Concentrations in quality control (QC) samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve. Inter-day repeatability was determined on three different days. Good agreement between the nominal and the back-calculated concentration for calibration samples was observed. The precision ranged from 2.1 to 7.4 % and mean accuracies were within 8 % of the nominal value for the two compounds.

The accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest (2.50 ng/mL), near the lowest (7.50 ng/mL), near the middle (200 ng/mL) and the highest (900 ng/mL) concentrations of the calibration range. Accuracy was determined by calculating the mean recovery for the determined concentrations in standard samples. Precision was assessed from the relative standard deviation (RSD) as a percentage of the mean recovery. The following validation criteria for accuracy and precision were used to assess method suitability: mean recoveries should be within 85–115 %, except at the lower limit of quantification (LLOQ), where it should not exceed 20 % (Guidance for Industry 2001). Series of five quality control samples were prepared at four different concentrations in the range of 2.50 (LLOQ)–900 ng/mL for benazepril and benazeprilat, by spiking drug-free plasma with the corresponding working solutions. It could be shown that the results met the acceptance criteria.

CRITICAL ASSESSMENT OF THE METHOD

The latter assay has several advantages in comparison to the first (which certainly is somewhat older):

The mass-selective detector is more specific and allows a lower limit of quantification than the nitrogen-phosphorus detector (NPD).

The use of trimethylsilyldiazomethane with methanol provides a less hazardous method for preparing methyl esters under mild conditions than ethereal diazomethane solution. Trimethylsilyldiazomethane is commercially available, obviating the need to synthesize diazomethane daily. It is a stable and safe substitute for either hazardous diazomethane or corrosive reagents containing boron trifluoride.

The procedure has a good throughput, with a combination of automated sample extraction with a 96-well disk plate using a Packard MultiProbe II roboter.

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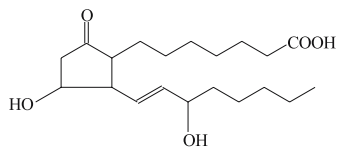
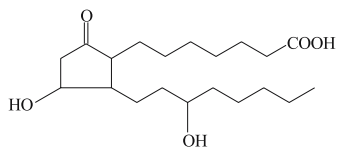
II.Q.3
Simultaneous Determination of Different Prostaglandins in Human Plasma Using Gas Chromatography/Negative Ion Chemical-Ionization Tandem Mass Spectrometry (GC-NICI-MS/MS)

PURPOSE AND RATIONALE

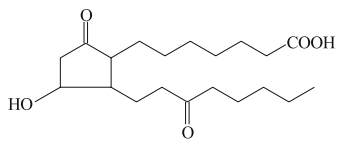
Prostaglandin E_1 (PGE_1) has been used therapeutically for the intravenous treatment of peripheral arterial occlusive disease (Hirai et al. 1986) and of erectile disfunction as an intracavernous (i.c.) injection (Porst 1996). $PGE_1$is rapidly converted to 15-keto-prostaglandin E_0 (15-keto-PGE_0), the major circulating metabolite, and prostaglandin E_0 (PGE_0), a metabolite with activity comparable with PGE_1. The following assay is used for the sensitive (in the low pg range) and selective simultaneous routine determination of prostaglandin E_1, prostaglandin E_0 and 15-keto-prostaglandin E_0, in human plasma by capillary gas chromatography-negative-ion chemical-ionization tandem mass spectrometry (GC-MS/MS) (Hammes et al. 1999).



Prostaglandin E_1 (PGE_1)



Prostaglandin E_0 (PGE_0)



15-keto-Prostaglandin E_0 (15-keto-PGE_0)

Note: Tandem mass spectroscopy has become to mean the mass spectrum of a mass spectrum; hence, *MS/MS*. Essentially in MS/MS, mass analysers are coupled together via an interface known as a collision cell. Ions of a selected mass (precursor ions) are transmitted by the first mass analyser into the collision cell. Here, they collide with the neutral atoms of an inert gas; generally argon is used. In this process a fraction of the translational energy inherent in the precursor ion is converted into internal energy. The internal energy is lost by the precursor ion decomposing or fragmenting into product ions. The product ions are transmitted through a second mass analyser and subsequently determined quantitatively (Venn 2000).

Reagents

PGE_1 was purchased from Arcos Organics (St. Augustin Germany), PGE_0 and 15-keto-PGE_0 from Cascade Biochem (Reading, UK). The deuterated internal standards D_6-PGE_1, D_4-PGE_0 and D_6-15-keto-PGE_0 were synthesized by the Chemistry Department (Schwarz Pharma, Monheim/Rhein, Germany). Ethyl acetate, acetonitrile, dichloromethane, hexane and methanol were obtained from Promochem (Wesel, Germany), ethanol and formic acid from Merck (Darmstadt, Germany) and pentaflourobenzyl bromide (PFBBr), N, N-diisopropylethylamine (DIPEA), MOX reagent (2 % methoxyamine hydrochloride in pyridine) and N, O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Oud Beijerland, Netherlands). Indomethacin was from Sigma (Deisenhofen, Germany) and Biostabil from Biotrans (Dreieich, Germany). All chemicals were of the highest grade available and used without further purification. Bond Elut C18/Si cartridges were obtained from ICT (Frankfurt/Main, Germany). Water was purified with the NANOpure system delivered by Werner (B.-Gladbach, Germany).

Assay

An analytical method for the determination of PGE_1, PGE_0 and 15-keto-PGE_0 in human plasma by capillary GC with tandem mass spectrometry was developed and validated. After addition of the deuterated internal standards, the compounds were extracted from plasma by solid-phase extraction using C18 cartridges, followed by derivatization to the pentafluorobenzyl (PFB) ester methoxime. After evaporation of the derivatization reagents to dryness, the samples were purified on Bond Elut Si-cartridges and converted to their trimethylsilyl (TMS) ethers. Quantitation was achieved by gas chromatography-negative-ion chemical-ionization tandem mass spectrometry. The lower limit of quantification (LLOQ) was 2 pg/mL for PGE_1 and PGE_0 and 10 pg/mL for 15-keto-PGE_0, extracted from 2 mL of human plasma.

PROCEDURE

1. To 2 mL plasma in a centrifuge tube, 200 pg D_6-PGE_1/D_4-PGE_0 and 500 pg D_6-15-keto-PGE_0 as internal standards are added to the sample and then adjusted to pH = 3.0–3.5 with 200 μL 3 % formic acid
2. the sample thus prepared is applied onto a C18 column – which has been preconditioned with methanol (6 mL) followed by redistilled water (6 mL) –, and slowly sucked through under vacuum
3. subsequently, the column is washed with 3 mL redistilled water and with 6 mL hexane and
4. the pressure drop is increased to 80 kPa and the column is sucked dry for 10 min
5. the analytes are eluted[3] with 3 mL ethyl acetate and
6. the eluate is evaporated to dryness at room temperature
7. the residue is incubated with PFBBr (10 μL) and DIPEA (20 μL) in acetonitrile (70 μL) for 10 min at 40 °C
8. the sample is evaporated to dryness and converted to the methoxime with 200 μL of MOX reagent for 30 min at 70 °C
9. After evaporation to dryness, the sample is transferred three times by 1 mL dichloromethane to a Bond Elut Si cartridge, pre-washed with 6 mL of dichloromethane
10. the cartridge is washed with ethyl acetate-dichloromethane (5:95, v/v) and
11. the analytes are eluted with 3 mL of ethyl acetate
12. the extract is incubated with 100 μL of BSTFA for 60 min at 50 °C
13. and then it is evaporated to dryness at room temperature
14. subsequently the sample is taken up in 40 μL of BSTFA, 1 μL of which is injected.

GC-MS/MS System

Finnigan TSQ 700 triple stage mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an Hewlett–Packard HP 5890 Series II gas chromatograph, an HP 7673A autosampler (Hewlett–Packard, Palo Alto, CA, USA) and an temperature-programmable split/splitless injector (Gerstel, Mühlheim/Ruhr, Germany)

Column: Fused-silica capillary column, Hewlett–Packard Ultra 2, 12 m × 0.20 mm inner diameter (ID);

Injection: Splitless mode;

Gases: Carrier gas (helium); inlet pressure 30 kPa.

Reagent gas (methane); ion source pressure 120 Pa.

Collision gas (argon); collision cell pressure 0.2 Pa.

Temperatures: Injection port (programmed) from 150 °C to 300 °C at 10 °C/sec (held for 5 min).

Oven program:

Initial temperature: 150 °C (held for 0.1 min)

Heating rate: 40 °C/min;

Final temperature 300 °C (7 min);

The transfer line, the manifold and the ion source were kept at 300 °C, 70 °C and 150 °C, respectively.

Detection: The mass spectrometer was operated in the negative ion mode with an emission current of 300 μA and an electron energy of 85 eV.

Sample size: 1 μL

Analysis time: about 9 min per sample.

For quantitation in the multiple ion detection (MID) mode, the precursor → product ions monitored in the negative ion mode were m/z 626.4 → m/z 346.2 = $[P–2(CH_3)_3SiOH]^-$ion for PGE_1, m/z 483.3 → m/z 393.2 $[P–(CH_3)_3SiOH]^-$ion for 15-keto-PGE_0 and m/z 528.4 → m/z 438.2 = $[P–(CH_3)_3SiOH]^-$ ion for PGE_0.

EVALUATION

Linear calibration curves were obtained over the concentration range 2–100 pg/mL (PGE_1 and PGE_0) and 10–500 pg/mL (15-keto-PGE_0) of human plasma. The LLOQ (Lower Limit of Quantitation) of the assay, i.e. the concentration with an accuracy and a precision ≤ 20 %, was 2 pg/mL (PGE_1 and PGE_0) and 10 pg/mL (15-keto-PGE_0). The precision and the accuracy of the method were determined by analysis of blank human plasma (2 mL) spiked with PGE_1/PGE_0 in the concentration range 4–200 pg and with 15-keto-PGE_0 in the concentration range 20–1000 pg. In any case, both the precision and the accuracy were < 17 % and indicated good reproducibility. The method has been applied successfully for the determination of PGE_1, PGE_0 and 15-keto-PGE_0 in human plasma after a 2-h i.v. infusion of 60 μg of PGE_1 in order to investigate the pharmacokinetics of PGE_1, PGE_0 and 15-keto-PGE_0 in healthy volunteers.

CRITICAL ASSESSMENT OF THE METHOD

A highly selective and sensitive routine method for the simultaneous determination of PGE_1, PGE_0 and 15-keto-PGE_0 in human plasma is described. Compared to the recently developed GC-NICI-MS/MS assay (Schweer et al. 1994), the described method with a modified purification step of the PFB ester

[3] Recovery over the concentration range validated: 95–100 % for PGE_1, 86–89 % for PGE_0 and 91–93 % for 15-keto-PGE_0)

methoxime derivatives by means of Bond Elut Si cartridges allows the processing of at least 24 samples per day applying the available solid-phase extraction unit.

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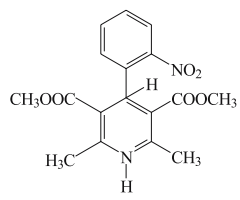
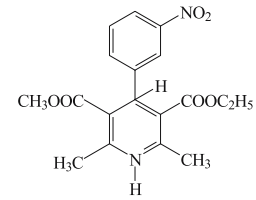
II.Q.4 Determination of Calcium Blocking Agents Using Gas Chromatography with Electron Capture Detection (GC-ECD)

PURPOSE AND RATIONALE

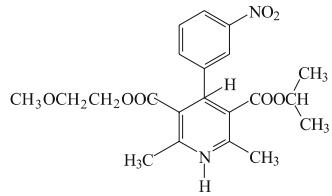
Nifedipine (NF), nitrendipine (NT), and nimodipine (NM) belong to the dihydropyridine group of calcium-blocking agents. The main indications are: Treatment of angina pectoris and for the treatment of arterial hypertension (Krebs et al. 1982); the main indication for NT is the treatment of hypertension (Stoepel et al. 1981). NM acts mainly on cerebral vessels (Langley et al. 1989). The assay was developed by Rämsch et al. (1986) to investigate the pharmacokinetics and the metabolism of calcium-blocking agents like nifedipine (NF), nitrendipine (NT), and nimodipine (NM) using capillary gas chromatography with electron capture detection (ECD).



nifedipine (NF)



nitrendipine (NT)



nimodipine (NM)

Note: The ECD detector consists of a sealed stainless steel cylinder containing radioactive ^{63}Nickel. The ^{63}Nickel element emits beta particles (electrons), which collide with the carrier gas molecules, ionising them in the process. This forms a stable cloud of free electrons in the ECD cell. When organic molecules that contain electronegative functional groups, such as halogens, phosphorus, and nitro groups pass by the detector, they capture some of the electrons and reduce the current measured between the electrodes. The ECD is by far more sensitive than the FID (Flame Ionization Detector) but has a limited dynamic range (about 10^4-fold) and finds its greatest application in analysis of halogenated compounds, like pesticides, herbicides, and drugs.

Reagents

The 1,4-dihydro-2,6-dimethyl-4-(2- or 3-nitrophenyl)-3,5-pyridine-dicarboxylate derivatives nifedipine (NF), nitrendipine (NT), and nimodipine (NM) were supplied by Bayer AG (Wuppertal, Germany). Analytical grade reagents were used at all times, toluene AR (Riedel-de-Haën AG, Seelze, Germany) was distilled over a 50 cm Vigreux column before use. Sodium hydroxide, 0.5 mol/L, was purchased from J.T. Baker Chemical Corp. (USA) and the amber screw-cap autosampler bottles (2 mL) were obtained from Pierce Biotechnology Inc. (Rockford IL USA) and rinsed with acetone before use.

Assay

Nifedipine, nitrendipine, or nimodipine were extracted from alkalised plasma directly into toluene containing the respective internal standard, nitrendipine or

nimodipine (NT for the quantification of NF and NM, and NM for the detection of NT). Hereby it is important to take into account that the samples have to be protected from daylight and from fluorescent light to prevent formation of photodecomposition products (Ahnoff et al. 1990 and Le Guellec et al. 1992). Under the action of daylight and of fluorescent light, e.g. nifedipine is converted into the corresponding nitrosophenylpyridine (Testa et al. 1979).

Subsequently the compounds were analysed by capillary gas chromatography using an electron-capture detector.

PROCEDURE

1. To 0.5 mL plasma in a 2-mL autosampler bottle, 50 μL 0.5 mol/L NaOH and 1 mL toluene containing 50 ng of the respective internal standard (NT for the quantification of NF and NM, and NM for the detection of NT) are added.
2. The bottle is covered with a piece of aluminium foil instead of the usual silicon or rubber disc and closed with the screw cap.
3. Subsequently the bottle is shaken for 5 min
4. The bottle is transferred without opening – or any centrifugation – directly into the autosampler
5. 2 μL of the upper toluene phase are injected into the gas chromatograph.

Using 300 μL micro-vials and one tenth of the reagents, 50 μL of plasma can be extracted to give the same sensitivity!

GC-System

Hewlett Packard 5840A gas chromatograph equipped with a ^{63}Ni electron capture detector (ECD), an HP 7672A autosampler (Hewlett–Packard, Palo Alto, CA, USA) and an temperature-programmable split/splitless injector (Gerstel, Mühlheim/Ruhr, Germany). The needle tip of the autosampler dipped only into the upper third of a sample bottle to prevent the injection of any of the aqueous phase into the GC column.

Column: Fused silica capillary column, Durabond DB-1 (0.1 μm film thickness), 30 m × 0.32 mm inner diameter (ID); (J. & W. Scientific, Folsom, CA, USA).

Injection: Splitless mode (1 min);

Gases: Carrier gas (helium), 3 mL/min.

Electron capture detector gas (argon/methane): 50 mL/min;

Temperatures: Injection port 250 °C;

Detector 300 °C

Oven program:

Initial temperature: 160 °C. Splitter closed; duration 1 min.

Heating rate: 10 °C/min;

Final temperature 270 °C (20 min);

Detection: ECD detector

Sample size: 2 μL

Analysis time: about 32 min per sample.

EVALUATION

The pharmacokinetics of the three dihydropyridine calcium blockers, nifedipine, nitrendipine, and nimodipine were investigated using the GC assay presented above. The retention times of the three compounds amounted to 18.6 min for NF, 20.8 min for NT and to 24.2 min for NM. The detection limits were at least 1 ng/mL, but for NT and for NM limits of 100 pg/mL could also be achieved.

In spite of the structural and physiochemical similarity of the three compounds, they differ in their pharmacokinetic behavior. NF, a relatively polar molecule with the lowest volume of distribution and highest plasma levels in relation to dose, has the greatest bioavailability. On the other hand, NT and NM, with a 3- to 4-fold lower solubility in water, have a low bioavailability, low plasma concentrations, and a larger volume of distribution (Rämsch et al. 1986).

CRITICAL ASSESSMENT OF THE METHOD

Analogous to the majority of dihydropyridines, nifedipine's, nitrendipine's, and nimodipine's chemical structures – a 2- or 3-nitrophenyl substituent in the 4-position combined with the dihydropyridine diester structure – results in a high response in electron capture detection (ECD), thus allowing high detection sensitivity and sufficient assay specificity towards endogenous compounds, metabolites or common co-medications (Mück et al. 1994).

However, because it contains 5 millicuries of ^{63}Nickel, the ECD is covered by a "General License" requiring a periodic "wipe test" and the filing of a form with the respective state's Department of Health or with another adequate authority. Its advantages are (Venn 2000):

- It is selective for halogens, nitro groups, peroxides, quinones
- It is extremely sensitive
- It is non-destructive (in contrast to the NPD, FID or MS).

Its disadvantages are:

- A limited dynamic range, 10^4-fold

- it is prone to contamination and
- it is radioactive; leakage must be checked regularly.

MODIFICATIONS OF THE METHOD

Since the calcium channel blockers are highly potent drugs, their concentration in human plasma and other biological fluids are generally low (high pg/mL- to low ng/mL range) thus requiring analytical techniques of high sensitivity and specificity. The numerous publications, which have appeared until the end of the eighties describing various set-ups of gas and liquid chromatographic procedures, have been thoroughly reviewed by M. Ahnhoff et al. (1990). Their conclusion may be summarized that a simple liquid/liquid extraction followed by gas chromatography with electron-capture detection (GC-ECD) or mass spectrometric detection (GC-MS, preferably in the negative ion chemical ionization mode (NICI) (Fischer et al. 1986) is usually an adequate and efficient way to provide drug concentrations for pharmacokinetic evaluation and therapeutic drug monitoring. If not restricted due to its generally inferior limit of quantification, high-performance liquid chromatography with UV or amperometric detection (HPLC-UV resp. HPLC-ELCD) can be an attractive alternative for the quantification of dihydropyridines in biological fluids (Mück et al. 1994).

However, as already mentioned earlier, high pressure liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) has evolved in the last few years as the major bioanalytical technique for the bioanalysis of analytes in biological matrices. This is reflected also in a number of LC-MS/MS assays for the determination of dihydropyridine calcium antagonists in biological fluids (Carvalho et al. 2001, Schug et al. 2002; Kang et al. 2004).

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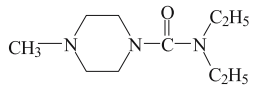
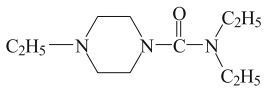
II.Q.5 Determination of Diethylcarbamazine (DEC) Using Gas Chromatography with Flame Ionization Detection (GC-FID)

PURPOSE AND RATIONALE

Single doses of diethylcarbamazine (DEC) in combination with albendazole have been found safe and efficacious for the treatment of *Brugia malayi* infection (Shenoy et al. 1999), and single dose treatment of DEC with ivermectin is effective against adult *Wuchereria bancrofti* (Dreyer et al. 1998). However, since DEC is a compound that lacks a chromophore, spectroscopic methods of analysis that utilize chromophores, like HPLC with UV detection, are not suitable for the bioanalytical determination of the compound. In order to achieve the sensitivity and specificity for the determination of DEC in plasma, a number of gas chromatographic methods have been developed (Bogan 1977; Nene et al. 1984; Lee et al. 1997), partly with nitrogen phosphorus detection. To overcome the regular maintenance that is required for the nitrogen–phosphorus detector (NPD), which made it difficult to apply the method to clinical pharmacokinetic studies with high sample throughput requirements, a sufficient sensitive, selective, accurate and reproducible gas chromatographic assay using flame ionization detection (FID) for the determination of DEC in human plasma was reported in 2001 by Miller et al.



diethylcarbamazine (DEC)



1-diethylcarbamyl-4-ethyl-piperazine (Istd.)

Note: The FID detector was the first successful universal GC detector to be developed and remains the most widely used. The effluent from the column is mixed with hydrogen and air, and ignited. Organic compounds burning in the flame produce ions and electrons, which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The current resulting from the pyrolysis of any organic compounds is measured. The FID is a useful general detector for the analysis of organic compounds[4]; it has high sensitivity, a large linear response range, a low noise and it shows a similar response for most analytes. It is generally robust and easy to operate, but because it uses a hydrogen diffusion flame to ionize compounds for analysis, it destroys the sample in the process.

Reagents

All solvents and chemicals were HPLC grade. Organic solvents, sodium carbonate (anhydrous), and sodium bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Carbonate buffer, pH = 10.0, was prepared using the sodium carbonate (anhydrous), and sodium bicarbonate. A solution of 0.1 % triethylamine in methanol was made for the elution solvent. Diethylcarbamazine citrate was obtained from Sigma, and used to make stock solutions (St. Louis, MO, USA). The internal standard, 1-diethylcarbamyl-4-ethylpiperazine (E-DEC) was synthesized by the Division of Medicinal and Natural Products Chemistry at the University of Iowa, College of Pharmacy. Ultrapure analytical grade Type I water was produced by a Milli-Q Plus water system (Millipore Corporation, Bedford, MA, USA). For the extraction of DEC and of its internal standard, Alltech Extract – Clean C18 cartridges, 500 mg with a 2.8 mL reservoir, and a SPE vacuum manifold (Alltech, Deerfield, IL, USA) were used.

[4]It responds to any molecule with a carbon-hydrogen bond, but not at all or poorly to compounds such as H_2S, CCl_4, or NH_3.

Assay

DEC and the internal standard (Istd), 1-diethylcarbamyl-4-ethyl piperazine HCl (E-DEC), were extracted from human plasma – that has been alkalised with carbonate buffer – after loading onto a conditioned C18 solid phase extraction cartridge, rinsed with water and eluted with methanol. After evaporation under a stream of nitrogen and reconstitution in methanol, 3 μL were injected into the GC system and detected using a flame ionization detector (FID). The retention time for DEC was 5.5 min and for the internal standard (E-DEC) it was 7.28 min.

PROCEDURE

1. To 0.5 mL human plasma in a disposable centrifuge tube, 25 μL of the working solution of the internal standard (21 μg E-DEC/mL) and 1 mL of carbonate buffer pH = 10.0 are added and the sample is mixed on a vortex mixer for 30 sec.
2. The sample thus prepared is applied onto a C18 column – which has been activated by aspirating 1 cartridge volume (~ 3 mL) methanol followed by 1 cartridge volume of HPLC grade water – and slowly sucked through under vacuum (at about 20 kPa pressure drop).
3. Subsequently, the column is washed with 2.5 mL of HPLC grade water at the same reduced pressure, and allowed to dry for 10 min under vacuum.
4. For sample elution, 2 mL of 0.1 % triethylamine in methanol is added to each cartridge and allowed to pass through the cartridge into 5 mL disposable centrifuge tubes under low vacuum.
5. After elution, the eluate is evaporated to dryness at 40 °C under a gentle stream of N_2 gas.
6. The residue is taken up in 50 μL methanol and mixed on a vortex mixer for 60 sec.
7. 3 μL of the reconstituted residue are injected into the GC.

GC-System

Hewlett Packard 5890 Series II Plus gas chromatograph equipped with a flame ionization detector (FID) and an HP 7673 autosampler (Hewlett–Packard, Palo Alto, CA, USA).

Column: Fused silica capillary column, Heliflex AT-35 capillary column (0.25 μm film thickness), 30 m × 0.32 mm inner diameter (ID); (Alltech, Deerfield, IL, USA).

Injection (*split/splitless*): No information available.

Gases: Carrier gas (helium): 1.5 mL/min; inlet pressure 11 p.s.i.g.

Detector make-up (helium): 25 mL/min;
Detector (hydrogen): 35 mL/min;
Detector (air): 420 mL/min;
Temperatures: Injection port 180 °C;
Oven: 160 °C
Detector: 240 °C;
Detection: FID detector;
Sample size: 3 μL
Analysis time: about 22 min per sample.

EVALUATION

Calibration curves for DEC in human plasma were linear using unweighted linear regression in the concentration range of 100–2000 ng/mL, with correlation coefficients greater than or equal to 0.9934 for all curves. The limit of quantification (LOQ) in human plasma was accepted as 70 ng/mL. Plasma samples were spiked to a nominal concentration of 70 ng/mL with DEC working solution and internal standard and carried through the extraction procedure. At the LOQ, the C.V. (n = 6) of the measured concentration was 4.5 %, and the deviation of the mean of the measured concentrations from the nominal value was –6.1 %.

Precision, accuracy, and recovery were evaluated by conducting repeated analysis (n = 6) of spiked plasma samples at three different concentration levels: 120, 1000, and 2000 ng/mL. For an intra-day run (n = 6), the coefficient of variation of DEC at 120, 1000, and 2000 ng/mL had been shown to be 4.5, 1.3, and 1.6 %, respectively. The deviation of mean values from nominal (n = 6) were –4.4, 2.0 and 1.4 % for DEC concentrations 120, 1000 and 2000 ng/mL, respectively. The CV results for inter-day precision at the same concentrations were all less than 7 % (n = 12). The deviation of mean values from nominal (n = 12) were –1.7, 2.6 and 0.8 % for DEC concentrations 120, 1000, and 2000 ng/mL, respectively.

Recovery was tested at low, medium and high concentration of DEC and internal standard. Absolute recoveries were determined by comparing the peak areas of extracted QC samples with the peak areas of recovery standards (unextracted equivalents of extracted QC samples). The mean recoveries for DEC and the internal standard were 84.8 and 85.5 %, respectively.

Autoinjector stability was carried out for over 13 h by repeated injection of the same extracted plasma sample at room temperature (nominally 25 °C); The results showed, that the extracted specimens remained stable over the course of the study. QC samples containing 120 and 2000 ng/mL DEC in plasma were subjected to 3 freeze/thaw cycles. Samples were frozen at –20 °C for 24 h and thawed unassisted at room temperature. When completely thawed the samples were transferred back to the original freezer and kept refrozen at least 24 h. Freezing and thawing of the QC samples appeared to have no effect on quantitation of the analyte. In addition, QC samples containing 120 and 2000 ng/mL DEC in plasma were subjected to storage at –20 °C for 12 weeks. Plasma samples (n = 6) were taken for DEC analysis at 0, 2, 4, 9 and 12 weeks. The QC samples stored in a freezer set to maintain –20 °C remained stable for the duration of the study period.

CRITICAL ASSESSMENT OF THE METHOD

The assay has been validated and the results of validation demonstrate that the standard curve is linear over the concentration range of 100–2000 ng/mL. The assay is reproducible and accurate, with recovery of the analyte and internal standard in the range of 80–90 %. The analysis requires 0.5 mL of plasma and has a limit of quantification of 70 ng/mL. The stability of plasma samples stored at –20 °C has been demonstrated for up to 12 weeks. Autoinjector stability has been demonstrated for over 13 h and freeze-thaw stability has been demonstrated for 3 freeze-thaw cycles. The procedure has a sample throughput of at least 30 specimens per day. The assay meets the guidelines for bioanalytical methods validation for human studies (Shah et al. 1991).

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Chapter II.R
Bioanalytical Assays – RIA/EIA

Heinz Jürgen Skrzipczyk
Patrick Verdier

INTRODUCTION

Immunoassays have become the most valuable analytical tool of medicinal in vitro diagnostics and are routinely employed for the detection of a wide range of analytes (e.g. hormones, peptides, proteins, viruses, pharmaceuticals). Further important areas of application are environmental pollutants and food analysis.

The radioimmunoassay (RIA) for the determination of insulin developed by Yalow and Berson (1959) combined for the first time the high sensitivity of a radioactively labeled compound with the high specificity of an immunological reaction by the formation of a thermodynamically stable antigen-antibody complex (dissociation constants are typically $10^{-9} - 10^{-12}$ M). In this way it was possible to detect quantitatively substances in the attomolar range even in the presence of an excess of other similar foreign substances without prior enrichment. The theoretical and practical aspects of RIA and related techniques including numerous examples are described by Moss et al. (1976), Jaffe et al. (1979), Travis (1979), Langone et al. (1981, Vol. 73/74), Langone et al. (1983), Odell et al. (1983), Chard (1990).

Antibodies are prepared by immunisation of animals (e.g. rabbits, guinea-pigs, sheep, goats) with the analyte of interest. However, direct immunisation is only possible with relatively large compounds (approx. > 1000 Da). Since small molecules are not capable to trigger an immune response, the immunisation is performed with hapten (compound)-carrier protein conjugates. Typical carrier proteins are keyhole limpet haemcyanin (MW = $4.5 \times 10^5 - 1.3 \times 10^7$ Da), bovine serum albumin (68 kDa) and ovalbumin (45 kDa). The haptens are coupled to the carrier proteins via amino or carboxylgroups using appropriate reagents.

The immunisation procedure (at least 12 weeks with booster injections, addition of oil based adjuvants to stimulate the immunogenic response) gives rise to an antiserum representing a mixture of polyclonal antibodies which are produced by different immunocompetent cells and thus have different binding sites and affinities.

Monoclonal antibodies generated by fusing mouse spleen cells to myeloma cells can be considered as a pure single reagent that is available in unlimited quantity. Drawbacks are high costs, clonal drift and often low affinity.

Further details to polyclonal/monoclonal antibodies are found in Langone et al. (1981, Vol. 73/74), Langone et al. (1983), Szentivanyi et al. (1986).

The use of a radioactive label (mainly ^{125}I) not only attributed to the excellent sensitivity of RIA but also to the excellent flexibility and precision of radiochemical labeling and its high robustness with regard to interferences because of the "hard" signal.

However, the disadvantages of radioactivity such as the required handling licenses, disposal costs,

short half-life (60 days for ^{125}I), short shelf-life of labeled reagents, restricted sensitivity due to limited signal emission during the measuring time of usually only 1 min led to the development of nonisotopic immunoassays (e.g. enzyme / fluorescence / chemiluminescence / bioluminescence immunoassay-EIA / FIA / CIA / BIA) described by Engvall and Perlmann (1971), Langone et al. (1981, Part B/C), Langone et al. (1983), Van Dyke (1985, Vol. I/II), Hemmilä (1989), Schaap et al. (1989), Stanley and Kricka (1991).

Nevertheless, RIA is still the first choice for research assays provided the required handling licenses are available and not the ultimate sensitivity is required.

Some of the new labels have not only facilitated replacement of radioisotopes, but also a breakthrough in sensitivity (zemptomolar range attainable).

The most wide-spread alternative to RIA is EIA (Tijssen 1985). Since the introduction of EIA at the beginning of the 1970s a tremendous number of techniques have become available by using different enzymes (horseradish peroxidase-HRP, alkaline phosphotase – AP, β-D-galactosidase – GAL, luciferase) and substrates (chromogenic, fluorogenic, luminogenic).

EIA has the potential for higher sensitivity than RIA because enzymes produce a lot of signal-generating species. In practice, however, the sensitivity is determined by the signal to noise ratio. Time resolved fluorescence detection is a powerful technique to overcome this problem by using fluorophores (rare earth chelates) with a long fluorescent time (μs – ms) compared to the background fluorescence (ns) – (Janssen 1997; Read 2001). In many enzyme immunoassays, specific signal and noise are amplified to a similar extent with the consequence that no improvement in sensitivity compared to RIA could be achieved. EIAs are in principle less robust than RIAs because enzymes are bulky and can disturb the immunological reaction. Furthermore, enzymes are prone to interferences. The implementation of sophisticated techniques and automation (assays are usually carried out in the 96/384 well format under controlled conditions) resulted in comparable assay quality independent of the label used.

In routine analysis, EIA and other nonisotopic immunoassays have displaced the RIA in many fields more and more during the past years.

Immunoassay Principles

A) Competitive immunoassay with analyte tracer

Analyte and analyte tracer (structurally similar to analyte, mostly ^{125}I-analyte) compete for a small number of antibody binding sites in an equilibrium reaction. The lower the concentration of the analyte, the more antibody-analyte tracer complexes can be formed. Selective measurement of the signal emitted from the antibody-analyte tracer complex requires prior separation of uncomplexed analyte tracer.

This assay principle can be applied for compounds of low and high molecular weight.

B) Competitive immunoassay with antibody tracer

The main difference compared to the method described before is that the antibody carries the label (antibody tracer). Analyte (from the sample) and an unlabeled analyte derivative (sometimes only analyte bound to a solid phase) compete for a small number of antibody tracer. The lower the concentration of the analyte, the more analyte derivative-antibody tracer complexes can be formed.

This assay principle is advantageous in cases when labeling of small antigens renders difficult or leads to different immunological properties compared to the unlabeled antigen.

C) Sandwich immunoassay

This principle requires two different antibodies (capture and tracer antibody).

Both antibodies bind to the analyte at different sites (epitopes) and thus form a sandwich complex. An excess of the two antibodies is employed in order to shift the equilibrium in favor of the sandwich complex.

Sandwich immunoassays have distinct advantages in comparison to competitive immunoassays: Higher specificity (two antibodies for analyte recognition) and better sensitivity (favorable equilibrium due to the excess of reagents). Theoretically, every analyte molecule present in the sample can be bound to a tracer antibody allowing extremely limits of detection. Often a straight-line calibration curve can be achieved over many orders of magnitude. In addition, higher and easier labeling of an antibody (in particular with nonisotopic labels) compared to a small analyte is possible.

The sandwich principle achieved its breakthrough when monoclonal antibodies became available because of the need of higher amounts of antibody compared to competitive assays.

The major disadvantage of this method is the restricted applicability to only analytes with MW > approx. 5 kDa), since two antibodies cannot bind to a small compound for steric reasons.

Separation Methods

The selective measurement of labeled immune complexes necessitate a prior separation of the unbound

analyte tracer (A), of antibody tracer not bound to the analyte derivative (B), or of unbound antibody tracer (C).

The first separation methods involved purification steps (chromatography or electrophoresis). Better manageable, but nowadays rarely found in commercial kits (however still used in research assays) are the methods in which the immune complexes are precipitated e.g. by addition of salts, organic solvents or a second antibody directed against the first antibody. All these techniques require a cumbersome centrifugation step.

Modern methods employ solid phases (e.g. tubes, particles, microtiter plates).

Based on solid phase technologies numerous fully automated immunoassay systems which allow the determination of a broad spectrum of analytes are nowadays commercially available. While very successful in the case of nonisotopic methods, attempts for automation in the RIA field more or less failed due to the properties of the radioactive label.

Alternatives for simple handling of immunoassays without the need of expensive instrumentation have been developed.

In the homogeneous immunoassays there is no separation step because they are based on a changing signal by formation of the immune complex. The first assay of this type was the EMIT method (enzyme-modulated immunoassay technology) described by Engvall et al. (1970). The main disadvantages of homogeneous assays are low sensitivity and a more pronounced susceptibility to interferences.

Dry tests and test strips described by Morris et al. (1987) and Litman (1985) contain all the reagents required for a quantifiable test on a strip or filter. Evaluation can be performed visually or be read-out by a pocket reflectometer. Automated systems for a higher sample throughput are also available. The sensitivity of these tests is rather low (mg-μg/mL range) but sufficient for application as quick tests in clinical chemistry.

Data Evaluation

Competitive immunoassays give a sigmoidal standard curve with a linear concentration-binding range of about two orders of magnitude. Due to the sigmoidal shape, sophisticated curve-fitting and interpolation software is required. 4-PL logistic fitting is the most widely proposed whatever immunoassay device manufacturers.

A straight-line calibration curve over many orders of magnitude can often be found in the case of sandwich immunoassays with deviations from linearity only in the lower and higher concentration range.

Special Features

1. Immunoassays can allow the quantification of enantiomers of a racemic chemical entity – by using a stereospecific tracer and a serum raised against the mixture of enantiomers.
2. The immunogen design generating an antibody for a product-class immunoassay – can also be applied for a wide spectrum immunoassay enabling high throughput – in research phase or in early stage of development for chemical series reaching the chemical optimisation phase. In principle, the "constant" scaffold of different compounds are being used as immunogen resulting in a serum containing antibodies for these compounds. In cases where high throughput combined with highest analytical sensitivity is required, a wide spectrum immunoassay (WSIA) could be the method of choice.

Future Trends in the Immunoassay Field

Chemical sensors utilize the immunological recognition principle by coupling with optical, electrochemical, or other transducer (signal transfer) described e.g. by Eggins (1996) and Rogers et al. (1998). A tendency to miniaturized formats ("chips") as part of the nanotechnology can be observed.

Phage libraries for antibodies enable the access to "monoclonal like" antibodies in unlimited quantities which can be produced within a period of two weeks without using animals (Marks et al. 1996). These antibodies are less immunogenic than monoclonal antibodies (mouse-human chimeras) because the amino acid sequences are entirely human.

A promising technique for the determination of small molecules in sandwich immunoassays is the use of monoclonal anti-idiotypic antibodies which recognize unique V region of antigen binding antibody and mimic internal image of antigen (Vogel M 2000). In recent years, antigen mimicry has been shown on molecular level for some xenoantigens.

Molecular imprinting is a technique in which the shape of a template molecule (analyte) is imprinted in a polymer, e.g. described by Kriz et al. (1997), Reid et al. (1998), Yano (1999), Yan (2002). The imprinted polymer can be used as an antibody mimic for an immunoassay.

Advantages of Immunoassays

The immunoassay can be considered as the most sensitive bioanalytical method.

Its selectivity is very high (but limitations with regard to metabolites).

Immunoassays are easy to handle and allow high throughput in contrast to HPLC-based methods. The throughput can be further increased by semi-or fully automated systems.

The running costs of immunoassays are distinctly lower compared to other sensitive bioanalytical methods.

Disadvantages of Immunoassays

The production of antibodies needs about 3 months / 6–12 months for polyclonal / monoclonal antibodies.

Matrix effects lead to a decrease in binding of analyte to antibody resulting in reduced sensitivity and falsely increased or decreased concentration depending on the assay principle. The matrix effects can be reduced or eliminated by diluting the sample.

Cross-reactivity (e.g. due to metabolites) can only be eliminated by collecting appropriate fractions from HPLC separations or applying solid phase extractions (SPE) preceeding the immunoassay procedure.

The high dose hook effect (HDH) evident as a decrease in signal with very high antigen concentration can be observed in sandwich immunoassays (in particular in single incubation assays). HDH can be prevented by diluting the samples or applying more steps in the assay.

A cross-validation with an accepted analytical method (e.g. GC, HPLC, LC-MS/MS) is required if the immunoassay could be subject to interference from matrix or metabolites.

Terms and Abbreviations

Terms and abbreviations used in the immunoassay field are sometimes ambiguous.

Radioimmunoasssay (RIA)

a) competitive immunoassay with a radioactive analyte tracer (“classical” RIA)

b) generic term for all immunoassays with a radioactive label.

Enzyme immunoassay (EIA)

see RIA, is distinguished only by the type of label used.

Enzyme-linked immunosorbent assay (ELISA)

a) excess reagent assay

b) generic term for all immunoassays with an enzyme label

2-site IXMA: A synonym for sandwich assay; “immuno-x . . . metric assay” (e.g. IRMA: immunoradiometric assay). The expression immunometric means that it is an assay with excess reagent.

1-site IXMA: Refers to a competitive assay with antibody tracer. The designation is not uniform because in 2-site IXMA immunometric means an assay with excess reagent.

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II.R.1
Competitive RIA of Apidra (Insulinanalogue) with Double Antibody Precipitation

PURPOSE AND RATIONALE

Apidra (HMR 1964) is a synthetic short acting insulin (polypeptide) which is structurally very similar to human insulin and has an approximate molecular weight of 5800 Dalton.

Quantitative insulin determination (Yalow et al. 1959) is useful for the diagnosis of diabetes mellitus and for monitoring therapy.

In the early development of Apidra a commercially available radioimmunoassay for the determination of rat insulin (Linco Research Inc. 14 Research RD, St. Charles, MO 6330, USA) was used for the analysis of Apidra in human serum because specific antibodies were not yet available.

Several RIAs for insulin had been compared before aiming at 100 % cross-reactivity with Apidra. The rat insulin RIA from Linco showed similar reactivity for Apidra and human insulin. Therefore, this assay was used with only slight modifications; rat insulin standards were replaced by Apidra standards in an appropriate human serum matrix for calibration.

The assay described below in more detail uses the principle of a competitive immunoassay with a double antibody precipitation (Chard 1990). The antibody (from guinea pig) bound tracer is separated from the free tracer by the second antibody present in the precipitating reagent (goat anti guinea pig antibody). After decanting the free tracer, the activity of the antibody-bound fraction is measured (B) and calculated as a fraction (B/B_0) of the non-competitive binding (B_0). A standard curve is set up with increasing calibrator concentrations by plotting the measured B/B_0 fraction against concentration, and from this curve, the concentration of antigen in unknown samples can be calculated.

PROCEDURE

Reagents

Reagents of the Linco rat insulin RIA are used except the standards and control sera.

The test kit comprises reagents for 250 determinations. It contains:

1 vial of ^{125}I-Insulin < 185 KBq, lyophilised

1 vial of guinea pig anti-rat insulin antiserum in assay buffer, 26 ml

1 vial of assay buffer (0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08 % sodium azide, and 1 % RIA grade BSA), 40 ml

1 vial of label hydrating buffer (assay buffer containing normal guinea pig IgG as carrier), 27 ml

1 vial of precipitating reagent (goat anti guinea pig IgG serum, 3 % PEG and 0.05 % Triton X-100 in 0.05 M Phosphosaline, 0.025M EDTA, 0.08 % sodium azide), 260 ml

Standards: stock solution: 20 ng/ml Apidra in insulin free human serum; standard matrix: insulin free human serum

Controls K1–K3 (low, medium, high): Apidra in human serum, concentration stated

Preparation of Reagents

^{125}I-insulin tracer is dissolved in entire volume of label hydrating buffer.

Antiserum, assay buffer, and precipitating reagent are ready to use.

Standards: The stock solution of Apidra is prepared by dissolving about 500 µg (exact weight) Apidra in 5 ml of 0.1 M Tris/HCI buffer, pH 9. This solution (100 µg/ml) is further diluted in insulin free human serum to a concentration of 20.0 ng/ml (nominal value). The standards (10, 5, 2.5, 1.25, 0.63, 0.31, 0.16 ng/ml) are prepared by serial dilution of the stock solution in insulin free human serum matrix (e.g. WBAG Resources GmbH).

Controls: Control samples are prepared by spiking human sera (insulin content below 0.5 ng/ml) with Apidra stock solution to a concentration of about 0.5, 1.5, 5.0 ng/ml.

Assay Procedure

100 µl of standard/control/sample is incubated in a tube with 100 µl of ^{125}I-insulin tracer and 100 µl of antiserum for 20–24 hours at 2–8 °C.

Separation of the antibody-bound and free radiolabeled ligand is done by dispensing 1 ml of cold precipitating reagent in each tube. The tubes are vortexed and incubated for 20 min at 2–8 °C. The precipitate is then centrifuged down at > 1500 g for 60 min at 2–8 °C. The supernate is decanted immediately and the remaining solution is allowed to drain by standing the tubes upside down on adsorbent tissue up to 15 seconds. Invert tubes only once. Pellets are fragile and slipping may occur.

The radioactivity is counted in a gamma counter for 1 min.

Total activity and non-specific binding (blank) are also determined in each run.

EVALUATION

Control/sample concentrations are read-off from the standard curve by use of the Immunoassay Data Management Program MultiCalc (Wallac Oy/Berthold).

Fitting algorithm = 4PL weighted

Blank correction is applied. NSB (non specific binding) counts are subtracted from each average counts, except total counts, prior to final data reduction.

Validation

Precision (within-day): 2.2–7.2 % (4.9 % near LOQ)

Precision (between-day): 1.6–4.7 % (2.3 % near LOQ)

Accuracy (between-day): 91.8–101.4 %

Linearity: The test shows a good linearity over the whole concentration range as shown by dilution of serum samples with assay buffer.

Limit of Quantitation (LOQ): 0.2 ng/ml (corresponding to ED-80 of the standard curve)

Specificity: No specific determination of Apidra (crossreactivity with human/rat/porc insulin is 80.2/106.9/102.2 %, respectively).

CRITICAL ASSESSMENT OF THE METHOD

The method described above could not differentiate Apidra from naturally occurring insulin/metabolites and precursors. This lack of specificity renders the characterisation of the pharmacokinetics difficult. Therefore, a specific assay for the determination of Apidra was required for its further development.

MODIFICATION OF THE METHOD

Aventis Pharma contracted Linco Research Inc., 14 Research RD, St. Charles, MO 6330, USA to design and produce a highly specific RIA for the accurate and precise routine determination of Apidra in human serum in commercial kit form ("Insulin Analog HMR 1964 RIA Kit").

This kit is very similar to the method described before with the exception that a highly specific antibody for Apidra (guinea pig anti-HMR 1964 antiserum prepared by Linco) is used. The antibody meets the specificity requirements (cross-reactivity e.g. to human insulin < 0.001 %) and the sensitivity requirements (< 0.2 ng/ml).

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II.R.2 Competitive RIA of Free Anti-Insulin Antibodies with PEG Precipitation (Semi-Quantitative Assay)

PURPOSE AND RATIONALE

Anti-insulin antibodies (AIA) develop in the serum of many patients who are receiving insulin treatment (Berson et al 1956). In addition, insulin auto antibodies (IAA) are detected in insulin dependent diabetes mellitus (IDDM) or Type I Diabetes before insulin therapy (Palmer et al 1986).

The determination of circulating anti-insulin antibodies is of clinical importance for the following reasons:

1. presence of free anti-insulin antibodies in serum interferes with the determination of insulin by immunoassays (Kuzuya et al. 1977)
2. high *titers* of AIA may induce a state of insulin resistence
3. AIA may influence the quality of the glycemic control in diabetic patients by prolonging the half life of insulin.

The RIA described (in-house assay) is intended for the semi-quantitative determination of free AIA in serum by the binding of the corresponding ^{125}I -Tyr-A 14-insulin tracer and precipitation of the complex by polyethylene glycol. It is useful for monitoring the development of AIA during immunogenicity studies in animals, toxicokinetic studies in animals and clinical studies with patients receiving (synthetic) insulin.

PROCEDURE

Reagents

Controls

A blank control (TO) containing no anti-insulin antibodies is used to determine the non-specific binding (NSB). Three titer controls (T1-T3) containing low, medium and high levels of anti-insulin antibodies (guinea pig anti-porc insulin antiserum, e.g. Scantibodies Laboratory Inc. T 531 B, Part. 3 AK 025 in buffer solution) are measured in each run.

Buffer: 0.05 M phosphate buffer pH 7.4; 0.5 % BSA, 0.4 % bovine IgG, 0.075 % NaN_3

Preparation of controls:

Stock solution: anti-insulin antiserum is diluted 1:100 in buffer

Titer control T3 (high): stock solution is diluted 1:100 in buffer

Titer control T2 (medium): titer control T3 is diluted 1:4 in buffer

Titer control T1 (low): titer control T2 is diluted 1:8 in buffer

Blank control TO: buffer

The titer controls are stored in polystyrene tubes at –20 °C. They are stable for at least 12 months.

^{125}I-insulin tracer

^{125}I-Tyr-A 14-insulin (e.g. human, porcine or bovine insulin) is prepared according to routine procedures. Aliquots in buffer solution (0.05 M phosphate buffer, pH 7.4, 1 % BSA, 0.05 % NaN_3) are lyophilized.

When stored at 2–8 °C the tracer can be used for at least 6 weeks.

Specification:

Specific activity: 13.320 MBq/mg (360 mCi/mg)

Activity: 37 kBq/ml (1~Ci/ml)

Concentration: approx. 2.8 ng/ml.

Assay Buffer

7.8 g $Na_2HPO_4 \times 2\,H_2O$ and 0.75 g KH_2PO_4 are dissolved in 800 ml of water. After pH-value has been adjusted to 7.4, 0.75 g NaN_3 is added and filled up to 1000 ml with distilled water. 5.0 g BSA is added and dissolved.

Dilution Buffer

For sample dilution assay buffer containing 4 mg IgG/ml (bovine immunglobulin) is used.

Storage:

Assay buffer can be stored at 2–8 °C for at least four weeks.

Dilution buffer can be stored up to 1 week at 2–8 °C.

PEG Solution

The PEG solution consists of 17.5 % (w/v) polyethylene glycol (PEG 6000) in phosphate buffer (0.05 M, pH 7.5) containing 0.075 % sodium azide and 0.5 % bovine serum albumin.

It can be stored at 2–8 °C for at least 6 months.

Preparation of Reagents

^{125}I-insulin tracer lyophilized, is dissolved in 20 ml H_2O bidest. It can be stored up to 24 hours at 2–8 °C after reconstitution.

The titer controls (T0–T3) are thawed and homogenized.

Assay buffer and PEG solution are ready to use.

Assay Procedure

100 µl of control/sample is incubated with 200 µl of ^{125}I-insulin tracer and 200 µl of assay buffer for 19–24 h at room temperature. Separation of the antibody-bound and free radiolabeled ligand is performed by dispensing 1 ml of PEG solution in each tube. The tubes are vortexed several times until mixing is complete. The rapidly formed precipitate is then centrifuged down at 1500 g for 15 min at room temperature. The supernate is decanted and the remaining PEG-solution is allowed to drain by standing the tubes upside down on adsorbent tissue. The radioactivity is counted in a gamma spectrometer for 1 min.

EVALUATION

Calibration

Due to the different nature of insulin antibodies that can be formed a defined analyte does not exist and a calibration is not possible. The tracer binding test is a semi-quantitative test without a standard curve. Three titer controls (T1–T3) containing low, medium and high levels of free anti-insulin antibodies and a blank control (TO) to determine the non-specific tracer binding (NSB) are measured in each run. Total activity of 200 µl tracer is also measured in each run.

Calculation of Results

The mean of duplicate determinations is calculated, rejecting obvious outliers. In cases CV > 15 % analysis is repeated. The binding percentage of ^{125}I-insulin is calculated, according to the formula:

$$B/T(\%) = (\text{sample counts/total counts}) \times 100.$$

Validation

Precision (within-day): 1.4–8.1 % (in the range from 5–90 % B/T in 10 determinations)

Precision (between-day): 4.9–11.1 % (for titer controls T1–T3, T0 and 2 human sera from 15 determinations on separate occasions

Accuracy: cannot be determined in a semi-quantitative assay

Linearity: cannot be tested since no standard curve exists

Matrix effects: cannot be tested since no standard curve exists

Specificity: due to structurally similarity of insulin analogues crossreactivity of antibodies is usually high

Limit of quantitation: cannot be defined for a semi-quantitative method (precision of double determinations is below 15 % down to the non-specific binding and between-day precision of the negative serum controls (about 4 % B/T) is below 10 %

Reference range:

The binding values of 20 healthy blood donors were determined e.g. with ^{125}I-human insulin tracer. It can be considered that a binding value higher than the mean value plus 3 standard deviations (e.g. 4.1 % B/T found with a tracer lot for human-insulin tracer binding) indicates the presence of antibodies.

For evaluation of antibody positive samples a negative control has to be determined in each individual run.

An absolute change in binding values > 10 % B/T from baseline to endpoint (in clinical studies) when measured in the same run is considered as significant.

Application data:

The binding values for Type II diabetic patients without prior insulin therapy ranged from 2.5–8.7 % B/T (mean 3.6 % B/T), whereas Type I diabetic patients that had been pretreated with insulin showed significantly elevated binding values before and after treatment ranging from 2.7–83.8 % B/T (mean 21.5 % B/T).

This is in agreement with published data that insulin antibodies are formed in IDDM (Type I diabetic patients).

Note: Insulin levels are determined to be lower in Type I compared to Type II patients since antibodies bind to the insulin administered and it is not available as free and acting insulin. On the other hand the results cannot be interpreted as free insulin concentration because antibodies also interfere with the immunological determination of insulin by competition with the antiserum. Determination of free insulin (not bound to antibody) requires a pretreatment of serum with PEG.

Method comparison:

A commercially available RIA for determination of free anti-insulin antibodies (CIS bio international) was used for method comparison with insulin tracer binding test for Type I diabetic patients. The CIS test uses a ^{125}I-porcine insulin tracer for insulin antibody determination. Linear regression analysis yielded a correlation coefficient of 0.94 for human insulin antibodies. The absolute binding values were 10–15 % lower in the commercial test than in the in-house test.

Note that absolute binding values cannot directly compared between different tests due to the semi-quantitative nature of the tracer binding test.

CRITICAL ASSESSMENT OF THE METHOD

A quantitative test for the determination of anti-insulin antibodies is not possible due to the different nature of insulin antibodies that can be formed. The criteria for evaluation of antibody positive samples are somewhat arbitrary, e.g. selected pool of healthy blood donors, definition of mean + 3 standard deviations, varying binding with different tracer batches, consider an absolute change in binding values > 10 % B/T in clinical studies from baseline to endpoint when measured in the same run as significant.

MODIFICATIONS OF THE METHOD

A slightly modified method (MDS Pharma Services Switzerland AG, CH-8602 Wangen) was used in the development of Apidra (a synthetic short acting insulin).

This method is based on the same principle as described above with only minor changes in the assay procedure.

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II.R.3 Immunoradiometric Assay (IRMA) of Ferritin with Bead Separation

PURPOSE AND RATIONALE

Ferritin, a macromolecule with a molecular weight of ~ 440 000 Da, is an iron storage protein which

occurs ubiquitously in the organism. A protein shell (apoferritin) surrounds a nucleus containing iron oxy-hydroxide phosphate. Every molecule can absorb up to 4000 atoms of iron and can contain up to 20 % of its weight as iron when saturated.

Functionally, ferritin, in conjunction with transferrin, regulates the movement of iron from the gastrointestinal tract to various tissues responsible for iron storage as well as to the bone marrow.

The ferritin found in each tissue has a characteristic structure, and exists as isoferritin. Of special interest is the serum ferritin, which is considered to be different from any specific isoferritin. The serum ferritin level reflects the iron status of the body.

More detailed information to ferritin is given by Crichton (1973) and Jacobs et al. (1975).

The RIA-gnost Ferritin kit (no more commercially available) from formerly Behringwerke AG, Radiochemical Laboratory described below uses the principle of an immunoradiometric assay (IRMA). It is a two-site solid phase assay of the sandwich type, based on a plastic bead as solid phase to which the anti-ferritin antibody adheres. The antibody-solid phase is incubated with standards or serum samples containing ferritin and in this process the ferritin in the solution is bound quantitatively to the solid phase via the antibody. The amount of ferritin bound to the solid phase is then determined by a reaction with ^{125}I-labeled anti-ferritin antibody. An antibody-ferritin-^{125}I-antibody complex is thus formed.

The RIA-gnost Ferritin kit uses anti-ferritin antibodies of two animal species, which have been raised against different organ isoferritins. Since the immunological characteristics of the ferritin circulating in the blood are not precisely known, it is necessary for the assay method to have broad specificity for organ isoferritins, measuring them all with practically equal potency. The antibodies used in RIA-gnost Ferritin meet this requirement.

It is typical for an immunoradiometric assay that a "high dose hook" effect occurs in the region of very high concentrations. Therefore, sera with concentration above the highest standard S7 have to be diluted with the dilution serum included in the kit.

PROCEDURE

Reagents

The kit consists of:

100 plastic beads with anti-ferritin immunoglobulin from sheep ("anti-ferritin beads")

1 anti-ferritin immunglobulin from rabbit iodine-125-labeled, < 4 μCi ("anti-ferritin-^{125}I-antibody"), lyophilised

8 ferritin standards, S0–S7, concentration in the range of 0–400 ng ferritin/ml (related to the protein fraction of the molecule), lyophilizates from serum

1 test serum, ferritin concentration declared, lyophilised

1 buffer, pH 8.6, protein carrier solution, lyophilised

1 diluent serum, lyophilised

1 pair of forceps.

Assay Procedure

- Number sufficient round-bottomed incubation tubes (3–5 ml) for 1 test serum, up to 41 serum samples and total activity. Assay duplicate samples
- Place one anti-ferritin bead in each tube (exception: total activity) by means of the forceps contained in the kit
- Add 200 μl buffer solution to each tube (exception: total activity)
- Dispense the standards, when completely dissolved, after mixing briefly on a rotary mixer, in aliquots of 100 μl into the tubes. Use a new pipette tip for each standard
- Dispense 100 μl of test serum / serum sample, after mixing on a rotary mixer, into the tubes. Use a new pipette tip for each serum sample
- Mix all tubes briefly on the rotary mixer; cover the whole test tubes rack with Parafilm and place the rack for the incubation on a horizontal shaker, shake for 2 hours at room temperature (22 ± 5 °C) with ~ 300 rpm (250–350 rpm)
- Carefully aspirate the incubates; it is best to use a Pasteur pipette (or a pipette tip) connected to a suction pump. The beads should remain in the tubes
- 2 ml destilled water are dispensed into each tube (exception: total activity) in such a manner that each bead is agitated. Aspirate the water.
- Dispense 300 μl of the anti-ferritin-^{125}I-antibody solution into each tube and shake the test tubes rack briefly by hand. Then cover the rack with Parafilm and place it on the horizontal shaker and shake for 3 hours at room temperature (22 ± 5 °C) with ~ 300 rpm (250–350 rpm)
- Carefully aspirate the incubates (exception: total activity); the beads should remain in the tubes
- Dispense 2 ml destilled water into each tube (exception: total activity) in such a manner that the bead is agitated. Aspirate the water.

- The tubes containing the beads and "total activity" are measured directly in a gamma scintillation counter for 2 minutes. Between 40 000 and 60 000 cpm are to be expected for the "total activity" tubes. This depends on the age of the kit and the efficiency of the counter.

EVALUATION

The cpm of the standards S0–S7 are calculated as a percentage of the cpm of the "total activity" and are plotted as individual values against the appropriate ferritin concentration. The "best fit" standard curve is drawn through these points.

The mean values are calculated for the two counts for the test serum and the patient serum samples. The ferritin concentrations per ml serum are read from the standard curve.

CRITICAL ASSESSMENT OF THE METHOD

The method described above was cumbersome in handling because of placing each individual anti-ferritin bead into the tubes by means of the forceps.

A data evaluation program with an appropriate fitting algorithm was not yet available.

MODIFICATION OF THE METHOD

Since the commercial distribution of the RIA-gnost Ferritin kit (beginning of the 1980s), improvements regarding easier handling, more sophisticated data evaluation and higher throughput have been made.

For instance, the next generation of RIA-gnost Ferritin was based on the coated tube technology which saved the manual transfer step of the anti-ferritin beads into the tubes. Further development led to semi- and fully automated assays based on enzyme / fluorescence and chemiluminescence immunoassays commercially available from several companies.

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II.R.4 Specific Radioimmunoassay of 314-d Isomer of Beraprost in Human Plasma

PURPOSE AND RATIONALE

Beraprost (*CAS*: 88475-69-8) is an equimolar mixture of two racemates (four isomers), APS-314d,l and APS-315d,l. Radioimmunoassays of the isomers of beraprost (an orally active prostacyclin PGI_2 analog which has anti-platelet and vasodilating effects) were carried out by combining a serum from a rabbit immunized with beraprost conjugated to bovine albumin and radioiodinated tracers prepared from each isomer. The main biological activity is contributed to the APS-314d isomer and the clinical daily dosing is in the range of 40 to 120 μg. APS-315d has a lower activity whereas APS-314l and APS-315l have almost no activity. The anti-beraprost serum contains three families of antibodies specific for the APS-314d and APS-315d isomers and for the mixture of the two l-isomers. The binding with either APS-314d, APS-315d or APS-315l tracers made it possible to determine the plasma concentrations of APS 314d isomer, APS 315d isomer and of the mixture of the two l-isomers during clinical studies (Mouren 1995).

Each assay method is specific with respect to the other stereoisomeric components of beraprost and related metabolites and also sensitive. The limit of quantification can be taken as 3 pg/ml for APS 314d, 12 pg/ml for APS 315d and 10 pg/ml for APS 314l + APS 315l, when applied to 0.1 ml of human plasma.

Reagents

- APS-314d Na
- sodium chloride (Prolabo)
- sodium azide (Merck)
- sodium dihydrogen phosphate monohydrate (Carlo Erba)
- anhydrous sodium hydrogen phosphate (Prolabo)
- powdered gelatin (Merck)
- triton X-100
- sodium salicylate (Prolabo)
- rabbit γ-globulins
- (Cohn's fraction II/III, G-0261, Sigma)
- Freund's complete and incomplete adjuvants (Difco)
- human blank plasma.

Preparation of Reagents

Immunogen and Rabbit Antiserum

Beraprost was linked to bovine serum albumin according to Erlanger B.F. et al. (1957).

A serum containing an antibody to APS-314d was produced in New Zealand rabbits. The immunogen (10 mg) was dissolved in 10 ml of a 0.9 % sodium chloride solution and emulsified with 10 ml of Freund's complete adjuvant by repeated passages through a syringe. Each rabbit received 1 ml of emulsion (500 μg of immunogen) subcutaneously in 10 sites on the back. Three booster injections were given at

4 week intervals with the same dose of immunogen but using Freund's incomplete adjuvant. Blood sera were collected 3 weeks after the last booster and checked for titer, affinity and specificity without any purification.

The sera can be stored, either undiluted at –30 °C for several years, or diluted 1:100 in a buffer at +4 °C for up to six months, without significantly losing activity. At the time of the assay, the selected serum is diluted 1:50 000 in assay buffer containing sheep anti-rabbit γ-globulins and rabbit γ-globulins.

Sheep anti-rabbit gammaglobulins for separation step
An antibody to rabbit γ-globulins was raised by immunizing sheep with rabbit γ-globulins.

Each sheep initially received 5 mg of protein in multiple injections at intervals of 5 weeks until a serum with a satisfactory precipitating potency was obtained. Generally, 2 booster injections were sufficient and blood was withdrawn 15 days after the last booster. The serum was purified by precipitation of the immunoglobulins with half a volume of a saturated ammonium sulfate solution and then dialysed for 4 days against a 0.9 % sodium chloride solution which was replaced every day. It was stored at –30 °C.

^{125}I-APS-314d
The radioactive tracer used was obtained by iodine 125-labeling of the conjugate of APS-314d and histamine, according to Hunter and Greenwood's method (1962). Stored at –30 °C the iodinated tracer was stable for at least 3 months without any loss of performance. It was diluted extemporaneously in assay buffer to obtain a solution containing 80 000 cpm per ml.

Assay Buffer
The assay buffer was prepared by dissolving 9 g of sodium chloride, 1 g of sodium azide, 1 g of gelatine and 2.5 g of sodium salicylate in 1 litre of a 0.1 M phosphate buffer containing 0.01 % (v/v) of triton X-100. It was stored at ambient temperature.

APS-314d Standard Solutions
A 100 μg/ml solution (M1) of APS-314d Na was prepared by dissolving the compound in demineralised water. This was stored at +4° C. Diluted 1:100 in phosphate-gelatine buffer, it yielded a solution (M2) of 1 μg/ml from which were obtained by 1:100 and 1:40 dilutions and then by successive 1:2 dilutions, solutions (A to J) containing 250 to 0.49 pg of APS-314d Na per ml of buffer. These solutions were prepared extemporaneously on the day of the assay.

PROCEDURE
Assays could be carried out with 0.1 ml of undiluted plasma samples or after dilution in human blank plasma. The standard curve was established in the presence of 0.1 ml of human blank plasma. The operating procedure is as follows:
For calibration: to 3 replicates of:
0.1 ml of assay buffer (maximum binding Bo)
or 0.1 ml of the M2 solution (non-specific binding)
or 0.1 ml of the standard solutions (i.e. 0.049 to 25 pg)
were added, in 12 × 65 mm glass test tubes, 0.1 ml of human blank plasma and 0.2 ml of assay buffer.
For plasma samples: to 3 replicates of:
0.1 ml of undiluted plasma samples
or to 0.1 ml of diluted plasma samples
were added, in 12 × 65 mm glass test tubes,
0.3 ml of assay buffer.

In all cases, the tubes were allowed to stand for 1 hour at room temperature, following which iodinated APS-314d (4 000 cpm) was added in 0.05 ml of assay buffer to all tubes, as well as to 3 tubes labeled as "total radioactivity". With the exception of the latter, the tubes were vortexed for a few seconds and then they received 0.05 ml of a mixture containing anti-beraprost serum (1:50,000 dilution) sheep anti-rabbit γ-globulins (200 μl/ml) and rabbit γ-globulins (200 μg/ml) (the quantity of rabbit γ globulins involved depends on the quality of the purified sheep anti-rabbit γ-globulins serum and must be checked with each preparation of this second antibody).

After mixing the reagents, incubation was carried out at +4° C for 3 days. The bound fractions were then sedimented by centrifugation for 30 min at 3000 rpm (about 2200 g) at +4 °C. The supernatant phases were eliminated by inverting the tubes which were then allowed to stand on cotton-wool for 30 min.

The total amount of radioactivity involved and the amounts bound to the antibody were measured for 2 min on a well-type gamma counter. The found values were corrected for non-specific binding and the concentrations of APS-314d in samples were obtained from the calibration curve smoothed by a 4-PL model.

Validation and Cross-reactivity Check
The assay method was validated and assay perfomance characterisation established:

Accuracy: the absolute value of the difference within and between-day was lower than or equal to 9.0 % and the limit of quantification can be taken as 3 pg/ml

Table 1

	Standard point	Samples	Human blank plasma	Assay buffer		Iodinated APS-314d	Mixture containing rabbit anti-Beraprost serum, sheep anti-rabbit γ-globulins and rabbit γ-globulins
Total radioactivity (3 replicates)					Allow to stand for 1 hour at room temperature	0.05	
Maximum binding Bo (3 replicates)			0.1	0.3		0.05	
Non-specific binding (3 replicates)	0.1 (M2)		0.1	0.2		0.05	0.05
Standard Curve (3 replicates)		0.1		0.3		0.05	0.05
Samples (3 replicates)		0.1		0.1		0.05	0.05
Quality controls (3 replicates)							

Precision: the within-assay coefficient of variation was between 4.0 and 10.1 % and the between-assay coefficient of variation was between 6.8 and 10.4 % over the range 3.13 to 100 pg/ml

Specificity: determined by Abraham's method (1974) at 50 % inhibition of maximum binding, the percentage cross-reactions of the APS-315d Na, APS-314l Na and APS-315l Na isomers in the assay of APS 314d Na, were 2.4, 0.5 and 0.1 % respectively. This establishes, by comparing to the results using the beraprost (4 isomers) as radiolabeled reagent, the complete fall down of the cross-reactivity and allows its use after administration of beraprost sodium in human.

CRITICAL ASSESSMENT OF THE METHOD

This way of using a single isomer labeled with high purity is an efficient alternative in order to achieve a stereospecific immunoassay. This immunoassay gives reliable informations in terms of safety pharmacokinetics. The application in pharmacokinetic studies of each isomer can be made. Only few amounts of isomer for tagged molecule and standard curves are requested.

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II.R.5 Enzyme Sandwich Immunoassay with Monoclonal Antibodies for the Detection of HBeAg and anti-HBe

PURPOSE AND RATIONALE

The commercially available Enzygnost HBe monoclonal kit (distributed by Dade Behring) can be considered as an example for an immunoassay which combines many favorable features, e.g. nonisotopic label, sandwich immunoassay principle, use of monoclonal antibodies, determination of antigen and anti-antigen antibodies in a single test, microtiter plate format, possibility for semi- and fully automation.

HBeAg and the corresponding antibody anti-HBe are found exclusively in connection with hepatitis B virus infection.

Seroconversion from "HBeAg positive" to "anti-HBe positive" is a favorable sign indicative of convalescence and an uncomplicated course.

Further information is found in Hollinger (1990), Bruss et al. (1988), Carmen et al. (1989), Brunetto et al. (1990).

The Enzygnost HBe monoclonal kit is based on the sandwich principle. HBe antigen (HBeAg) in the sample binds to monoclonal anti-HBe antibodies to the surface of the walls in the microtitration plate. Unbound sample constituents are then removed by washing, peroxidase-conjugated monoclonal antibodies to HBeAg are added and bind to the remaining free antigen determinants.

The excess conjugate is washed out, hydrogen peroxide substrate with chromogen is added and reacts with the bound peroxidase producing a blue color. This enzymatic reaction is stopped by the addition of a stopping solution and the resulting yellow color is measured. The resultant color intensity is proportional to the concentration of HBeAg in the sample.

In the test for anti-HBe, anti-HBe antibodies in the sample block the HBeAg pipetted with the positive HBeAg reagent. When the resulting mixture is then used in the HBeAg test, no HBe antigen or minimal HBeAg is detected if the sample is positive for anti-HBe. The color intensity of the sample is inversely proportional to the concentration of anti-HBe.

PROCEDURE

Reagents

Enzygnost HBe monoclonal (test plate): microtitration plate coated with monoclonal antibodies to HBe antigen

Anti-HBe/POD Conjugate: Monoclonal anti-HBe, conjugated with peroxidase (POD),

preservative: phenol (max. 1 g/L)

HBeAg reagent, positive: genetically engineered HBe antigen, stabilized with bovine serum albumin and Synperonic, lyophilised, preservative: phenol (max. 1 g/L)

Anti-HBe Control, positive: Human serum containing monoclonal antibodies to HBe antigen,

preservative: phenol (max. 1 g/L)

HBe Control, negative: Negative human serum for the HBeAg and anti-HBe tests,

preservative: phenol (max. 1g/L)

Washing Solution POD (concentrate): Phosphate buffer solution (90 mmol/L) containing Tween (18g/L),

preservative: phenol (max. 1 g/L)

Buffer/Substrate TMB: Hydrogen peroxide (approx. 0.1g/L in acetate buffer solution (25 mmol/L)),

preservative: 1-butanol (max. 10 mL/L)

Chromogen TMB: Tetramethyl benzidine dihydrochloride (5 g/L)

Stopping Solution POD: 0.5 N sulphuric acid

Preparation of the Reagents

For each test plate, dilute 20 mL washing solution POD to 400 mL with destilled or demineralised water.

Working Chromogen Solution: For each test plate, dilute 1 mL Chromogen TMB with 10 mL Buffer/Substrate TMB in the empty plastic bottle supplied with the kit and store closed and protected from light.

Working solution of HBeAg reagent, positive: Before the test reconstitute a vial of of HBeAg Reagent, positive with 13 mL destilled or demineralised water. Shake gently to mix and ensure that the reagent has completely dissolved before use (approx. 30 min).

Equipment

Incubator: Covered water bath (+37 ± 1 °C) or comparable incubation methods

BEP II: For automatic dispensing of reagent and washing

BEP III: For automatic processing of the test after dispensing the samples as well as for evaluation

BEP 2000: For fully automatic processing and evaluation of the test

Assay Procedure

Procedure for HBeAg test using the BEP II (detection of HBeAg):

1. Dispense samples: Pipette 100 µL/well HBe Control, negative into 4 wells, 100 µL of HBeAg Reagent, positive into the next well and then fill the following wells with 100 µL of undiluted sample. At the end of the series / plate pipette 100 µL of HBeAg Reagent, positive once more and cover with foil.
2. Incubate: Incubate at 37 ± 1 °C for 60 ± 2 min.
3. Wash: Remove the foil, aspirate the wells and wash 2 × with approx. 0.3 mL/well of diluted washing solution. Proceed immediately to the "Dispense conjugate" step.
4. Dispense conjugate: Fill each well with 100 µl of Anti-HBe/POD Conjugate, cover with fresh foil and immediately place into the incubator.
5. Incubate: Incubate at 37 ± 1 °C for 60 ± 2 min, then proceed immediately to the "Wash" step.
6. Wash: Remove the foil and wash 4 times as described in "3. Wash".
7. Dispense substrate: Pipette 100 µL of the Working Chromogen Solution into each well and cover the plate with fresh foil.

8. Incubate: Incubate at +18 to + 25 °C for 30 ± 2 min, protected from light.
9. Stop reaction: Remove the foil and add 100 µL of Stopping Solution POD to each well, keeping to the same timing as in "8. Dispense substrate".
10. Read: At 450 nm within one hour.

The use of a photometer with two wavelengths (measurement and reference beams) is to be recommended. The absorbances of the control samples and patient samples are to be measured at a wavelength of 450 nm. The wavelength recommended for the reference reading is 650 nm (if necessary between 615 and 690 nm).

Procedure for Anti-HBe Test Using the BEP II (Detection of Anti-HBe)

1. Dispense samples: Pipette 25 µL/well HBe Control, negative into 4 wells, 25 µL of Anti-HBe Control, positive into the next well and then fill the following wells with 25 µL of undiluted sample. At the end of the series / plate pipette 25 µL of Anti-HBe Control, positive once more.
2. Binding of the anti-HBe: Directly after dispensing the samples, add 75 µL of HBeAg Reagent, positive, (working solution) into each well containing the 25 µL of sample or control, then seal with foil.

Perform the subsequent steps as described for the HBeAg test, i.e. continue processing starting at "2. Incubate".

Test Procedure Using the BEP III

Before using the BEP III, perform the sample dispensing steps (Section 1 from "procedure using the BEP II"). Immediately afterwards place the uncovered test plates into the BEP III. The test is then processed fully automatically.

Test Procedure Using the BEP 2000

The sample dispensing steps and the subsequent processing of the test are performed fully automatically by the analyser.

EVALUATION

The evaluations are performed automatically if the BEP III or BEP 2000 is used. The following sections apply if the measurements are carried out without a software.

HBe Test

Calculate the mean absorbance value of the negative controls, then calculate the cut-off value by adding 0.050:

$A_{(neg)} + 0.050 = \text{cut-off}$

Based on the criteria of the test, the samples are classed as follows:

1. $A_{sample} <$ cut-off-10 % = HBeAg negative
2. cut-off-10 % $\leq A_{sample} \leq$ cut-off + 10 % = HBeAg equivocal
3. $A_{sample} >$ cut-off + 10 % = HBeAg positive

Anti-HBe Test

Calculate the mean absorbance value of the negative controls, then calculate the cut-off value by multiplication with 0.5:

$A_{(neg)} \times 0.5 = \text{cut-off}$

Based on the criteria of the test, the samples are classed as follows:

1. $A_{sample} >$ cut-off + 10 % = anti-HBe negative
2. cut-off-10 % $\leq A_{sample} \leq$ cut-off + 10 % = anti-HBe equivocal
3. $A_{sample} <$ cut-off-10 % = anti-HBe positive

Samples with equivocal results must be retested in double determinations. If in the retest the mean value of double determination is greater than or equal to the cut-off, or less than the cut-off, the initial equivocalresult can be ignored and the sample to be considered reactive or negative as appropriate.

HbeAg

Precision (within-day): 3.8–6.4 %

Precision (between-day): 8.7–11.4 %

Anti-Hbe

Precision (within-day): 3.2–7.4 %

Precision (between-day): 3.4–6.3 %

CRITICAL ASSESSMENT OF THE METHOD

The method involves a lot of incubation and washing steps which is due to the use of the enzyme label. In addition, the incubation conditions (time and temperature) have to be kept very strictly to meet the product specifications. These requirements can hardly be fulfilled for longer sample batches by a manual procedure. Therefore, automation at least for the processes of dispensing of reagents and washing is recommended. The best results, however, will be obtained with the

analyzers optimised for product performance ("closed systems" with reagents and instrumention from the same manufacturer). User defined modifications are not supported by the manufacturer and may affect the assay results.

The example described above supports the general statement made in the introduction that EIAs which have to fulfill high quality requirements with regard to sensitivity and robustness likewise need sophisticated techniques and/or automation.

MODIFICATIONS OF THE METHOD

The number of incubation and washing steps can be greatly reduced by employing "direct" instead of "indirect" labels (enzyme generates a signal only by reaction with a substrate). In addition to the radioactive label, nonisotopic alternatives with improved features (e.g. time-resolved fluorescent, chemiluminescent, electrochemiluminescent labels) have become available. Immunoassays based on these labels show an excellent performance even by manual procedure, automation is only required for a higher sample throughput. The advantages of luminogenic compared to chromogenic substrates (e.g. higher sensitivity and dynamic range of signal) are often used to replace the chromogens by luminophors in already available tests and systems.

An example for an anti-HBe test with a direct label is the electrochemiluminescence immunoassay "ECLIA" from Roche Diagnostics (automated on "Elecsys" immunoassay analyser), whereas the Immulite anti-HBe test from Diagnostics Products Corp. represents an enzyme-amplified chemiluminescence immunoassay with sustained signal (automated on the Immulite chemiluminescent system).

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Chapter II.S
Clinical Studies – Typical Designs

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INTRODUCTION

Drug efficacy and response is a function of drug concentration over time. In clinical pharmacokinetic studies, aspects of drug absorption, distribution, metabolism, and excretion over time are assessed. In the early clinical development the pharmacokinetics of a drug is studied in healthy subjects followed by studies in patient population(s) with the aim to find the relevant dose in the target population(s). Particular pharmacokinetic studies in special populations assess the necessity of a dose adjustment from the planned/established clinical dose for patients.

In the following sections, typical clinical pharmacokinetic studies are presented with results and conclusions, along with special emphasis on the individual study rational, objective, design, and evaluation technique. In addition, a critical assessment of the method, as well as potential modifications and alternatives are presented.

For the purposes of simplicity, the description of each study is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety (and pharmacodynamic) parameters are also studied.

The protocols were developed with consideration of the current good clinical practices and conducted in compliance with the protocol that had received prior independent ethics board approval.

The principles and practices concerning the clinical conduct with particular emphasis on ethical aspects are stated in guidelines. These principles have their origins in 'The Declaration of Helsinki'.

The design and conduct of the clinical studies presented in the examples below was in conformance with these principles. Subjects were included only after informed consent was given. All studies were part of a sound clinical development plan of the sponsor.

The protocols were subject to critical review, and it was assured that the information they contain is consistent with the actual risk-benefit evaluation of the investigational product. The respective internal

review boards of the sponsor had approved them before finalization.

Assays used for bioanalytical measurements were validated, as the complete evaluation, assessment, and reporting of these clinical pharmacokinetic studies followed international and scientific quality standards.

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II.S.1 Exploratory Assessment of Drug Dose Linearity/Proportionality and its Use for Study Optimization

PURPOSE AND RATIONALE

The first tolerability studies in early clinical development always provide pharmacokinetic (PK) data over a considerable dose range. Especially the explorative first-in-man study with escalating single doses, or an explorative proof of principle study with escalating multiple doses provides a valuable basis for an exploratory assessment of dose linearity/ proportionality of drugs in humans. In addition such an assessment can directly help within the same study to optimize the dose selection and dose progression. Already in this early phase of the development, these data are going to support exposure-response relationships, and thus a potential submission (US FDA 2003, ICH E4 1994).

Information on dose linearity/proportionality in humans is required during later development, if the dosage form is to be modified (EU CPMP 1999), and especially if several strengths are in use (EU CPMP 2001).

A priori, however, animal data can provide hints with respect to the likelihood of observing a non-linear relationship of the administered dose and the resulting exposure as reflected by PK parameters. But even if doses were compared on a 'per kg' basis, animal studies might be misleading, due to the different physiology and extrinsic factors in animals and man.

Dose Linearity/Proportionality typically is assessed, based on exposure-related PK-parameters measured in the systemic circulation (blood), like the AUC for a specified time-interval, or sometimes also based on the peak values (C_{max}), but can also be estimated based on PK-parameters which are expected to be dose independent, like Clearance (CL), (terminal) Half-Life ($t_{1/2z}$), or Volume of Distribution (V). And for a drug which is mainly eliminated via the renal route, this assessment of dose-proportionality can also be based on the amounts of drug excreted in urine over a specified time, or extrapolated to infinity. In both cases, it is important to use an assay with a sensitivity sufficient to quantify the amounts of drug completely.

PROCEDURE

The design of an exploratory assessment of dose linearity/proportionality during the conduct of a first-in-man study for candidate drug (XYZ1234) is presented below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety parameters are in the main focus.

Protocol Outline

Single oral dose-escalation study, to study safety, tolerability, pharmacokinetics and pharmacodynamics of XYZ1234 in healthy male subjects (first-in-man study).

Secondary Objective

One of the secondary objective of the study was to determine the pharmacokinetics (PK) of ascending single oral doses of XYZ1234.

Study Design

The study had a single center, double-blind, placebo-controlled, randomized, single oral dose-escalation study design. Eight healthy male volunteers per dose step were to be randomized and treated with up to 2 of the following escalating dose steps: 10, 25, 50, 100, 200, 300, and 400 mg XYZ1234, or placebo, with a washout period of at least 2 weeks between the treatments. Six subjects in each dose step were randomized to XYZ1234 and 2 subjects to placebo.

Starting with the lowest dose, each of the subsequent doses was administered only if the preceding dose was safe and well tolerated. The decision to proceed to the next higher dose was based on the full range of safety parameters, and pharmacokinetic data.

Eligible subjects entered the study unit the evening before study drug administration, and were to be assessed for their baseline characteristics on the morning of the day of drug administration. After each oral dose, subjects remained in the study unit for 48 hours.

Inclusion Criteria

Healthy male volunteers, aged between 18 to 45 years, inclusive.

Treatments

In this study, a clear, colorless solution of varying concentrations of active substance XYZ1234 was used, to provide the same dosing-volume for the solution in each dose step. The escalating, double-blind doses of XYZ1234 oral solutions were administered at doses of 10, 25, 50, 100, 200, 300, and 400 mg, or placebo.

The decision to proceed to the next higher dose was taken, based on the full range of safety parameters, and the bioanalytical data (with pharmacokinetic parameters for the last-but-one dose step, which had to be available and acceptable for the next planned dose step, based on an adequate extrapolation). This could result in the planned higher dose step(s) being skipped, or conversely, additionally to include a dose below the starting dose of 10 mg. The doses to be administered might have to be modified (decreased or interim steps be defined) as needed, according to the ongoing benefit risk assessment during the conduct of the study. The dose progression was to be continued until the maximum tolerated dose had been attained, or if derived from bioanalytical data, other findings were to be expected which prohibited further dose-increases (e.g. prohibitive non-linearity, or the AUC being too large compared to the AUC from toxicology).

Pharmacokinetic Data

Concentrations of XYZ1234 in urine and plasma were determined, and used for calculation of the PK-parameters.

If feasible and adequate, an explorative investigation on potential metabolites in plasma and urine was to be performed.

EVALUATION

Due to the small sample size, all variables were only presented descriptively for the different bioanalytical data and pharmacokinetic parameters calculated.

Individual plasma concentrations of XYZ1234 were tabulated together with standard descriptive statistics. Individual and median profiles were presented graphically.

PK Parameters

If possible the following PK parameters were to be determined, based on plasma concentrations of XYZ1234 using non-compartmental, or if adequate, compartmental procedures: at least maximum concentration (C_{max}), time to maximum concentration (t_{max}), area under the concentration-time curve from time of drug administration to t hours ($AUC_{(0–t)}$), area under the concentration-time curve from time of drug administration extrapolated to infinity (AUC_{inf}), terminal elimination half-life ($t_{1/2z}$), mean (residence) time (M[R]T), as well as relative total oral clearance (CL/f) and relative volume of distribution during the terminal phase (Vz/f)).

For urine data, the individual and mean fractional and cumulative urinary excretion of XYZ1234 were calculated; the cumulative excretion profiles were represented graphically; fractional and total urinary excretion ($Ae_{(t2\text{-}t1)}$; $Ae_{(0\text{-}48\ h)}$), urinary recovery (% of administered dose), and renal as well as non-renal clearance (CL_R, CL_{NR}) were determined.

Explorative dose-proportionality was to be investigated in parallel to the progress of the dose escalation, using dose normalized values (on a dose per body weight basis) for AUC, C_{max}, and Ae (characterized by an additional suffix "$_{norm}$") and/or by adequate regression analyses of all parameters. Predictions for the next dose steps were to be derived, based on these findings.

CRITICAL ASSESSMENT OF THE METHOD

As rationale for selection of the dose range, doses providing a pharmacological effect (active doses) and doses being at the upper dose limit (NOAEL doses) were used. The dose-progression was to be continued until the MTD has been attained, or derived from bioanalytical data, until other prohibitive findings (prohibitive non-linearity, area under the plasma-concentration time curve (AUC) exceeding that AUC from the toxicological studies) arise.

The described evaluation provides a tool, also called 'online PK', which allows adjusting the dose in this 'first-in-man' study on a very flexible basis. Consequently, this flexible dose-scheme is described already in the study protocol. The main prerequisite is, besides an adjustable dosing form, an immediate shipping and evaluation of the bioanalytical samples.

On an explorative basis, the relationship of concentrations resulting from the different doses can be conveniently studied, using an evaluation as described here. Typically, this evaluation is part of the first study in humans (which is always a single dose study), but could also be applied for early multiple dose studies in analogy.

As mentioned earlier, this type of supportive study provides explorative data related to dose linear-

ity/proportionality. In the context of the described study, these data are then used to predict the exposure for further planned dose steps, (inside) and especially outside the dose range investigated so far.

At least if a notable non-linear effect is seen by this explorative evaluation, then a more elaborate study will need to be performed.

MODIFICATIONS OF THE METHOD

Typically, a dose-proportional increase of exposure (AUC) cannot be expected, if a concentration-dependent mechanism of distribution and elimination exists. Especially the renal clearance of a drug is quite often concentration dependent, if strong binding to blood constituents (proteins, cells) plays a major role. In those cases it is recommendable to use free fractions of the drug instead, in order to find a parameter independent of the total concentrations, which then might be predictive for dose-proportionality.

If a pivotal investigation of dose linearity/proportionality becomes necessary, a dedicated study has to be conducted. It will typically be a study where the subjects get at least three different doses in a randomized, intra-individual crossover. The dose steps should cover the clinical recommended dose range, and – if possible – should also include the maximum tolerated dose; they should ideally increase in a geometric progression.

However, this registration study should best be performed when the therapeutic dose range is clearly established, and the final drug formulation is available.

REFERENCES

EU CPMP (1999) Note for Guidance on Modified Release Oral and Transdermal Dosage Forms: Section II (Pharmacokinetic and Clinical Evaluation) July 1999

EU CPMP (2001) Note for Guidance on the Investigation of Bioavailability and Bioequivalence. July 2001

ICH-E5 (1998) Ethnic Factors in the Acceptability of Foreign Clinical Data. March 1998

US FDA (2003) Draft Guideline for Industry: Pharmacogenomic Data Submissions. November 2003

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above under "Procedure" is presented below. It should be noted that in this specific example-study for the first application in man, only a liquid formulation was available, which – due to low solubility of the drug – generated notable inter- (and intra-) individual variability.

Results

Only at the higher 200 and 300 mg doses, XYZ1234 could be detected in plasma 48 h after administration. Following the lower doses of 10 and 25 mg, the last quantifiable concentration was detected after 3 and 4 h respectively. The quantification-limit of the analytical method might have impeded somewhat the reliability of the PK parameters obtained for the two lowest doses (10 and 25 mg). For example, the low values of the calculated $t_{1/2z}$ for these two doses (see Table 1) probably resulted from not having quantifiable concentrations at time points following 3 and 4 h p.o., with the consequence of having smaller $AUC_{inf,\ norm}$ values for the lower doses (see Figure 1), and a further increase up to the 300 mg dose. This is also the reason why in this case linearity was not studied with the usual statistics (bioequivalence criteria for a dose-2/dose-1 ratio). AUC_{inf} nevertheless exhibited in average a dose-proportionality, the 95 % confidence range includes the origin of the coordinates (see Figure 2).

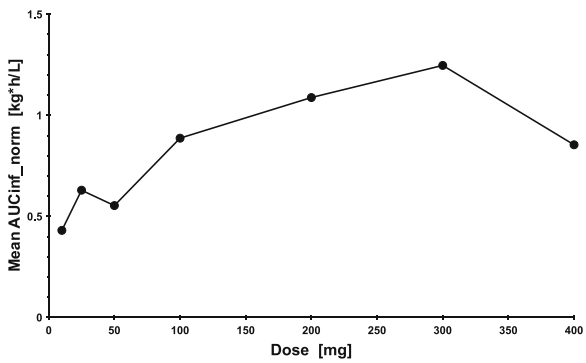

Fig. 1. Relationship of 'dose per body weight'-normalized AUC_{inf} values for XYZ1234 versus dose.

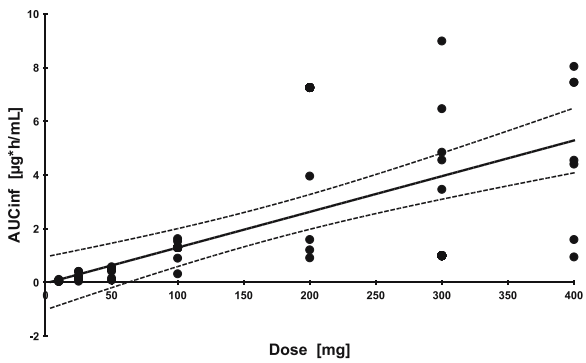

Fig. 2. Relationship of observed AUC_{inf} values for XYZ1234 plasma-profiles versus dose,with linear regression (*bold line*), and the 95 % confidence range (*dashed line*).

Table 1 Synopsis of key PK parameters. AUC_{inf}, C_{max} (including dose normalization to 100 mg), and the terminal half-life.

Dose	N	AUC_{inf} [µg*h/mL]				C_{max} [µg/mL]				$t_{1/2z}$ [h]		
Step		Mean	CV %	Median	Med/ 100 mg	Mean	CV %	Median	Med/ 100 mg	Mean	CV %	Median
10 mg	4	0.056	70.9	0.054	0.535	0.034	65.7	0.035	0.350	1.3	69.5	0.8
25 mg	6	0.208	57.7	0.199	0.794	0.109	49.7	0.111	0.442	2.1	51.2	1.8
50 mg	5	0.353	61.7	0.426	0.852	0.107	60.4	0.116	0.232	4.2	74.6	2.4
100 mg	5	1.129	47.3	1.278	1.278	0.252	55.0	0.223	0.223	4.9	44.1	4.0
200 mg	5	2.840	94.8	1.430	0.715	0.424	47.1	0.347	0.174	14.0	82.6	8.1
300 mg	6	4.828	56.4	4.544	1.515	0.472	50.9	0.448	0.149	12.2	52.6	11.0
400 mg	6	4.486	65.1	4.495	1.124	0.434	89.7	0.272	0.068	7.4	39.0	7.1

Total relative plasma clearance (CL/f) was found to decrease for the reasons mentioned above, leveling off at the 100 mg dose.

C_{max_norm} gradually declined until to the 400 mg dose. C_{max} does not always increase linearly with dose, however this finding might indicate that the rate of oral absorption is declining with increasing doses. AUC_{inf_norm} declined at the 400 mg dose, though the median values for the non-normalized AUC_{inf} were almost identical for the 300 and 400 mg doses (see Table 1). This might indicate that the extent of absorption is maximal and is leveling off at the 300 mg dose.

Comparing the data from the seven dose groups synoptically, it can be seen that the dose normalized exposure [AUC_{inf}] shows much fewer differences for the medians than the dose normalized peak values [C_{max}] (see Table 1). The variability [CV %] is greater than 50 % for nearly all of these observations.

As described, the assessment of the dose linearity/proportionality is hampered in this case by the fact that for the lower doses the profile is not complete assessable, that obviously at the highest dose the resorption is limited, and that the used suboptimal formulation generates a lot of variability. Nevertheless, the example shows that even in this case predictions to support dose escalation were possible, and were even very helpful in that specific study.

II.S.2 Exploratory Evaluation of Time-invariant Steady-state Pharmacokinetics

PURPOSE AND RATIONALE

The first tolerability studies early in a clinical development always provide pharmacokinetic (PK) data over a range of doses, mostly with a single-dose approach. In situations where an explorative proof of principle study with escalating multiple doses in patients is needed as the GO/NO GO criterion for further development, the assessment of steady state conditions in a highly standardized, i.e. a healthy population, has proven to provide meaningful data. Under specific circumstances information from these studies has a pivotal character for a submission (US FDA 2003, ICH E4 1994). The definition of dose linearity/proportionality of drugs in humans after repeated dosing has a crucial impact on the design of large-scale studies in patient settings, including special patient populations (see other contributions in this section).

PROCEDURE

The design of an exploratory assessment of dose linearity/proportionality during the conduct of a multiple-dose study for candidate drug (XYZ1234) is presented below. In this case study a drug was investigated that had shown a terminal half-life of about 24 hours (h) in single-dose trials. In the first clinical studies with different solid formulations, high inter- and intra-subject variability was observed in plasma concentrations and derived pharmacokinetic variables. Data from a study that compared a new formulation to a conventional one and to an oral solution showed that intra-subject variability was markedly reduced. As in that study only one dosage strength had been tested, we administered different dosage strengths to assess dose linearity/proportionality at steady state.

Protocol Outline

Pharmacokinetics, safety and tolerability of multiple oral doses of 25, 50 and 75 or 100 mg XYZ1234 given once daily over 7 days as capsules in healthy men in an open-label study.

Primary Objective

To assess pharmacokinetics of multiple oral doses of 25, 50 and 75 or 100 mg XYZ1234 given once daily over 7 days as capsules.

Study Design

Open label study with three treatment groups, with multiple oral doses of 25 mg (Treatment Group I) and 50 mg (Treatment Group II) once daily, immediately after intake of a standard breakfast in a parallel-group design. Safety information and bioanalytical data were reviewed to determine the dose for Treatment Group III (multiple oral doses of 75 or 100 mg XYZ1234).

Inclusion Criteria

Caucasian men between 30 and 65 years of age, with Body Mass Indices of 18–29 kg/m^2. Volunteers were to be healthy, non-smoking, and not receiving regular medication.

Treatments

Treatment A: Multiple oral doses of 25 mg XYZ1234 as capsules, given once daily immediately after intake of a standard breakfast for seven days.

Treatment B: Multiple oral doses of 50 mg XYZ1234 as capsules, given once daily immediately after intake of a standard breakfast for seven days.

Treatment C: Multiple oral doses of 75 or 100 mg XYZ1234 as capsules, given once daily immediately after intake of a standard breakfast for seven days.

Pharmacokinetic Data

Derived from concentrations of XYZ1234 in plasma and urine before and at predefined times after dosing.

EVALUATION

All bioanalytical data, derived PK data, and safety data were listed and descriptive statistics calculated. Individual and median data were plotted. The log-transformed PK parameters AUC and C_{max} were analyzed for dose proportionality. The PK parameters $AUC_{(0–24)}$ and C_{max} were also descriptively analyzed for accumulation ratio.

CRITICAL ASSESSMENT OF THE METHOD

For the definition of the lowest dose, exposure data from animal pharmacological effect models and from single dose studies in man were used. In addition, information from a food screen and the elimination half-life were applied for the design of the study. The assumptions made were a) moderate effect of a high-fat high-calorie breakfast, thus provide a standard continental breakfast on each dosing day and b) elimination half-life of almost 24 h lets one expect that steady state is reached after 5 days of once daily dosing. To confirm the latter expectation, pre-dose and 12 h post-dose plasma concentrations were analyzed on days 2 to 7 and compared to each other. An algorithm for the selection of the highest dose was predefined on the basis of 'online' safety and bioanalytical data, i.e. C_{max}.

MODIFICATIONS OF THE METHOD

If the active moiety of a drug exhibits half-lives that are greater than 2 days, the evaluation of steady state conditions might become problematic in a study with healthy subjects. In these situations the insertion of PK profiling days in patient studies might prove to be helpful. Whereas in single-dose studies a cross-over design might be the proper choice to assess intra-individual variability, a parallel-group or sequential design has to applied in multiple dose settings.

REFERENCES

US FDA Guidance for Industry: Exposure-Response Relationships – Study Design, Data Analysis, and Regulatory Applications. April 2003

ICH E4: Dose-Response Information to Support Drug Registration. March 1994

EXAMPLE

To illustrate the type of data that can be obtained using the study discussed, a summary of the pharmacokinetic results obtained from the study described above under "Procedure" is presented below.

Results

The primary objective of this study was to investigate the pharmacokinetics of XYZ1234 after multiple dosing for seven days at three dose levels. Pharmacokinetic profiles were derived both on day 1 (up to 23.5 h post dose) and on day 7 (up to 119.5 h post dose) and samples were also taken on days 2 to 6 to monitor the approach to steady-state.

Mean C_{max} and $AUC_{(0–24)}$ (= AUC_τ) both on day 1 (C_{max}: 0.46, 1.16 and 3.29 μg/mL; AUC_τ: 7.12, 16.37 and 39.85 μg*h/mL) and on day 7 ($C_{max,ss}$: 0.91, 2.31 and 5.95 μg/mL; $AUC_{\tau,ss}$: 14.17, 33.55 and 74.48 μg*h/mL) increased with increasing dose although the increases were marginally higher than strict dose proportionality would predict: the deviation from linearity was however minor. The median time to reach maximum plasma concentration was 6 h on both profile days and at all doses. The terminal phase half-life of XYZ1234 on day 7 was quite constant with mean values between 19 to 23 h: a slight reduction in half-life (accompanied by a reduced terminal phase volume of distribution) was observed at the higher doses.

Table 2 Pharmacokinetic parameters of XYZ1234 (Day 1).

PK Parameter	MEAN (geometric mean): DAY 1 Treatment A (25 mg)	Treatment B (50 mg)	Treatment C (100 mg)
$C_{max,init}$ [μg/mL]	0.46 (0.45)	1.16 (1.12)	3.29 (3.14)
$T^*_{max,init}$[h]	6.0*	6.0*	6.0*
$AUC_{\tau,init}$[μg*h/mL]	7.12 (6.97)	16.37 (15.87)	39.85 (38.59)

* median values reported

Table 3 Pharmacokinetic parameters of XYZ1234 (Day 7).

PK Parameter	MEAN (geometric mean): DAY 7 Treatment A (25 mg)	Treatment B (50 mg)	Treatment C (100 mg)
$C_{max,ss}$ [μg/mL]	0.91 (0.89)	2.31 (2.26)	5.95 (5.71)
$T^*_{max,ss}$[h]	6.0*	6.0*	6.0*
$C_{av,ss}$[μg/mL]	0.59 (0.58)	1.40 (1.37)	3.10 (3.02)
$AUC_{\tau,ss}$ [μg*h/mL]	14.17 (13.92)	33.55 (32.88)	74.48 (72.34)
$AUC_{(0\text{-}t),ss}$ [μg*h/mL]	24.88 (24.13)	56.00 (54.40)	112.62 (107.12)
$AUC_{(0\text{–}inf),ss}$ [μg*h/mL]	27.55 (26.85)	58.60 (56.88)	116.20 (110.55)
$t_{1/2,ss}$[h]	23.28 (22.86)	20.40 (20.21)	18.76 (18.18)
$V_{z,ss}$ [L]	61.35 (59.22)	45.04 (44.34)	37.92 (36.24)

*median values reported

The extent of exposure to XYZ1234 in this study generally increased in a dose proportional fashion over the dosing range of 25 to 100 mg, although there was a slight trend to higher values at the higher doses. Times to reach maximum concentration were constant at all doses and after both single and multiple dosing: terminal phase half-lives were also fairly constant at all doses after multiple dosing. After 7 days of repeated dosing, the observed accumulation of XYZ1234 (based on AUC_τ and C_{max}) showed a factor of about 2, agreeing well with the theoretical expectation: this suggests linear pharmacokinetic behavior of XYZ1234 between single and repeated dosing.

Median $C_{max,ss}$ values of 0.79, 2.3 and 5.0 μg/mL at 6 h post dose were observed: slightly higher increases were apparent than strict dose proportionality would predict.

From the mean trough concentrations of XYZ1234 on days 2 to 7, it is apparent that steady-state conditions were attained at all doses after 7 days of repeated dosing, in line with the observed terminal phase half-life of the compound.

Descriptive statistics for the primary pharmacokinetic parameters of XYZ1234 on days 1 and 7 are shown in Tables 1–4.

The mean data for $C_{max,init}$ (and to a lesser extent $AUC_{\tau,init}$) on day 1 confirm the descriptive observation already made that the increases with increasing dose were slightly higher than strict dose proportionality would predict. Median $T_{max,init}$ was very constant (6 h) throughout the dose range studied.

The analysis of the dose adjusted values confirm the faster than linear increase of $C_{max,init}$, $C_{max,ss}$ and $AUC_{(0\text{–}24)}$,init with increasing dose.

The same trend for $C_{max,ss}$ and $AUC_{\tau,ss}$ was observed on day 7 as on day 1 i.e. slightly higher than dose proportional increases were recorded $AUC_{(0\text{–}t),ss}$ and $AUC_{(0\text{–}inf),ss}$ on the other hand showed increases very closely related to the increases in dose. As for the single dose data, $T_{max,ss}$ remained very stable over the dose range investigated, with median values of 6 h. There was an indication for slightly reduced terminal phase half-life times at the higher doses which may explain the reduced differences in $AUC_{(0\text{–}t),ss}$ and $AUC_{(0\text{–}inf),ss}$. This was also accompanied by a lower volume of distribution associated with the terminal phase at the higher doses.

Dose proportionality could be shown for the dose dependent parameters $AUC_{(0\text{–}inf)}$, $AUC_{(0\text{–}t)}$ and all the three possibilities of treatment comparisons(B vs A, C vs A, C vs B). The 95 % confidence interval contains all of the expected ratios.

For the parameter $AUC_{(0\text{–}24)}$ only the expected ratio of the comparison of C and A is not contained in the confidence interval. So dose proportionality could be shown for the two other comparisons.

For the parameter C_{max} dose proportionality could not be shown for all of the three comparisons. The three expected comparisons are not contained in the confidence intervals.

A comparison of geometric mean values for C_{max} and AUC_τ between day 7 and day 1 indicate ratios of

Table 4 XYZ1234 PK parameters – dose proportionality.

Parameter	Comparison	Estimated ratio	Expected ratio if dose proportional	95% CI
AUC_{τ} [μg*h/mL]	50 mg:25 mg	2.362	2.000	1.97, 2.84
	100 mg:25 mg	5.197	4.000	4.33, 6.24
	100 mg:50 mg	2.200	2.000	1.83, 2.64
$AUC_{(0–inf)}$ [μg*h/mL]	50 mg:25 mg	2.119	2.000	1.69, 2.66
	100 mg:25 mg	4.118	4.000	3.28, 5.17
	100 mg:50 mg	1.943	2.000	1.55, 2.44
$AUC_{(0–t)}$ [μg*h/mL]	50 mg:25 mg	2.254	2.000	1.79, 2.85
	100 mg:25 mg	4.439	4.000	3.51, 5.61
	100 mg:50 mg	1.969	2.000	1.56, 2.49
C_{max} [μg/mL]	50 mg:25 mg	2.544	2.000	2.07, 3.13
	100 mg:25 mg	6.442	4.000	5.24, 7.92
	100 mg:50 mg	2.532	2.000	2.06, 3.11

Table 5 Observed accumulation ratios of geometric mean parameters for XYZ1234.

Ratio	MEAN (geometric mean): DAY 7: DAY 1		
	Treatment A (25 mg)	Treatment B (50 mg)	Treatment C (100 mg)
$C_{max,ss}/C_{max,init}$	1.98	1.99	1.81
$AUC_{\tau,ss}/AUC_{\tau,init}$	1.99	2.05	1.87

approximately 2 at all dose levels: the ratios are given in the following table.

These results agree well with the theoretical accumulation anticipated according to the formula:

Accumulation $R=1/1–2^{-\epsilon}$ where $\epsilon = \tau/T_{1/2}$ and τ is the dosing interval.

Thus, for a dosing interval of 24 h and a half–life close to 24 h, the predicted accumulation factor is 2, close to the observed value found in this study. This is a good indication for linear kinetic behavior of XYZ1234 between single and repeated dosing.

The peak to trough ratio (data not shown) increased with dose, reflecting the higher than dose proportional increase in $C_{max,ss}$.

The course of the median trough plasma concentrations (Figure 3) shows a saturation beyond day 5, which is an indicator for reaching the steady state.

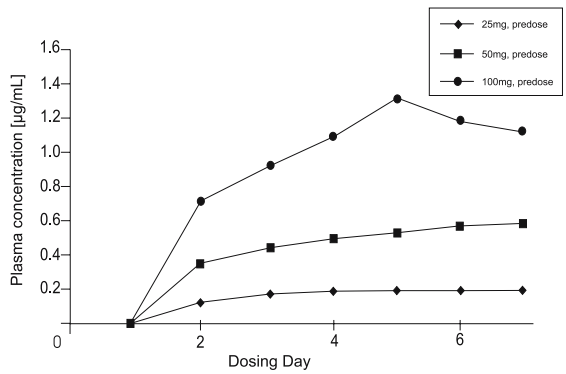

Fig. 3. XYZ1234 Trough plasma concentrations

II.S.3 Special Populations: Exploratory Profiling of the Impact of Gender, High Age, and Food Intake, on Drug Disposition

PURPOSE AND RATIONALE

In early clinical development the steps before entering into larger patient studies are typically an explorative first-in-man study with escalating single doses in healthy volunteers, an explorative proof-of-principle study with escalating multiple doses in healthy volunteers, and then a proof-of-concept with escalating multiple doses in a population as close as possible to the target population for the phase IIb/III development program. Additional supportive studies are required to justify the fastest possible broadening of the population in the proof of concept study, and thus to accelerate patient recruitment into the larger patient studies. Over and above this industry need, the principles of ICH and US Federal Drug Agency (FDA) guidance (US FDA November 1989, ICH-E7 August 1994, US FDA July 1993, US FDA December 1978) also require that the pharmacokinetics and safety of a candidate drug is studied in the range of populations likely to receive the drug during the clinical development and later, once the drug is marketed.

The principles of US FDA and European Agency for the Evaluation of Medicines Products (EMEA) guid-

ance also recommend an early profiling of food effects on bioavailability to help guide and select the formulations for further development (US FDA December 2002, EMEA CPMP/EWP/560/95 December 1997).

Experience has shown that the exploratory profiling of the impact of gender, high age, and food intake (in the form of a food screen for worst case food effects) on drug disposition (pharmacokinetics) and safety can be conveniently done in one study with the design described in this article. If a notable effect of these parameters is/are seen, then confirmatory studies need to be performed.

A priori animal data can provide hints with respect to the likelihood of observing some impact of gender, high age, and food intake on candidate drug pharmacokinetics.

The observation of gender-related differences in drug pharmacokinetics in multiple-dose animal studies is one hint that a gender effect might be expected, although gender effects observed in rodent should be viewed with caution. Data from in-vitro metabolism assays based on liver fractions from men and women could also suggest gender related differences in metabolism.

Animal studies indicating that the drug candidate is primarily eliminated renally and/or physicochemical characteristics making a renal elimination likely are flags suggesting the need to profile candidate drug pharmacokinetics in elderly – since some degree of renal impairment is often observed in this population.

Animal studies reporting an effect of food intake on drug pharmacokinetics, although suggestive, tend to be of low predictivity. This lack of predictivity can be explained by the different physiology and feeding behaviors in animals and man. Thus, if oral dosing is planned, since compliance with dietary restrictions could be an issue in outpatient studies, a screen for notable food effects in man should be included early during clinical development. Food effects are generally greatest when drug product is administered immediately after a meal is ingested. The nutrient and caloric content of the meal can also influence drug availability differently in different drugs, whereby meals that are high in calories and fat content are more likely to result in an effect. The time to dosing and fluid intake can also influence drug disposition.

PROCEDURE

The design of a study providing the suggested exploratory profiling of the impact of gender, high age, and food intake on the pharmacokinetics of candidate drug (Drug XYZ) in a single clinical study is presented below. For the purposes of simplicity, the study description is limited to the collection, handling, and interpretation of pharmacokinetic data although other parameters were also studied.

In this explorative study candidate drug is given immediately after ingestion of a high-calorie/high-fat meal (US FDA December 2002). Since the impact of different food compositions and times to food intake are not tested, this aspect of the study is referred to as a food screen.

Protocol Outline

Explorative pharmacokinetics of drug XYZ (200 mg, given as tablet), including a food screen, in younger and elderly, obese, but otherwise healthy, adults.

Objectives

Primary: The primary objective of the study was to investigate and compare the pharmacokinetics of Drug XYZ, including a food screen, in younger adult and elderly obese, but otherwise healthy, men and women.

Secondary: The secondary objective of the study was to assess the safety of single doses of Drug XYZ in younger and elderly obese, but otherwise healthy, adults.

Study Design

A single-center, open-label, randomized, balanced, single-dose, two-treatment (fed versus fasted), two-period crossover design. In one trial period subjects were dosed after fasting (= fasted trial period), and in the other trial period subjects were dosed after consuming a high fat breakfast (= fed trial period). Subjects were randomized to the sequence of trial periods. The washout period between trial periods was at least 5 days which approximated to $>$ 10 Drug XYZ apparent terminal half-lives.

Number of Subjects

Twenty-four (24) subjects (12 men/12 women, per gender 6 younger adult subjects, 6 elderly subjects) were recruited.

Due to the variability in the pharmacokinetic parameters seen in earlier studies, no formal sample size justification was given for 12 subjects per population. However, according to the draft guidance for industry by the US FDA 12 subjects is the typical minimum sample size for a bioavailability comparison (US FDA December 1978, US FDA December 2002). This was fulfilled here for each of the strata male, female, younger, elderly.

Inclusion Criteria

The following inclusion criteria were met: adults aged between 18 and 45 years (young adults) and aged 65 years or older (el-

derly); with a body mass index (BMI) of ≥ 30 and < 40 kg/m^2; women who are either post-menopausal or surgically sterile or willing to use prescribed barrier contraceptive methods; who are healthy for the purpose of the study and not receiving regular medication in the month preceding the study; with normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant; who are non-smoking or light smokers; elderly subjects with creatinine clearance of $>$ 50 mL/min; young adults with creatinine clearance of $>$80 mL/min.

Treatments

A single-dose of Drug XYZ (200 mg) was given orally as tablets. In the fasted trial period, subjects remained fasted until 2 h after dosing. In the fed trial period, subjects were dosed orally after consuming a high fat breakfast whose composition conformed to US FDA guidance (US FDA December 2002).

Pharmacokinetic Data

Plasma concentrations of Drug XYZ, before and at predetermined times post dose were measured.

Concentrations of Drug XYZ in urine collected over the profiling period were measured. Volumes of urine collected over each profiling period were recorded.

The volume of urine collected over 24 h and the concentration of creatinine were determined to allow the subjects creatinine clearance to be calculated.

EVALUATION

The data pertinent to the assessment of the explorative profiling of issues related to age, gender, and the impact of food intake when dosing from study described above, was evaluated as follows:

Due to the investigational nature of this study, and the small sample size, all variables were only presented descriptively. Where appropriate, individual data were presented together with descriptive statistics.

Descriptive pharmacokinetic parameters (standard parameters including peak concentrations (C_{max}), time of C_{max} (T_{max}), area-under-the-curve (AUC) between time 0 and time t where t = 24 h post dose ($AUC_{0\text{-}t}$), AUC after extrapolation to infinity ($AUC_{0\text{-}\infty}$), apparent terminal half-life ($t_{1/2\_z}$)) for plasma Drug XYZ were calculated using a non-compartmental analysis employing a linear/log trapezoidal method as implemented in WinNonlin (Pharsight Corp.) protocols. For urine Drug XYZ data, the descriptive pharmacokinetic parameters included fractional/cumulative urinary excretion and renal clearance of Drug XYZ were calculated using SAS for Windows protocols.

If appropriate, pharmacokinetic parameters were compared descriptively between age groups (with/without stratification), between genders (with/without stratification), and between fasted and fed subjects (with/without stratification and individually). Although not intending to show bioequivalence, the 90 % confidence intervals (CI) for the differences in the log transformed exposure measurements were calculated.

CRITICAL ASSESSMENT OF THE METHOD

The impact of gender, high age, and food intake on candidate drug pharmacokinetics and safety can be conveniently done in one explorative study with the design described in this article. Typically this study would run shortly after key data are available from the first-in-man study and employs a dose in the upper third of the dose range tested in the first-in-man study. The highest safe dose from the first-in-man study is not usually chosen unless a notable safety margin is given because food intake can cause substantial increases in exposure.

As the clinical development progresses, the dose chosen in this study may prove to be higher or lower than the actually required therapeutic dose. Similarly, the drug formulation available at this early time-point may differ from that eventually marketed. These differences may have a notable influence on the magnitude of food effect seen. In the experience of the author, the observed food effects ranged from a 50 % reduction in AUC to a 20-fold increase in AUC when different oral formulations of the same drug were dosed immediately after the same high fat/high calorie food.

As mentioned earlier, this type of supportive study provides explorative profiling. If a notable effect of these parameters is seen, then suitable confirmative studies will need to be performed. However, these studies should ideally be performed when the therapeutic dose range and the final drug formulation are known.

Although the impact of gender and high age on the pharmacokinetics of the developmental drug can be studied based on single-dose data as described, the use of steady-state data can be required if there is reason to believe that the pharmacokinetics of the drug studied are not accurately predictable from single-dose data.

MODIFICATIONS OF THE METHOD

The type of study described in this section provides explorative profiling of issues related to age, gender, and

Table 6 Summary of key Drug XYZ PK parameters; young versus elderly, fasted versus fed

Parameter	Statistic	All PK subjects (n = 24)		Young (n = 12)		Elderly (n = 12)	
		Fasting	Fed	Fasting	Fed	Fasting	Fed
C_{max} (μg/mL)	Geometric mean	1.23	0.96	1.24	0.87	1.22	1.06
	Range	0.73–2.50	0.58–1.96	0.75–1.72	0.58–1.39	0.73–2.50	0.66–1.96
	Geometric mean ratio (90%CI: upper-lower)	0.7816 (0.6969–0.8767)		– – – –		– – – –	
T_{max}(h)	Median	1.0	3.0	1.0	3.0	1.0	3.0
	Range	0.5–2.0	1.0–5.0	0.5–2.0	1.0–4.0	1.0–2.0	1.0–5.0
$AUC_{0-\infty}$ (μg.h/mL)	Geometric mean	9.26	8.99	7.80	7.44	11.0	10.86
	Range	3.52–34.61	3.63–40.06	3.52–14.89	3.63–17.0	6.03–34.61	3.94–40.06
	Geometric mean ratio (90%CI: upper-lower)	0.9714 (0.8985–1.0502)		–		–	
$t_{1/2z}$ (h)	Geometric mean	12.4	12.0	12.0	11.4	12.7	12.8
	Range	6.8–30.2	6.7–39.4	8.6–18.5	8.5–17.0	6.8–30.2	6.7–39.4

the impact of food intake when dosing. If appropriate, the same principle could be applied to address other variables. For example, to investigate the impact of different types of food composition and/or time of food ingestion using a relatively small population. Typically this type of evaluation would be extended during the later clinical development by performing a population pharmacokinetic/pharmacodynamic assessment of the impact of gender and high age on the drug disposition during the phase II/III studies.

The most common impact of high age is via a reduced hepatic and/or renal function. In the example given, the study inclusion criteria deliberately allow the inclusion of elderly subjects with mildly impaired renal function, i.e. subjects presenting creatinine clearances of $>$ 50 mL/min. No measures were taken to ensure that the number of enrolled elderly volunteers with normal and mild renal impairment was balanced. Such measures would have allowed an explorative insight into the impact of mildly impaired renal function, at the expense of delaying recruitment, since the recruitment of healthy elderly with mild renal impairment is more complicated. If renal elimination is believed to play a notable contribution to developmental drug elimination, then the complication of recruitment may be warranted.

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EXAMPLE

To illustrate the type of data that can be obtained using the study discussed, a high level summary of the pharmacokinetic results obtained from the study described above under "*Procedure*" is presented below.

Results – Pharmacokinetics

The data from the study described above, as summarized in Tables 6 and 7 and Figure 4, can be summarized as follows:

1. A trend was seen in all analyzed groups towards a slightly lower Drug XYZ plasma C_{max} under fed conditions than after fasting. Drug XYZ plasma C_{max} was also reached later under fed conditions than after fasting in all analyzed groups.
2. From 6 h post-dose until at least 48 h post-dose, the Drug XYZ plasma concentrations (median values) were slightly higher in the elderly than in young adults and also slightly higher in female than in male subjects, both under fed and under fasting conditions.
3. Urinary excretion of unchanged Drug XYZ was low: 48 h after administration approximately 1 %

Table 7 Summary of key Drug XYZ PK parameters; male versus female

Parameter	Statistic	Male (n = 12)		Female (n = 12)	
		Fasting	Fed	Fasting	Fed
C_{max} (μg/mL)	Geometric mean	1.31	0.95	1.15	0.97
	Range	1.03–1.60	0.66–1.52	0.73–2.50	0.58–1.96
T_{max}(h)	Median	1.0	3.0	1.0	3.0
	Range	0.5–2.0	1.0–5.0	1.0–2.0	1.0–5.0
$AUC_{0-\infty}$(μg.h/mL)	Geometric mean	8.12	7.46	10.55	10.84
	Range	3.52–34.61	3.63–32.86	6.03–30.62	5.30–40.06
$t_{1/2z}$ (h)	Geometric mean	11.5	11.1	13.3	13.1
	Range	6.8–22.3	6.7–25.4	9.7–30.2	8.7–39.4

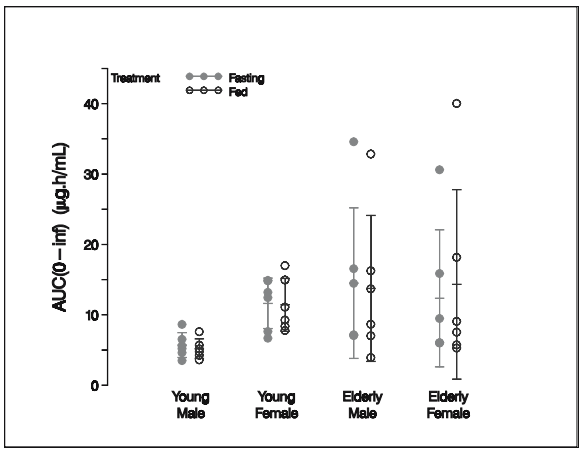

Fig. 4. Individual $AUC_{0\infty}$ values per stratum, with arithmetic mean ± 1 SD

of the administered dose was excreted unchanged. There was a trend in all analyzed groups towards a slightly lower cumulative Drug XYZ excretion in urine under fed than under fasting conditions. Cumulative excretion seemed slightly higher in the elderly than in the younger adults and in women than in men, both under fed and under fasting conditions.

4. Drug XYZ showed delayed absorption due to food intake. The overall geometric mean Drug XYZ plasma C_{max} was 1.23 mg/L under fasting and 0.96 mg/L under fed conditions. Overall median T_{max} was 1 h under fasting and 3 h under fed conditions. Similar differences in C_{max} and T_{max} under fasting and fed conditions were seen in all populations analyzed.
5. Overall, no differences were seen in AUC parameters and elimination half-life under fasting and fed conditions.
6. A trend was seen towards a smaller $AUC_{0-\infty}$ in the young than in the elderly (geometric mean after fasting 7.8 and 11.0 mg.h/L, respectively) and in the male than in the female subjects (geometric mean after fasting 8.12 and 10.55 mg.h/L, respectively).
7. There was a trend towards a shorter $t_{1/2\_z}$ value in the young than in the elderly (geometric mean after fasting 12.0 and 12.7 h, respectively) and in the male than in the female subjects (geometric mean after fasting 11.5 and 13.3 h, respectively).
8. An exploratory analysis was performed using a four-factor ANOVA model, with treatment, period, and sequence as fixed factors and subject within sequence as random factor. The results from the ANOVA were used to calculate the back-transformed 90 % confidence intervals (CI) for the differences between the fed and fasted condition in the log-transformed exposure measurements (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$). For C_{max} the difference between fasting and fed conditions was found to be statistically significant while this was not the case for the AUC parameters.

II.S.4 Profiling of Drug Absorption, Distribution, Metabolism and Elimination in Man: the hADME Study

PURPOSE AND RATIONALE

The aim of this kind of study is to characterize the Absorption, Distribution, Metabolism and Elimination of the investigational product in humans (hADME study), following an administration of the compound in a radiolabeled form. The use of a radiolabel allows identifying metabolites, which were not known beforehand, and to characterize them. In addition, using a radiolabel is – in most cases – the only way to establish a complete balance of the drug and its metabolites, which is required to validate the completeness and predicitvity of the results.

A typical hADME study includes an overall balance of excretion of the administered radioactivity

(mass balance), protein binding, metabolic profiles in plasma, urine and feces, and if possible the determination of descriptive pharmacokinetic parameters for the radioactivity, the parent compound, and identified metabolites in plasma and in urine. If feasible and adequate, structures of the observed metabolites will be elucidated.

Knowledge of the absorption, distribution, metabolism and elimination in man, beyond being a prerequisite for assessing the consequences of dosing in target patient population in Phase II studies, will also aid the prediction of potentially relevant drug–drug interactions and of high-risk populations for dosing with the investigational drug.

Presently there are no official, general guidelines for this kind of study available; the different aspects are covered instead by dedicated guidelines, e.g. metabolism, related to drug interaction (US FDA 1999, US FDA 1997, EU CPMP 1997), or for the related preclinical studies (EU EUDRALEX 3BS11a 1994), or only in a high level form (EU EUDRALEX 3CC3a 1988).

This type of hADME studies using radioactive-labeled drug should be run unless phase I studies show that $\geq$ 90 % of the dose is excreted unchanged in urine. In this case, a hADME study may not be required. For those drugs, a urine assay should be the prerequisite for phase I trials and then mass balance may be established in trials where quantitative urine collections are performed. If phase I data indicate that 'cold mass balance', i.e. $\geq$ 90 % recovery in urine cannot be obtained, a hADME study has to be scheduled for the development program.

PROCEDURE

The design of a typical hADME study for a candidate drug (XYZ1234) is presented below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety parameters are also studied.

Protocol Outline

Absorption, distribution, metabolism and elimination of XYZ1234 in healthy men following oral administration of ^{14}CXYZ1234 (100 MG, 4 MBq) as solution.

Objective

Primary objective: To investigate the absorption, distribution, biotransformation and elimination of XYZ1234 in healthy men following oral administration of ^{14}CXYZ1234. This includes: (i) overall balance of excretion of the administered radioactivity (mass balance), (ii) metabolic profiles in plasma, urine and feces, (iii) if possible, structures of the observed metabolites, (iv) determination of descriptive pharmacokinetic parameters for XYZ1234 and its active metabolite (XYZ5678) in plasma and in urine, and (v) characterization of protein binding in plasma.

Study Design

A single center, open-label, single-dose, study design. Subjects entered the clinical site on the morning one day before dosing, and remained at the study site at least 7 days after dosing. In case radioactivity measurement showed that recovery in the excreta (urine plus feces) was still below 95 % of the administered dose on day 7, or that the activity in the last plasma sample was still above 1 Bq/mL, then the subjects were requested to stay in the clinic until one of the levels had reached the mentioned limits. The maximum prolongation of the stay in the clinical center was 14 days post-dosing. If necessary, after that time the subjects had to continue daily collection of urine and feces at home until the radioactivity in the excreta samples reached definite threshold criteria (1 Bq both per mL of urine and per homogenate of 100 mg feces respectively).

Sample size/Population

Six white men aged between 45 and 65 years, with Body Mass Indices (BMI) of 19 – 29 kg/m^2.

Treatments

After overnight fasting, a single oral dose of ^{14}CXYZ1234 (100 mg, 4 MBq) administered as solution.

Pharmacokinetic Data

- Radioactivity administered orally, and radioactivity recovered in whole blood, plasma, urine, feces and expired air
- Concentrations of XYZ1234 and its active metabolite (XYZ5678) in plasma and urine
- Metabolic profiles: number of, and radioactivity attributable to, specific peaks in analyses (chromatograms) of the radioactivity in plasma, urine and feces
- Structures of observed metabolites (if possible)
- Protein binding in plasma (in vitro only).

EVALUATION

Where appropriate, individual data were presented together with the descriptive statistics.

If measured radioactivity for plasma, blood and urine was related to the weight of a sample, the result were converted to the volume of the sample, in order

to generate the values as concentrations, comparable to the 'normal' bioanalytical data.

In the case of urine for this conversion a constant specific weight of 1.02 g/mL is assumed for all subjects. In the case of plasma and whole blood for this conversion a constant specific weight of 1.03 g/mL (plasma) and of 1.05 g/mL (whole blood) respectively is assumed for all subjects. In the case of the radioactivity measurements, results are then also listed in terms of measured radioactivity per mL sample and as "concentrations" (i.e. given in μg equivalents per mL sample (μg-eq/mL)), representing the sum of original compound and/or radiolabeled metabolites.

Due to the small sample size, all variables were only presented descriptively for the different bioanalytical data and pharmacokinetic parameters calculated: number of relevant observations, geometric mean, geometric standard deviation, arithmetic mean, standard deviation, coefficient of variation, median, minimum and maximum.

CRITICAL ASSESSMENT OF THE METHOD

The required information can be generated in this explorative study with a design described as above. The threshold criteria to stop collecting urine and feces (as described under 'study design') guarantee that the combined daily excretion reached a value below 0.1 % of the administered dose. Further collection would not significantly contribute to the overall balance, and has to be outweighted to the burden to the subject involved. Using fixed specific weights per matrix for the calculation of concentrations in the respective – originally weighted – samples (urine, plasma, and blood), had proven to be superior to an individual determination of the specific weights: the variability of the results was reduced, without a hint for any reduction of the accuracy of the resulting concentration values.

This study can already be conducted after key data are available from the first-in-man study, but should use a dose in the upper third of the range of therapeutic doses, and a formulation similar to the therapeutic one. This type of hADME studies are increasingly being performed as early as possible in the clinical development, in order to support the assessment of the consequences of dosing in the target patient population, or special populations, and in order to support the prediction of potentially relevant drug–drug interactions and high-risk populations for dosing with the investigational drug. These advantages of conducting the study early, have to be balanced against possible uncertainties related to the therapeutic dose and the formulation during early development; timing of the hADME study has therefore to be decided on a project-specific basis.

MODIFICATIONS OF THE METHOD

The example shown comprises protein-binding in vitro only (pre-dose samples), since the parent compound in plasma was known massively to exceed all metabolites. If this is not the case, additional samples to determine the protein binding ex vivo are required. Depending on the kind of measurement (radioactivity vs. specific), these samples can then indicate a general hint for protein binding across all labeled molecules (weighted by their occurrence in plasma), if only the ex-vivo radioactivity is determined, or can provide protein binding data for metabolites, if a specific assay is available.

This type of hADME study will always have an explorative character, and typically comprises less than the normal minimum of 12 subjects for a PK study. If the compound is expected to show different and unpredictable ADME characteristics in special populations, then it might be necessary to include these populations in addition to normal, healthy subjects. Or, if other conditions might influence ADME in an unpredictable manner (e.g. food effects), then it might even be necessary to run the study in a cross-over design. The inclusion of females (being not of childbearing potential) was discussed several times, but it seems to be a rare exception for hADME studies.

Furthermore, if non-linearities are observed for the ADME characteristics instead of a single dose, multiple dose studies may be needed, where the radio labeled drug is administered under steady-state conditions.

hADME studies preferentially use compounds labeled with C-14; labeling with H-3 provides in many cases less stability, and requires a "wet/dry" comparison of the samples during analysis, in order to detect free tritiated water. Similar precautions, and tests for the in vivo stability of the label, are necessary if other nuclides are used as radiolabel.

A promising alternative to the conventional radioanalytical measurement of C-14 in hADME studies is Accelerated Mass Spectrometry (AMS), which can operate as a detector for C-14 with an increase of sensitivity by a factor of 1000 (Garner 2000). This technique is complex and presently not used routinely. In addition, as an alternative, the last-generation, high-sensitivity liquid scintillation beta counters show an increase of sensitivity by a factor of 10–20. In those cases where the dosimetric calculations indicate that a traditional dose (2–4 MBq) would result in an

unacceptable radiation-burden (e.g. due to an intensive and long lasting binding to melanopherous tissues), then both techniques can certainly provide useful alternatives, since using these radioanalytical tools it would allow administration of much lower doses of the radionuclide.

If drug development has to be done exclusively in compromised patients (some anticancer, anti-aids drugs) which may make a comprehensive hADME trial using a radioactive drug difficult to conduct, then other means of establishing mass balance and metabolism information may have to be negotiated.

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EXAMPLE

To illustrate the type of data that can be obtained using the study under discussion, a high level summary of the pharmacokinetic results obtained from the study described above under "Procedure" is presented below.

Results

(i) Balance of excretion: On average, 34.6 % of the administered radioactivity was excreted in urine and 60.6 % was excreted in feces. No quantifiable radioactivity was found in expired carbon dioxide. The total excretion averaged 95.2 % after 17 days. The urinary excretion of the parent drug averaged 8.85 % of the dose and the urinary excretion of the metabolite XYZ5678 averaged 11.7 %. There was still 14.1 % of the drug that was eliminated in the urine in a form different than XYZ1234 or XYZ5678, as one or several other metabolites.

(ii) Metabolic profile: The metabolites in urine with the largest contribution were the demethyl product (P7) (26 %), and the hydroxylated isomers (P3, P4) (19 % and 6 %). An unidentified metabolite with a molecular weight of 450 g/mol (P5) represented 19 % followed by the unchanged product (P9) (joint contribution of ca. 18 %).

The contribution of each peak to the total radioactivity in feces was calculated, and the results showed that the metabolites in feces with the largest contribution were the hydroxylated isomers (P3, P4; 38 % and 11 % respectively) and unknown "450 g/mol" metabolite (P5) (24 %), while the unchanged drug (P9) was found with a contribution of 7 %.

In plasma, XYZ1234 remained mainly unchanged. Only trace levels of the demethyl compound XYZ5678 (P7), glucuronide (P6), and the unidentified "450 g/mol" metabolite (P5) were found.

(iii) Structure of metabolites: the parent drug XYZ1234 had three main metabolites: XYZ5678, 2-OH XYZ1234, and XYZ1234 glucoside. The XYZ5678 had a metabolite, 2-OH XYZ5678.

(iv) Pharmacokinetic parameters in plasma and urine: ^{14}C-XYZ1234 solution was rapidly absorbed from the gastro-intestinal tract. The peak radiocarbon concentration in plasma averaged 1.61 μg-eq/mL and occurred between 0.5 and 4 h. $AUCinf_{inf}$ of plasma radiocarbon was on average 36.9 μg-eq.h/mL. Elimination of radiocarbon was characterized by a mean half-life of 20.5 h.

Whole blood concentrations represented approximately 80 % of the plasma concentrations. C_{max} = 1.33 μg-eq/mL, $AUCinf_{inf}$ = 32.7 μg-eq.h/mL. The mean half life, 20.2 h, was very close to the plasma half-life. There was radioactivity in the red blood cells and it declined with an elimination half-life similar to the half-lives in plasma and whole blood radiocarbon concentrations.

The peak concentration of XYZ1234 averaged 1.54 μg/mL and was reached between 0.5 and 4 h after dosing. $AUCinf_{inf}$ averaged 37.2 μg.h/mL. The elimination half-life (19.8 h) was similar to the half-life observed for radiocarbon.

The renal clearance of XYZ1234 averaged only 4 mL/min, this being small compared to the non-renal clearance, which was 10 fold higher (42 mL/min). Whereas the renal clearance of the metabolite XYZ5678 averaged 144 mL/min.

The C_{max} of XYZ1234 and XYZ5678 summed was 99 % of the C_{max} of radiocarbon pharmacokinetic in plasma. The t_{max} for radiocarbon and for XYZ1234 were similar, the t_{max} of XYZ5678 appearing a few hours later. The difference in average urinary excretion between the sum of XYZ5678 and XYZ1234, and the radiocarbon, showed that 14.1 % of the drug was eliminated in the urine in a form different than

XYZ1234 or XYZ5678, as one or several other metabolites. This was also confirmed by the metabolic profiles.

(v) Plasma Protein Binding. The mean protein binding was 91.3 %, no major interindividual differences occurred.

II.S.5 Assessment of the Relative and/or Absolute Bioavailability of Drugs

PURPOSE AND RATIONALE

The assessment of a drug's bioavailability (BA) is the most important information on its PK. Consequently numerous guidelines primarily focus on this issue as from the exposure efficacy as well as safety for the patient is deduced.

Bioavailability is defined as the rate and extent by which the active moiety becomes available at the site of action. Because neither concentrations nor amounts can generally be determined at the site of action, plasma/serum concentrations are used as a surrogate to determine the rate and extent of bioavailability. Provided that the pharmacokinetics of the drug considered is linear and time-invariant, the area under the curve (AUC) is a measure for the fraction of the dose available according to Dost's law of corresponding areas. The AUC after intravenous or intraarterial administration is set to 100 % availability.

Consequently, bioavailability is defined for a formulation not for a drug.

Bioavailability studies quantify rate and extent of absorption. They compare the efficiency of the disposition of several drug formulations, e.g. immediate-release vs. extended-release or capsule vs. tablet or tablet A vs. tablet B etc., or they compare the disposition of different routes of administration, e.g. oral vs. subcutaneous or oral vs. intravenous. According to the definition, a comparison to the intravenous bolus injection yields the 'absolute' bioavailability.

Bioavailability figures should always be given for the active moiety of a drug.

The criterion of bioequivalence applies if there is a similarity in bioavailability (statistically proven) that is unlikely to result in clinically relevant differences in efficacy and/or safety.

The bioavailability of a drug formulation is best described by C_{max}/T_{max} (rate) and Area under the systemic concentration-time curve AUC (extent).

Details on the design of and the interpretation of data from bioavailability studies are given in the literature.

PROCEDURE

The design for an absolute bioavailability study for drug XYZ1234 is presented below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although safety parameters were also in the focus.

Protocol Outline

A phase I, open-label, randomized, cross-over study to investigate the bioavailability, safety, tolerability and pharmacodynamics following single oral administration of XYZ1234 as capsule and single intravenous administration of XYZ1234 in healthy men.

Primary Objective

To characterize the bioavailability of XYZ1234 drug substance (25 mg) as a capsule formulation following a single oral administration in fasting conditions in healthy male adult volunteers, using 10 mg of intravenously administered XYZ1234 as reference formulation.

Study Design

This was an open-label, single-dose, randomized, 2-period cross-over study with a minimum washout period of 7 days. Each treatment group received treatment A (10 mg XYZ1234, intravenously administered) and treatment B (25 mg XYZ1234 as capsule, orally administered), once each under fasting conditions.

Inclusion Criteria

Healthy male subjects, aged 18–45 years (inclusive), with a Body Mass Index between 18 and 27 kg/m^2 (inclusive), normal or clinically irrelevant abnormal findings (in the opinion of the investigator) in the medical history and physical examination, laboratory values, ECG, blood pressure and pulse rate, negative serology (HIV antibody, hepatitis B surface antigen, hepatitis C antibody) and urine screen for drugs of abuse.

Treatments

Regimen A (Reference Treatment):

Intravenous (IV) administration of XYZ1234 (10 mg, administered over thirty minutes)

Regimen B (Test Treatment):

Oral (PO) administration of XYZ1234 (25 mg, as a capsule formulation).

Pharmacokinetic Data

Concentrations of unconjugated XYZ1234 and Cystein (CYS)-conjugated XYZ1234 in plasma were measured pre-dose and at predetermined times up to 48 hours post-dose.

The primary analysis examined pharmacokinetic parameters calculated from plasma concentrations of CYS-conjugated XYZ1234 using non-compartmental techniques. The secondary analysis examined the pharmacokinetic parameters of unconjugated XYZ1234.

EVALUATION

The primary analyses consisted of characterizing the bioavailability of oral XYZ1234 using intravenous XYZ1234 as the reference. Determination of bioavailability was to be based on the plasma concentrations of CYS-conjugated XYZ1234. Descriptive statistics and formal statistical analysis were used to summarize and analyze the pharmacokinetic parameters of unconjugated XYZ1234 and CYS-conjugated XYZ1234 in all evaluable subjects.

The secondary analyses consisted of assessing the safety, tolerability and pharmacodynamic responses after administration of XYZ1234 and XYZ1234 in plasma and urine using descriptive statistics.

CRITICAL ASSESSMENT OF THE METHOD

For the oral route of administration the dose was selected according to the experience from the FIM study, where this dose was safe and well tolerated and was in the dose-proportional range. The dose for the intravenous route of administration was adjusted according to the results from animal bioavailability studies where the absolute bioavailability was in the range of 50 %.

Bioavailability/Bioequivalence studies are usually conducted in healthy subjects. Although the inclusion of women is now being encouraged, we enrolled only men. The study was the second clinical trial in this project.

As the bioequivalence rules are clearly defined, the study population must ensure a high level of standardization, making it sometimes difficult to extrapolate to patient settings. Typical enrolment criteria are:

- Non-smoking males between 18 and 45 years
- Normal for weight and BMI
- (Clinically) healthy
- Not using any medication
- Massive dietary and general restrictions
- No hypersensitivities
- No history or presence of any condition that might interfere with the absorption, distribution, metabolism or elimination of the drug under investigation.

MODIFICATIONS OF THE METHOD

In this example, an oral formulation has been compared to an intravenous one, aiming at 'absolute' bioavailability. More often, the relative bioavailabilities of different oral formulations are assessed in BA studies. The reference formulation in these studies usually is either a marketed (solid) product or an oral solution.

If the drug under investigation has a toxic potential BA studies have to be conducted in the patient setting the drug is intended for use.

Deviations from the a.m. high level of standardization might become necessary depending on the properties of the compound.

Almost all clinical study types described in this section deal in any way with bioavailability and/or bioequivalence questions. Specifics are mentioned there.

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EXAMPLE

To illustrate the type of data that can be obtained using the study discussed, a high level summary of the pharmacokinetic results obtained from the study described above under "*Procedure*" is presented below. Due to the anticipated mode of action of the drug (blood pressure lowering) in this example, instead of an intravenous bolus injection an intravenous infusion over 30 minutes was chosen.

Results – Pharmacokinetics

The calculated bioavailability on basis of the AUC_{last} of conjugated XYZ1234 was 38 %. However, this could be a slight underestimation of the bioavailability since this AUC_{last} could only be determined until 6 h post-dose. A calculation of the bioavailability on basis of the AUC_{last} or $AUC_{0\text{-}inf}$ of unconjugated XYZ1234 yielded a slightly higher bioavailability of 45–47 %.

Table 8 Summary of pharmacokinetic parameters in plasma

Treatment	C_{max}(ng/mL)	T^{a}_{max} (h)	AUC_{last} (ng.h/mL)	AUC_{0-inf} (ng.h/mL)	$t_{1/2}$ (h)
unconjugated					
10 mg IV	49.5 (30.4–96.7)	0.50 (0.50–0.58)	81.8 (42.7–149.2)	207.4[b] (85.1–602.4)	85[b] (36–165)
25 mg PO	15.1 (8.8–24.3)	1.00 (0.50–4.00)	85.0 (60.5–136.4)	215.9[b] (127.8–337.8)	94[b] (61–192)
conjugated					
10 mg IV	280.8 (198.0–420.6)	0.50 (0.50–0.75)	367.1 (256.5–1344.5)	nd	nd
25 mg PO	98.1 (45.9–209.9)	1.50 (1.00–4.00)	317.1 (139.0–704.7)	nd	nd

nd = not determined
[a] for T_{max}, the median (range) is given instead of the geometric mean (range)
[b] n = 14

Table 9 Results of bioavailability analysis

Analyte	Parameter	Treatment ratio (PO/IV)	90% CI
conjugated	AUC_{last}	0.38	0.33–0.43
unconjugated	AUC_{0-inf}	0.47	0.38–0.59
	AUC_{last}	0.45	0.43–0.47

Note: Data were dose corrected

A summary of the pharmacokinetic parameters in plasma is presented in Table 8.

After oral treatment with 25 mg XYZ1234, plasma concentrations of the parent compound were low as a result of a rapid metabolization into the first metabolite XYZ1234. For unconjugated and conjugated XYZ1234, C_{max} was reached on average 1–1.5 h after oral treatment, after which a rapid initial elimination phase and a slow terminal elimination phase was observed, with a terminal elimination half-life of 3.5–4 days (unconjugated XYZ1234). This long half-life led to a small carry-over effect in Period 2 for unconjugated XYZ1234. Due to the relatively high lower limit of quantitation (LLQ) of the assay for conjugated XYZ1234, the terminal elimination phase for this analyte could only be observed for one subject. The concentrations of conjugated XYZ1234 in plasma were 5–10 fold higher than of unconjugated XYZ1234. The AUC_{last} (both analytes) and AUC_{0-inf} (unconjugated XYZ1234 only) were similar after treatment with XYZ1234 25 mg PO and XYZ1234 10 mg IV.

In summary, the calculated bioavailability on basis of the AUC_{last} of conjugated XYZ1234 was 38 %. However, this could be a slight underestimation of the bioavailability since this AUC_{last} could only be determined until 6 h post-dose. A calculation of the bioavailability on basis of the AUC_{last} or AUC_{0-inf} of unconjugated XYZ1234 yielded a slightly higher bioavailability of 45–47 %.

II.S.6 Drug–Drug Interaction Studies

PURPOSE AND RATIONALE

During pre-clinical developments numerous flags can arise which indicate that the drug under study has the potential to interact, i.e. its pharmacokinetics or pharmacodynamics can be altered by concomitant medications, dietary factors and/or social habits such as tobacco or alcohol in the target population (US FDA 1999 and references therein). Regulatory guidance suggests that if appropriately performed in-vitro studies indicate the lack of such an interaction, then a specific clinical study is not compulsory. However, if the claim, "No clinically relevant interaction with Drug X" is desired in the product label, then a confirmatory clinical study is compulsory even if in-vitro studies indicated the lack of an interaction (EMEA CPMP/EWP/560/95 1997).

When discussing interactions it is important to differentiate between "detectable" and "clinically relevant" interactions. It is accepted that for compounds with a wide therapeutic margins pharmacokinetic drug interactions may have little clinical relevance. An interaction is considered clinically relevant when the therapeutic and/or toxicity of a drug is changed to such an extent that a dosage adjustment or medical intervention may be required, or when concomitant use of two interacting drugs could occur when both

are used as therapeutically recommended (EMEA CPMP/EWP/560/95 1997).

The basis of an interaction can be pharmacokinetic, pharmacodynamic, or a combination thereof. Pharmacodynamic interactions may be caused by a wide variety of mechanisms; hence detailed guidance for pharmacodynamic studies is limited and the study design must be chosen on a case-by-case basis.

The scope of mechanisms causing pharmacokinetic interactions may include alterations in one or more of the absorption, distribution, metabolism, and elimination processes. The alterations may reflect effects of the developmental drug on the pharmacokinetics of the potential interaction partner, and vice versa.

Extensive guidance has been published by regulatory agencies on in-vitro and in-vivo drug–drug interactions studies, and how the results obtained can impact the drug dosing and labeling (US FDA 1999, EMEA CPMP/EWP/560/95 1997, US FDA 1997). Some of the limitations of this guidance are discussed in a recent review that also provides a summary of current industry practice (Bjornsson et al. 2003). However, there is clearly a need for a further harmonization of study designs and marker substrates employed, and in the manner in which the data obtained is interpreted, for example by the development of classification systems. In addition, while existing guidance mainly covers P450-mediated drug interactions, the importance of other mechanisms such as those related to transporters has been recognized, and should also be addressed.

Typically drug–drug interaction studies include some form of comparison of the bioavailability of some marker substrate, for the example given below of ethinylestradiol, when dosed with or without concomitant dosing with the investigational drug, in this example Drug XYZ. Ethinylestradiol is one active component of oral contraceptives, and thus a very common concomitant medication for Drug XYZ whose target population is largely younger women. From earlier in-vitro and animal in-vivo studies Drug XYZ was known to be potent inducer of both phase I and phase II metabolizing enzymes, including those enzymes reportedly involved in the clearance of ethinylestradiol. Since a clinically relevant drug–drug interaction between Drug XYZ and oral contraceptives would impact the product label and probably also impact the market value of the drug, the study described below was performed.

PROCEDURE

The design of a typical drug–drug interaction study is presented below. For the purposes of simplicity, the description of this example is limited to the collection, handling, and interpretation of data pertinent to the assessment of potential pharmacokinetic and pharmacodynamic interactions, although other parameters were also studied.

Protocol Outline

Effects of repeated once daily Drug XYZ doses on the safety, pharmacodynamics and pharmacokinetics of ethinylestradiol after dosing with monophasic oral contraceptives containing ethinylestradiol in healthy overweight or obese women.

Objectives

Primary: The primary objective of the study was to study the effects of repeated once–daily Drug XYZ doses on the pharmacokinetics of ethinylestradiol after dosing with monophasic oral contraceptives containing ethinylestradiol in healthy overweight or obese women.

Secondary: The secondary objective of the study was (i) to assess whether Drug XYZ affects the contraceptive effect of the oral contraceptives containing ethinylestradiol as reflected by changes in serum progesterone and 17-β-estradiol levels, and (ii) to evaluate the safety, tolerability and pharmacokinetics of repeated once-daily oral doses of Drug XYZ.

Study Design

Single-center, double-blind, randomized crossover study in young healthy overweight or obese women. Subjects crossed-over with respect to Drug XYZ or placebo that was given double-blinded. The study ran over two menstrual cycles.

Cycles 1 and 2 involved dosing with either Drug XYZ or matching placebo on days 6 to 20, dosing with the subjects normal ethinylestradiol containing oral contraceptive on days 8 to 28, hospitalization on days 19 to 21, and visits to the study site (lunch times) on days 1 (cycle 1 only), 6, 12, 16, 24, and 28.

Day 1 was the first day of the stop week, i.e. the first day after completing the previous cycle: (menstruation generally starts on day 2 or 3, dosing with the oral contraceptive starts on day 8). Sexually active subjects used double barrier contraception during cycles 1 and 2, and for 28 days after completing cycle 2. Subjects were advised to continue use of these measures for at least 28 days after completing cycle 2. Dropouts were not to be replaced.

Number of Subjects

Based on published variability in pharmacokinetic studies of ethinylestradiol in lean subjects, taking confidence intervals of 80–125 %, residual variance ranged between 10 and 33 %. Based on these residual variance values, calculated samples sizes ranged between 6 and 30 (subjects). For example, based on a residual variance value of 17.5 %, a sample size of 14 was calculated.

The chosen sample size reflects (i) the formal sample size calculation (using a residual variance value of 17.5 %) based on the pharmacokinetics of ethinylestradiol in lean subjects, (ii) published sample sizes in other studies of this type of study ranged between 12 and 34, (iii) that this study will include overweight and obese subjects, a population who have been suggested to show a higher variability in their pharmacokinetics and in their menstrual cycles, and finally (iv) the plan not to replace dropouts.

Based on the planned Analysis of variance on log-transformed data, 90 % confidence intervals for AUC ratios ethinylestradiol + Drug XYZ and ethinylestradiol alone, 20 subjects had to complete the study as planned.

Inclusion Criteria

The following inclusion criteria were met: women aged between 18 and 35 years; with body mass index (BMI) of $25.0 \leq 35.0$ kg/m^2; who are either post-menopausal or surgically sterile and willing to use prescribed barrier contraceptive methods; who – apart from being overweight or obese – are healthy for the purpose of the study and not receiving regular medication in the month preceding the study (with the exception of oral contraceptives); who are using monophasic contraceptives containing ethinylestradiol as the estrogen compound; who present normal gynecological histories and normal, regular menstrual cycles (within the previous 12 months); without contraindications for treatment with oral contraceptives; who are not pregnant or lactating; with normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant for the study; who are non-smoking or light smokers.

Treatments

Cycles 1 and 2: Oral administration of (i) the subjects' normal oral contraceptive once daily (mornings before breakfast) on days 8 to 28 of each cycle, and (ii) Drug XYZ or matching placebo, once daily (mornings before breakfast) on days 6 to 20 of each cycle (15 doses in total).

Pharmacokinetic/Pharmacodynamic Data

Plasma concentrations of ethinylestradiol (EE) on day 20 in cycles 1 and 2.

Plasma concentrations of Drug XYZ, before and at predetermined times post dose were measured on days 6, 12, 16, 20, 24, and 28 in cycles 1 and 2.

Serum concentrations of 17-β-estradiol and progesterone on days 6, 12, 16, 20, 24, and 28 in cycles 1 and 2.

EVALUATION

The data pertinent to the assessment of potential pharmacokinetic and pharmacodynamic interactions from the study described above, was evaluated as follows:

Where appropriate, individual data were presented together with descriptive statistics including mean, standard deviation, standard error of the mean, coefficient of variation (in %), median, minimum, maximum, and the number of relevant observations.

Where applicable, pharmacokinetic parameters (C_{max}, T_{max}, $AUC_{0\text{-}24}$, $t_{1/2}$) were calculated using a non-compartmental analysis employing a linear/log trapezoidal method.

Plasma EE: Descriptive statistics and comparison of plasma EE concentrations in cycles 1 and 2. Analysis of variance on log-transformed data, 90 % confidence intervals for AUC ratio of EE + Drug XYZ and EE alone ($AUC_{EE+Drug\ XYZ}/AUC_{EE}$).

Analysis of variance was performed on the log-transformed $AUC_{0\text{-}24}$ of ethinylestradiol to estimate intra-subject variability. The intra-subject variability was subsequently used to estimate the 90 % confidence interval of the $AUC_{EE+Drug\ XYZ}/AUC_{EE}$ ratio.

Plasma Drug XYZ: Individual plasma concentrations were tabulated together with standard descriptive statistics for each variable.

Serum progesterone: Descriptive statistics and comparison of serum progesterone concentrations in cycles 1 and 2. Descriptive comparison of the proportion of subjects who ovulated whilst receiving Drug XYZ and contraceptive concomitantly and the proportion of subjects who ovulated whilst receiving contraceptive alone. Ovulation was assumed if serum progesterone levels exceeded 1.4 ng/mL on day 20 of a menstrual cycle. Individual and mean/median profiles were presented graphically.

Serum 17-β estradiol: Descriptive statistics and comparison of serum 17-β estradiol concentrations in cycles 1 and 2. Individual serum concentrations of 17-β estradiol were tabulated.

CRITICAL ASSESSMENT OF THE METHOD

The study described above could have been powered to study the effect of Drug XYZ on pharmacodynamic effects of ethinylestradiol, in this case, to study effects on ovulation.

The study described above did not study interactions with other active components of oral contraceptives whose pharmacokinetics could also be altered by concomitant dosing with Drug XYZ.

The study described above did not study whether or not the observed effects on the pharmacokinetics of ethinylestradiol were reversible, and if so, the time-course thereof.

The lack of prior knowledge of the time course of any Drug XYZ mediated induction of phase I and II metabolizing enzymes, complicated the interpretation of the data obtained from the example study. Based on previous clinical studies the Drug XYZ dosing regimen was chosen to ensure that enzyme induction was maximal at the time shortly before ovulation. However, it was not known whether or not the observed enzyme induction would fade after prolonged exposure to Drug XYZ, or whether on re-challenge the same magnitude of enzyme induction would be seen, or if the observed induction was reversible.

The example study would typically be followed by further profiling of this drug–drug interaction potential with other substrates, representing a range of concomitant medication in the target patient population, for example using population pharmacokinetic approaches and definitive studies designed to support clear labeling statements.

MODIFICATIONS OF THE METHOD

The following general issues and approaches should be considered (for a more detailed discussion see references). In the following discussion, the term substrate (S, in our example ethinylestradiol) is used to indicate the drug studied to determine if its exposure is changed by another drug, which is termed the interacting drug (I, in our example Drug XYZ). Depending on the study objectives, the substrate and the interacting drug may be the developmental drugs or approved products.

Study Design

Clinical drug–drug interaction studies are generally designed to compare substrate levels with and without the interacting drug and thus many of the principles applying to comparative bioavailability studies also apply here. Because a specific study may consider a number of questions and clinical objectives, no one correct study design for studying drug–drug interactions can be defined.

The following considerations may be useful when choosing a study design:

1. Interpretation of findings from these studies will be aided by a good understanding of dose/concentration and concentration/response relationships for both desirable and undesirable drug effects. In certain instances, reliance on endpoints other than pharmacokinetic measures/parameters may be useful
2. The inhibiting/inducing drugs and the substrates should be dosed so that the exposure of both drugs is relevant to their clinical use
3. The time at steady state before collection of endpoint or pharmacokinetic observations depends on whether inhibition or induction is to be studied. Inducers can take several days or longer to exert their effects, while inhibitors generally exert their effects more rapidly. Thus if induction is to be assessed, a more extended profiling period after attainment of steady state for the substrate and interacting drug may be necessary
4. When attainment of steady state is important, long half-lives of the substrate, interacting drugs, and/or their metabolites should be considered
5. When a substrate and/or an interacting drug are to be studied at steady state, documentation that near steady state has been attained is important
6. Studies can usually be open label (unblinded), unless pharmacodynamic endpoints subject to bias are part of the assessment of the interaction
7. For a rapidly reversible inhibitor, administration of the interacting drug either just before or simultaneously with the substrate on the test day might be the appropriate design to increase sensitivity.

Study Population

Clinical drug–drug interaction studies may generally be performed using healthy subjects, on the assumption that findings in this population should predict findings in the target patient population. Safety considerations, however, may preclude the use of healthy subjects. In certain circumstances, inclusion of patients from the targeted patient population may offer certain advantages, including the opportunity to study pharmacodynamic endpoints.

Choice of Substrate and Interacting Drugs

Substrates for a Developmental Drug

When testing inhibition, the substrate selected should generally be one whose pharmacokinetics is markedly altered by co-administration of known specific inhibitors of the affected enzyme systems (i.e., a very sensitive substrate should be chosen). If the initial study is positive for inhibition, further studies with other substrates representing a range of substrates based on the likelihood of co-administration may be useful. If the initial study is negative with the most sensitive substrates, it can be presumed that less sensitive substrates will also be unaffected.

Developmental Drug as Substrate

When testing a developmental drug for the possibility that its metabolism is inhibited or induced (i.e., as

a substrate), selection of the interacting drugs should be based on a priori knowledge of the enzyme systems that metabolize the developmental drug. The choice of interacting drug should then be based on known, important inhibitors of the pathway under investigation. If the study results are negative, then absence of a clinically important drug–drug interaction for the metabolic pathway could be claimed.

Route of Administration

For a developmental drug used as either an interacting drug or substrate, the route of administration should generally be the one planned for in product labeling.

Dose Selection

For both the substrate and interacting drug, testing should maximize the possibility of finding an interaction. For this reason, the maximum planned or approved dose and shortest dosing interval of the interacting drug (as inhibitors or inducers) should be used. Doses smaller than those to be used clinically may be needed for substrates on safety grounds and may be more sensitive to the effect of the interacting drug.

Sample Size and Statistical Considerations

For both developmental drugs and approved drugs, when used as substrates and/or interacting drugs in drug–drug interaction studies, the desired goal of the analysis is to determine the clinical significance of any increase or decrease in exposure to the substrate in the presence of the interacting drug. Assuming unchanged PK/PD relationships, changes may be evaluated by comparing pharmacokinetic measures of systemic exposure that are most relevant to an understanding of the relationship between dose (exposure) and therapeutic outcome.

Results of drug–drug interaction studies should be reported as 90 % confidence intervals about the geometric mean ratio of the observed pharmacokinetic measures with (S+I) and without the interacting drug (S). Confidence intervals provide an estimate of the distribution of the observed systemic exposure measure ratio of S+I versus S alone and convey a probability of the magnitude of the interaction.

When a drug–drug interaction is clearly present, the sponsor should be able to provide specific recommendations regarding the clinical significance of the interaction based on what is known about the dose-response and/or PK/PD relationship for either the investigational agent or the approved drugs used in the study.

The sponsor may wish to make specific claims in the package insert that no drug–drug interaction is expected. In these instances, the sponsor should be able to recommend specific no effect boundaries, or clinical equivalence intervals, for a drug–drug interaction. No effect boundaries define the interval within which a change in a systemic exposure measure is considered not clinically meaningful.

REFERENCES

Bjornsson TD, Callaghan JT, Einolf HJ et al. (2003) The conduct of in vitro and in vivo drug-drug interaction studies: a pharmaceutical research and manufacturers of America (PhRMA) perspective. Drug Metabolism 31:815–832

EMEA CPMP/EWP/560/95 (1997) Note for guidance on the investigation of drug interactions. December 1997

US FDA (1993) Guideline for the study and evaluation of gender differences in the clinical evaluation of drugs; Notice

US FDA (1999) Guidance for industry: In vivo drug metabolism/drug interaction studies – study design, data analysis, and recommendations for dosing and labeling. November 1999

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above under "Procedure" is presented below.

Results

The data pertinent to the assessment of potential pharmacokinetic and pharmacodynamic interactions from the study described above, as given in Table 10, can be summarized as follows:

In this study nine different brands of oral contraceptives were used. The dose of ethinylestradiol per pill most commonly taken was 30 μg (by 17 subjects) and ranged from 20 to 50 μg across all 24 subjects who completed the study as planned.

Pharmacokinetics: When EE was administered in combination with Drug XYZ, arithmetic mean $t_{1/2}$ and T_{max} values were comparable for both treatments. Geometric mean C_{max} and $AUC_{0\text{-}24}$ values were approximately 20 % to 30 % lower, respectively, than when EE was administered with placebo. For $AUC_{0\text{-}24}$, the lower and upper limit of the 90 % CI were below the pre-defined 90 % CI of 0.8–1.25. Therefore, a pharmacokinetic interaction between Drug XYZ and EE can be concluded. The mechanism for this statistically significant reduction in systemic exposure to EE is unknown, however, since Drug XYZ is known to induce CYP 1A2 in man, and CYP 1A and phase II enzymes in animals, the observed effect could reflect a metabolic interaction between Drug XYZ and EE.

Table 10 Summary statistics of pharmacokinetic parameters for ethinylestradiol.

Parameter	EE + placebo				EE + Drug XYZ			
	n	Arith. mean	CV (%)	Geo. mean	n	Arith. mean	CV (%)	Geo. mean
$\mathbf{C}_{max}$ (pg/mL)	24	98.3	39	91.1	24	79.4	43	72.5
$\mathbf{AUC}_{0-24}$ (pg.h/mL)	24	933	34	879	24	672	32	637
	Treatment ratio and 90% confidence interval of $EEAUC_{0-24}$ (EE + Drug XYZ over EE + placebo)0.73 (0.68–0.78)							
$t_{1/2}$ (h)	24	22	27	21	24	21	28	20
T_{max} (h)	24	1.2	55	–	24	1.2	54	–

Pharmacodynamics: Serum progesterone and 17-β-estradiol levels are considered reliable indicators for the occurrence of ovulation. Since progesterone and 17-β-estradiol levels were comparable for both treatments, and progesterone serum concentrations did not exceed 1.4 ng/mL (as defined in this study, progesterone concentrations above 1.4 ng/mL on day 20 of a menstrual cycle indicated ovulation) for both treatments, it was concluded that Drug XYZ administration did not affect the contraceptive effect of ethinylestradiol-based oral contraceptives and that no ovulation occurred in any of the subjects.

II.S.7 Profiling the Effect of Food on Drug Bioavailability

PURPOSE AND RATIONALE

Drugs intended for oral administration have to pass through the gastrointestinal tract before they can enter the blood stream and eventually reach their target site of action. Often already profiled using a food screen in the first-in-man study, the assessment of the influence of food intake on the bioavailability of a drug belongs to the most important steps when describing the drug pharmacokinetics in early clinical development. The outcome of such food screens or formal food interaction studies has a considerable impact on the design of ensuing studies and forms the basis for later labeling recommendations as well as for the package insert (US FDA Guidance for Industry 2002).

Food can alter the bioavailability of drugs either by direct physical or chemical interaction or by the physiological response. Such effects are most prominent when the drug product is administered shortly after a meal. The composition of such a meal should lead to the greatest possible physiological reaction. Consequently, a high-fat, high-calorie breakfast after overnight fasting is recommended. Details on the composition of such a meal and the design of those studies can be found in US FDA Guidance for Industry 2002 and EU CPMP 1999.

PROCEDURE

The design of an exploratory food interaction bioavailability study with drug XYZ1234 is presented below. In this given project, the food interaction study was initiated parallel to a hADME study just after completion of the first-in-man study. In this study, the collection, handling, and interpretation of pharmacokinetic data were in the main focus.

Protocol Outline

Study of the effect of food on the pharmacokinetics of film-coated tablets (3 × 200 mg) of XYZ1234 in healthy men.

Primary Objective

To assess the effect of food on the pharmacokinetics of 600 mg XYZ1234 in healthy men.

Study Design

An open, randomized, 4-period crossover study. There were four sequence groups of 5 subjects each. Each sequence group received the treatments A, B, C and D (different time intervals between food intake and medication), in different sequential order (Williams design). Washout periods were at least 4 days.

Inclusion Criteria

Healthy men aged between 40 and 65 years. Body weights within −15 % and +10 % of the normal weight according to Broca.

Treatments

Treatment A: Single dose of 3 × 200 mg XYZ1234 on an empty stomach (overnight fasting) and start of high fat food intake 4 h later.

Treatment B: Single dose of 3 × 200 mg XYZ1234 together with high fat food (drug administration 15 minutes after start of high fat food intake).

Treatment C: Single dose of 3 × 200 mg XYZ1234 2 h after start of high fat food intake.

Treatment D: Single dose of 3 × 200 mg XYZ1234 on an empty stomach (overnight fasting) 1 hour before start of high fat food intake.

Pharmacokinetic Data

Concentration of XYZ1234 in plasma before and at predefined times after dosing.

EVALUATION

Descriptive Statistics of all Variables

Analysis of Variance (ANOVA) with treatment, subject (nested within sequence) and period as main factors were performed for C_{max} and $AUC_{0\text{-}inf}$. The 90 % confidence intervals of the point estimates of the ratio of C_{max} and $AUC_{0\text{-}inf}$, and of the difference between treatments for t_{max} were determined. Pairwise comparisons to treatment A were made with treatment A versus treatment B being the primary comparison.

CRITICAL ASSESSMENT OF THE METHOD

The information originating from preceding studies is needed for the proper design of a food interaction study: safety and tolerance data has to be considered as well as the PK results including a food screen. The terminal half-life of the drug or its active metabolite(s) will provide the basis for the washout periods. Single dose linearity/proportionality will help to define the dose. Safety and tolerance data will justify the dose. The PK comparison in a food screen (FIM study, see below) will influence the sample size.

It depends on the target indication, e.g. acute or chronic use, and on the intended dosing regimen, e.g. once, twice or three-times daily, whether a given food effect is acceptable for justification of further development or if it defines the 'knock-out'.

The study design described here is a quite complex approach. A simplification would limit the study conditions to both extremes: a high fat, high calorie breakfast starting 0.5 hour before the drug administration versus fasting overnight (at least 10 h) + at least 4 h after drug administration.

MODIFICATIONS OF THE METHOD

There is a tendency to include a so-called food screen already in a FIM study. The limitation of such an approach is the non-availability of information mentioned in the previous chapter. On the other hand, changes in the formulation during the drug development phase, a switch from an immediate-release to an extended-release formulation might necessitate a repetition.

Only if the conditions for a waiver apply, a food interaction study is not needed for a submission package. Under all other circumstances, where drug products are administered orally for systemic exposure, this kind of study is a must. And it must be conducted with the drug product that is intended for the market authorization.

REFERENCES

EU CPMP (1999) Note for Guidance on Modified Release Oral and Transdermal Dosage Forms: Section II (Pharmacokinetic and Clinical Evaluation) July 1999

US FDA Guidance for Industry: Food-Effect Bioavailability and FED Bioequivalence Studies December 2002

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the pharmacokinetic results obtained from the study described above under "Procedure" is presented below.

Results – Pharmacokinetics

The XYZ1234 concentration-time profiles show that food caused a delay in drug absorption and a lower peak concentration. This effect was most pronounced when food and medication were taken at the same time. A summary of the pharmacokinetic parameters in plasma is presented in Table 11.

A summary of the point estimates of the treatment ratios or of the difference between treatments for XYZ1234 is presented in Table 12. The $AUC_{0\text{-}inf}$ was similar for all treatments. When compared to treatment A the $AUC_{0\text{-}inf}$ of the other treatments was within the 80–125 % equivalence limits.

The point estimates show that taking food 2 h before, or at the same time as receiving medication delayed absorption and lowered the maximum plasma concentration. The relative bioavailability, however, was similar for all treatments. The lower limit of the 90 % CI for C_{max} was marginally outside the predefined equivalence range for treatment B and C but C_{max} was within the equivalence range for treatment D.

The intake of a high fat meal before or at dosing lowered the maximum plasma concentration (C_{max}) and increased the absorption time (t_{max}).

The relative bioavailability, however, was equivalent when medication was taken with food or under fasting conditions.

Table 11 Summary of the pharmacokinetic parameters in plasma.

	PK parameter (geometric mean + range)				
	C_{max}	t_{max}*	$t_{1/2}$	AUC_{0-inf}	CLtot/F
Treatment	(μg/mL)	(h)	(h)	(μg.h/mL)	(L/h)
A (n = 19)	4.51	0.75	2.78	11.0	51.4
food 4 h after dosing	(2.00–8.02)	(0.25–2.50)	(2.05–4.98)	(4.96–17.0)	(33.4–115)
B (n = 19)	3.60	1.50	2.77	11.4	49.8
food with dosing	(1.72–8.57)	(0.75–6.00)	(1.78–4.22)	(5.62–19.0)	(29.9–101)
C (n = 19)	3.52	1.50	3.10	9.89	57.4
food 2 h before dosing	(2.02–5.57)	(0.75–2.50)	(1.86–5.98)	(4.12–17.3)	(32.9–138)
D (n = 19)	5.41	0.50	3.05	11.8	48.3
food 1 h after dosing	(2.98–8.65)	(0.50–1.50)	(2.40–4.45)	(6.13–18.2)	(31.2–92.7)

* for t_{max} the median + range is presented

Table 12 Summary of the point estimates of the treatment ratios or of the difference between treatments.

PK parameter	Treatment	Point estimate of treatment ratio*	90% CI
C_{max}	B/A	0.79	0.69–0.91
	C/A	0.78	0.68–0.89
	D/A	1.20	1.04–1.37
AUC_{inf}	B/A	1.03	0.94–1.12
	C/A	0.89	0.81–0.98
	D/A	1.07	0.97–1.17
t_{max}* (h)	B–A	1.13	0.88–1.63
	C–A	0.94	0.63–1.25
	D–A	0.00	–0.13–0.13

* Point estimate of the treatment difference (h) is presented for t_{max}

II.S.8 Exploratory Profiling of Enzyme Induction on Drug Disposition

PURPOSE AND RATIONALE

Concomitantly administered drugs have the potential to interact, i.e. their pharmacokinetics or pharmacodynamics can be altered (US FDA 1999 and references therein). Regulatory guidance suggests that if appropriately performed in-vitro studies indicate the lack of such an interaction, then a specific clinical study is not compulsory. However, if the claim, "No clinically relevant interaction with Drug X" is desired in the product label, then a confirmatory clinical study is compulsory even if in-vitro studies indicated the lack of an interaction (EMEA CPMP/EWP/560/95 1997).

Drug–drug interactions mediated by enzyme induction are less common than those mediated by enzyme inhibition. Drug–drug interactions due to enzyme induction are also less likely to cause safety issues, except toxic if a toxic metabolite is formed by metabolite activation, however, they may affect the activity of the developmental drug itself and of concomitant medications.

Pre-clinical profiling for enzyme induction is complicated by the observation that, contrary to that seen for enzyme inhibition, the enzyme inductive potential of a drug in man is difficult to assess pre-clinically, especially in non-human systems. There, are however, numerous flags which can indicate that the drug under study has some activating effect on drug metabolizing enzymes such as the cytochrome P 450 isozymes (CYP) 1A2, 3A4 etc. These flags can include a drop in systemic exposure to developmental drug after multiple dosing, increases in animal liver weights after multiple dosing in toxicology studies, class characteristics, and positive signals in animal and human in-vitro enzyme induction screens.

If one or more of these flags are observed, especially positive signals in human in-vitro enzyme induction screens, then the in-vivo inductive potential of the developmental drug is typically studied in suitable explorative clinical studies. Given the expectation that enzyme induction is more likely to cause a reduced efficacy of developmental drug itself and/or of concomitant medications than to cause safety issues, the profiling of enzyme induction during early clinical development man is typically included as secondary objective in other studies. Such explorative clinical studies employ non-indication specific, but metabolically well characterized, marker drugs or compounds. If a notable inductive potential is seen in these explorative clinical studies, then more specific studies with drugs with a narrow therapeutic index critical and/or frequent usage in the target population are usually performed.

PROCEDURE

The design of a study providing the suggested exploratory profiling of the effect of Drug XYZ on

CYP 1A2 and CYP 3A4 mediated metabolism, is presented in the Protocol Outline below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of data pertinent to the assessment of potential drug mediated effects on CYP 1A2 and CYP 3A4 mediated metabolism, although other parameters were also studied.

Protocol Outline

Safety, tolerability and the effect on CYP 1A2 and CYP 3A4 mediated metabolism, of single and repeated oral doses of 400 mg and 1200 mg drug XYZ or placebo in overweight or obese, but otherwise healthy men.

Objectives

Primary: The primary objective of the study was to investigate in overweight or obese, but otherwise healthy men: (i) the safety and tolerability of single and repeated oral doses of 400 mg and 1200 mg Drug XYZ, and (ii) the effect of Drug XYZ on CYP 1A2 and CYP 3A4 mediated metabolism.

Secondary: The secondary objective of the study was to investigate the pharmacokinetics of Drug XYZ after single and repeated oral doses of 400 mg and 1200 mg Drug XYZ in overweight or obese, but otherwise healthy men.

Study Design

The study had a single-center, single- and repeated-dose, single-trial period, and double-blind design.

Subjects received single doses of 150 mg caffeine, once before starting treatment with Drug XYZ (Day 1) and then again together with Drug XYZ dosing on Day 10. In addition, subjects received a single daily dose of 400 mg and 1200 mg Drug XYZ or matching placebo on Day 2 and then again on Days 4–10.

On Days 1 and 10 CYP 1A2 activity was monitored using the plasma concentrations of caffeine and paraxanthine and CYP 3A4 activity was monitored using the urinary excretion of free cortisol and 6-β-hydroxy-cortisol.

The treatment period between trial periods was at least 10 days which approximated to > 10 Drug XYZ apparent terminal half-lives.

Number of Subjects

The inclusion of eight subjects was considered to be in line with common industry practice for this type of explorative study.

Inclusion Criteria

The following inclusion criteria were met: Men aged between 18 and 55 years; with body mass index (BMI) of 25–35 kg/m^2; who – apart from being overweight or obese – are healthy for the purpose of the study and not receiving regular medication in the month preceding the study; with normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant; who are non-smokers.

Treatments

Single (Day 2) and repeated (Days 4–10) oral doses of 400 mg and 1200 mg Drug XYZ or matching placebo after fasting. Single doses of 150 mg caffeine, once on Day 1 and then again on Day 10.

Pharmacokinetic Data

Plasma concentrations of Drug XYZ, caffeine and paraxanthine, before and at pre-determined times post dose were measured.

Concentrations of Drug XYZ, 6-β-hydroxy-cortisol and free cortisol in urine collected over the profiling period were measured. Volumes of urine collected over each profiling period were recorded.

The volume of urine collected over 24 h and the concentration of creatinine was determined to allow the subjects creatinine clearance to be calculated.

EVALUATION

The data pertinent to the assessment of potential drug mediated effects on CYP 1A2 and CYP 3A4 mediated metabolism from study described above, was evaluated as follows: Due to the investigational nature of the study, and the small sample size, all variables were only presented descriptively. Where appropriate, individual data were presented together with descriptive statistics.

Plasma caffeine and paraxanthine: Descriptive pharmacokinetic parameters (standard parameters including peak concentrations (C_{max}), time of C_{max} (T_{max}), area-under-the-curve (AUC) between time 0 and time t where t = 24 h post dose (AUC_{0-t}), AUC after extrapolation to infinity ($AUC_{0-\infty}$), apparent terminal half-life ($t_{1/2\_z}$), total clearance (CL)) for plasma caffeine and paraxanthine on Days 1 and 10 were calculated using a non-compartmental analysis employing a linear/log trapezoidal method as implemented in WinNonlin (Pharsight Corp.) protocols. Changes from baseline (Day 10–Day 1) in caffeine clearance and the area-under-the-curve (AUC) ratio paraxanthine/caffeine were presented individually and with corresponding descriptive statistics. The ratio of paraxanthine AUC/caffeine AUC was calculated.

Urinary 6-β-hydroxy-cortisol and free cortisol: The following pharmacokinetic variables were derived from urine concentration data for 6-β-hydroxy-cortisol and free cortisol on Days 1 and 10 using SAS for Windows protocols: amount excreted during each collection interval for 6-β-hydroxy-cortisol and free

cortisol; total amount excreted (mg) during 12 h ($Ae_{0–12}$) and 24 h ($Ae_{0–24}$) for both compounds; the ratio of $Ae_{0–24}$ of 6-β-hydroxy-cortisol/$Ae_{0–24}$ of free cortisol; the ratio of $Ae_{0–12}$ of 6-β-hydroxy-cortisol/$Ae_{0–12}$ of free cortisol.

CRITICAL ASSESSMENT OF THE METHOD

Ideally, such a study should profile the dose-dependence, the time-dependence, and the reversibility of the enzyme induction. Also the enzyme substrates used as markers for the enzyme activity should be drugs with a narrow therapeutic index used by the target population and/or frequent usage in the target population.

In the study described the time-dependence and reversibility of the enzyme induction was not studied. Also the enzyme substrate used as a marker for the CYP 1A2 activity was caffeine, which although frequently encountered in the target population and commonly used as a marker for CYP 1A2 activity, is not a drug with a narrow therapeutic index used by the target population. The enzyme substrate used as a marker for the CYP 3A4 activity, urinary 6-β-hydroxy-cortisol and free cortisol, although readily amenable to inclusion in studies, is not a drug and is also known to be a relatively insensitive marker for CYP 3A4 induction. Also urinary 6-β-hydroxy-cortisol and free cortisol does not differentiate between intestinal and liver CYP 3A4 activities.

MODIFICATIONS OF THE METHOD

The design of studies profiling of enzyme induction are typically case-specific since the time and dose-dependence of enzyme induction differs between the enzyme(s) being induced and the drug causing the induction. There is no clearly defined regulatory guidance on enzyme induction studies beyond the recommendation that study designs should be science based.

It is generally accepted that the dosing regimen should minimally ensure that the anticipated therapeutic steady-state exposure is maintained (or exceeded) for some days since although some inducible enzymes respond rapidly, others require longer exposure before responding. Ideally, such a study should profile the dose-dependence, the time-dependence, and the reversibility of the enzyme induction. Also the enzyme substrates used as markers for the enzyme activity should be drugs with a narrow therapeutic index used by the target population and/or frequent usage in the target population.

The study described in this article provides explorative profiling of potential enzyme induction after dosing over two weeks at two dose levels and in comparison to placebo. This relatively comprehensive design reflects the combination of (i) the clear expectation that a clinically relevant enzyme induction would be observed based on numerous flags from pre-clinical studies and experience with other members of this chemical class characteristics for this drug, (ii) the chance ability to build in the planned investigation into a tolerability study due to the use of innocuous (caffeine) or endogenous enzyme markers (urinary 6-β-hydroxy-cortisol/free cortisol), and (iii) the opinion that a clinically relevant enzyme induction would severely impact the market value of Drug XYZ and thus should be profiled as early as possible.

In practice, the explorative profiling of enzyme induction is even less elaborate than performed for Drug XYZ because (i) it requires the use of enzyme substrates which are less amenable to inclusion in tolerability studies, and (ii) risk benefit considerations do not justify studies a with a range of dose levels.

Instead specific confirmatory clinical studies employing drugs with a narrow therapeutic index used by the target population and/or frequent usage in the target population, are typically implemented in the late clinical development as part of the range of drug–drug interaction studies used to support the drug label.

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Table 13 Mean (SD) plasma caffeine and paraxanthine pharmacokinetic variables (ng.h/mL) by treatment.

Analyte Variable Day	N	Treatment group Placebo	400 mg	1200 mg
Caffeine				
AUC_{0-t}				
Day 1 (Baseline)	8	26626.1 (8971.9)	16261.7 (3908.3)	21977.9 (4309.7)
Day 10	8	24996.9 (5393.2)	6464.0 (2052.7)	6580.1 (1493.6)
$AUC_{0-\infty}$				
Day 1 (Baseline)	8	43222.8 (23538.3)	20585.0 (8236.6)	33769.3 (11680.6)
Day 10	8	37447.4 (14356.4)	6776.0 (2326.7)	6793.7 (1572.8)
Paraxanthine				
AUC_{0-t}				
Day 1 (Baseline)	8	8258.1 (2036.8)	7430.9 (1166.8)	7809.7 (2228.7)
Day 10	8	8886.6 (2549.1)	5624.1 (770.9)	6054.2 (1192.0)

Table 14 Mean (SD) ratio of urinary 6-β-hydroxy-cortisol/free cortisol excreted by treatment.

Analyte Variable Day	N	Treatment group Placebo	400 mg	1200 mg
Ratio 6-β-hydroxy-cortisol/Free cortisol				
Ae_{0-12} Day 1 (Baseline)	8	8.75 (2.3)	11.4 (7.9)	9.28 (3.7)
Ae_{0-24} Day 10	8	11.3 (2.0)	11.4 (4.7)	12.7 (6.1)

US FDA (1999) Guidance for industry: In vivo drug metabolism/drug interaction studies – study design, data analysis, and recommendations for dosing and labeling. November 1999

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the data from the study described above is presented below.

Results – Pharmacokinetics

The data pertinent to the assessment of potential drug mediated effects on CYP 1A2 and CYP 3A4 mediated metabolism from study described above, as given in Tables 13 and 14, can be summarized as follows:

- The mean $AUC_{0-\infty}$ for caffeine on Day 10 was decreased by approx. 67 %, and approx. 80 % when compared to Day 1 for subjects treated with 400 mg and 1200 mg Drug XYZ, respectively, and was more pronounced than for caffeine and paraxanthine mean AUC_{0-t} values, which also decreased substantially
- Administration with placebo showed no notable change in caffeine or paraxanthine levels
- On Day 10, the mean caffeine clearance [dose/$AUC_{0-\infty}$] was increased by approx. 3-fold in subjects treated with 400 mg Drug XYZ and 5-fold in subjects treated with 1200 mg
- All the caffeine / paraxanthine results suggest that Drug XYZ induced CYP 1A2 in humans and that the extent of induction was dependent on the dose administered
- Given that a natural approx. 20-fold variation in CYP 1A2 activity has been reported in the literature, the observed induction was considered to be clinically irrelevant
- The ratio of 6-β-hydroxy-cortisol/free cortisol excretion was about 11 for all treatments on both day 1 and 10 suggesting that there was no noticeable effect on CYP 3A4 activity.

II.S.9 Formulation Interactions

PURPOSE AND RATIONALE

The reasons why individuals differ in their responsiveness to drugs in medical products are manifold, and include age, gender, genetics, disease, and drugs given concomitantly.

The focus of interaction studies has changed from ad hoc observational studies to rationally designed studies. Depending on the structural and physicochemical characteristics and on animal and human in vitro data, selective in vivo studies are performed. Based on the results of such studies the risk of clinically relevant interactions may be predicted. As a result, essential informa-

tion on formulation interactions has become an integral part of the labeling document of a drug product.

Formulation interactions can be of many different kinds, such as formulation interactions with packaging material (compatibilities), interactions between the active drug and exipient(s), or interactions between formulations (e.g. when mixing the drug product with other (parenteral) drug products). Details about how to deal with changes in components or composition of drug products are described in published regulatory guidance (Guidance for Industry 1999), while no formal guidance exists that in particular covers all type of formulation interactions. It is important to differentiate between detectable interactions and clinically relevant interactions. A clinically relevant interaction for example is the concomitant administration of two drugs that interact to such an extent that a dosage adjustment may be required. Details about clinically relevant (drug–drug) interactions and assessment of equivalence of formulations can be found. (Guidance for Industry 2001, Steinijans, Hauschke 1997, CPMP/EWP/QWP/1401/98 2002, CPMP/EWP/560/95 1998)

While formulation interactions often are subject to in vitro investigations, the section below presents a particular example of an in vivo formulation interaction study (CPMP/EWP/QWP/1401/98 2002): a potential interaction of a drug in medical practice frequently given concomitantly with another drug (i.e. both mixed in a syringe) was subject to a clinical study which is illustrated below. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety and pharmacodynamic parameters were also studied.

PROCEDURE

The design of a typical formulation interaction study is presented below.

Protocol Outline

Pharmacokinetics of HMR1964 (insulin glulisine) syringe-mixed versus simultaneosly injected 0.1 IU/kg HMR1964 (insulin glulisine) and 0.2 IU/kg NPH insulin in healthy subjects using the euglycemic clamp technique.

Objective

To investigate the pharmacokinetics of insulin glulisine following subcutaneous (s.c.) administration of insulin glulisine immediately premixed in a syringe with NPH insulin versus separate simultaneous injections of insulin glulisine and NPH insulin.

Design, Treatment and Sample Size

This was a single-dose, randomized, open-label, two-way, crossover study. 0.1 IU/kg insulin glulisine and 0.2 IU/kg NPH insulin separate and simultaneous s.c. injections in the abdominal area or 0.1 IU/kg insulin glulisine and 0.2 IU/kg NPH insulin by s.c. injection in the abdominal area, immediately after being premixed in the syringe.

Population Treated

Thirty-two (32) healthy male subjects, aged 18–45 years, with body mass index between 18 and 26 kg/m^2, with a minimum body weight of 65 kg were treated.

Pharmacokinetic interaction studies are suggested to be performed in healthy individuals. Furthermore, it was anticipated that any interaction, should it occur, would be present in this population.

EVALUATION

Statistical Procedures Pharmacokinetics

The interpretation of the pharmacokinetic variables C_{max}, AUCs and MRT of insulin glulisine was based on 95 % confidence intervals, after ln-transformation of the data. These 95 % confidence intervals were calculated for the respective mean ratios of pair-wise treatment comparisons. In addition, the "test" treatment was compared to the "reference" treatment with respect to the pharmacokinetic variables using an ANOVA with subject, treatment and period effects, after ln-transformation of the data. The subject sum of squares was partitioned to give a term for sequence (treatment by period interaction) and a term for subject within sequence (a residual term). Due to the explorative nature of the study, no adjustment of the α-level was made for the multiple testing procedure.

The time to maximum insulin glulisine concentration (T_{max}) was analyzed by non-parametric analyses. 95 % non-parametric confidence intervals for the respective median difference in treatment ("test-reference") were calculated according to the literature. Pair-wise treatment comparison was made for the pharmacokinetic variables.

CRITICAL ASSESSMENT OF THE METHOD

Introduction

HMR1964 (INN: insulin glulisine) is a human insulin analogue for the treatment of Type I and Type II patients with diabetes mellitus. Combinations of insulin preparations that differ both in their time of onset and duration of action are used optimally to control blood glucose in patients with diabetes mellitus. The most commonly used insulin regimens include a long-acting in-

sulin to provide basal insulin requirements and control fasting and pre-prandial blood glucose in combination with a short-acting insulin to control prandial blood glucose excursions. These two insulins are sometimes premixed in the syringe prior to injection. HMR1964 (insulin glulisine) has a more rapid onset and shorter duration of action than regular insulin when administered subcutaneously.

Study Rationale

The purpose of the study was to compare the time concentration profiles of syringe-premixed versus simultaneously, subcutaneously injected 0.1 IU/kg insulin glulisine and 0.2 IU/kg NPH insulin. This is intended to address the use of insulin glulisine in a basal bolus treatment regimen, when it might be injected together with NPH insulin to avoid the need for 2 separate injections, with the aim of determining whether mixing the 2 insulin preparations might compromise the rapid-acting properties of insulin glulisine.

Dosing Recommendation/Therapeutic Dose-Range

A single dose of 0.1 IU/kg HMR1964 (insulin glulisine) was chosen for this study, as this dose is well within the range of recognized average prandial insulin dose in Type I and Type II patients with diabetes mellitus. Based on the primary pharmacokinetics of HMR1964 (insulin glulisine), a single dose study was anticipated to be adequate.

MODIFICATIONS OF THE METHOD

Individual Bioequivalence

In contrast to the standard average bioequivalence approach for which the regulatory requirements (Guidance for Industry 2001, CPMP/EWP/560/95 1998) have been internationally harmonized, this is by no means the case for the more recent concept of individual bioequivalence (Steinijans and Hauschke 1997). The main reason for introducing more complex replicate designs and bioequivalence criteria are the highly variable drugs, for which the setting of suitable bioequivalence ranges poses a major problem and scaling of the bioequivalence criteria by the intra-subject variability has been suggested. The shortcoming of the present two-treatment, two-period (2×2) crossover design to detect subject-by-formulation interaction provides a second argument in favor of the more complex replicate designs. A unified approach of proposed statistical procedures for the replicate design has been given by Schall et al. 1996. However, the availability of these methods and understanding of them seems to be limited to a small working group, so a broader international awareness of the problems and potential solutions is desirable.

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EXAMPLE

To illustrate the type of data that can be obtained using the study under discussion, a high level summary of the data from the study described above under "Procedure" is presented below.

Thirty-two healthy male subjects with demographics as described above completed the study according to the protocol.

The insulin glulisine concentration-time profiles are reflected in the following results:

Table 15 Pharmacokinetic parameters of HMR1964 (insulin glulisine). Separate simultaneous (Treatment A) versus immediately premixed (Treatment B).

Variable	Geometric mean (arithmetic mean) (N = 32) Simultaneous (A)	Premixed (B)	Point estimate/(95% CI)[1] Premixed (B)/Simultaneous (A)
$AUC_{(0-clamp\ end)}$ [$\mu IU.min.mL^{-1}$]	9261.79 (9401.83)	8251.20 (8843.44)	89.1%(78.1; 101.6%)
C_{max}[μIU/mL]	69.98 (71.80)	51.32 (55.39)	73.3% (64.3; 83.6%)
MRT [min]	92 (96)	118 (121)	127.3% (120.3; 134.7%)
T_{max} [min]	47[2]	50[2]	3.9 min (–3.3; 12.4 min)[3]

[1]Point estimates and 95% confidence intervals for the ratio oftreatment means, based on ln-transformed data.
[2]Median values.
[3]Point estimates and 95% confidence intervals for therespective median differences from non-parametric data analysis.

The total systemic insulin glulisine availability as presented by [$AUC_{(0\text{-clamp end})}$] was similar for the two modes of administration of insulin glulisine and NPH insulin, mixed in a syringe immediately before injection (treatment B), and the separate simultaneously injections (treatment A).

The maximum insulin glulisine concentration C_{max} was somewhat attenuated, being 27 % less, when insulin glulisine was premixed with NPH insulin as compared to the separate simultaneous administration. Nevertheless, the time to C_{max} did not differ between the two treatments with T_{max} values of 47 and 50 minutes (min).

The MRT of insulin glulisine was somewhat longer when insulin glulisine was premixed with NPH insulin (118 min) compared to their simultaneous administration (92 min), corresponding to an average increase of 27 %.

In summary, the total insulin glulisine availability [$AUC_{(0\text{-clamp end})}$] was similar for the two modes of administration and further, the time to maximum concentration [C_{max}] was not affected when insulin glulisine was mixed with NPH insulin with T_{max} values of 47 and 50 min. C_{max} was somewhat attenuated being 27 % less when insulin glulisine was immediately premixed with NPH insulin as compared to the separate simultaneous administrations of these study medications.

Overall these data support the possibility of mixing of insulin glulisine with NPH insulin in a syringe immediately prior to administration.

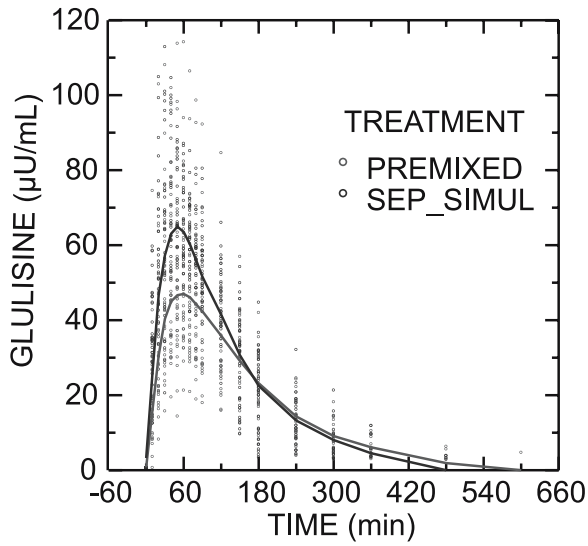

Fig. 5. Insulin glulisine concentration time profiles: individual profiles (*dotted lines*) and the fitted average mean profiles (*solid line*).

ABBREVIATIONS
ANOVA: Analysis of variance
AUC: Area under the curve
C_{max}: Maximum concentration
IU: International units
INN: International nonproprietary name
MRT: Mean residence time
NPH: Neutral protamine Hagedorn (isophane insulin)
s.c.: Subcutaneous(ly)
T_{max}: Time to maximum concentration

II.S.10 Special Population: Subjects with Renal Impairment

PURPOSE AND RATIONALE

A pharmacokinetic (PK) study in individuals with impaired renal function is recommended when renal impairment is likely significantly to alter the disposition of a drug/or its active metabolite(s) such that a dose adjustment may be needed. In the main, this is the case primarily for drugs that are mainly eliminated (excretion and/or metabolism) by the kidneys and/or if a drug has a narrow therapeutic window. However, severe re-

nal impairment may lead to an accumulation of a drug through diverse mechanisms, thus also low renal clearance drugs may significantly be affected by renal impairment.

Most drugs are cleared by elimination of unchanged drug by the kidney and/or by metabolism in the liver. For a drug eliminated primarily via renal excretory mechanisms, impaired renal function may alter its pharmacokinetics (and pharmacodynamics) to an extent that the dosage regimen needs to be changed from that used in patients with normal renal function.

A study should also be considered when a drug or an active metabolite exhibits a combination of high hepatic clearance (relative to hepatic blood flow) and significant plasma protein binding. In this setting, renal impairment could induce a significant increase in the unbound concentrations after parenteral administration due to a decreased plasma protein binding coupled with little or no change in the total clearance (decrease in unbound clearance).

More details about when such studies may be or may not be important and other aspects like study design and methods can be found in CPMP/EWP/225/02 2004 and Guidance for Industry 1998. Although the most obvious type of change arising from renal impairment is a decrease in renal excretion, or possibly renal metabolism of a drug or its metabolites, renal impairment has also been associated with other changes, such as changes in absorption, hepatic metabolism, plasma protein binding, drug distribution, drug accumulation. These changes may be particularly prominent in patients with severely impaired renal function and have been observed even when the renal route is not the primary route of elimination of a drug. Thus, for most drugs that are likely to be administered to patients with renal impairment, pharmacokinetic characterization should be assessed in patients with renal impairment to provide rational dosing recommendations. It may not be feasible to conduct the study in patients with the condition for which a drug is intended. An acceptable alternative is to use volunteers with different degree of renal function. It may also be necessary to study the influence of dialysis on the pharmacokinetics of a drug. In this case it is recommended to study this end stage renal diseased sub-group separately from the renal impaired individuals, as different types of clinical study protocols are suggested to be applied for those undergoing dialysis and those who are not. Renal impaired individuals not undergoing dialysis are subject to the example that is discussed below.

It is also central to distinguish between explorative studies conducted during the investigational phase before the confirmatory safety and efficacy trials, aimed to give appropriate dosage recommendations for the patients with reduced renal function and confirmative studies (usually conducted in parallel to the confirmatory safety and efficacy trials) when assessing the influence of renal impairment on the pharmacokinetics of a drug, as for instance study design and timing of study conduct may be different.

In summary, the primary goal of a study in individuals with impaired renal function is to determine if the pharmacokinetic is altered to such an extent that the dose and/or dosing regimen of a drug should be adjusted from that established in the confirmatory safety and efficacy trials.

The following sections present a particular study in renal impaired individuals with result illustration and conclusion. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data, although clearly safety parameters were also studied.

PROCEDURE

The design of a typical renal impairment study is presented below.

Protocol Outline

Pharmacokinetics of HMR1964 (insulin glulisine) after subcutaneous injection of a single dose of 0.15 IU/kg in non-diabetic subjects with different degrees of renal function in an open, parallel group, single dose, multi center study.

Objective

To investigate the pharmacokinetics of HMR1964 (insulin glulisine) in non-diabetic subjects with different degrees of renal function.

Design, Population, Treatment

Single-dose, open, three-parallel-group, study in non-diabetic subjects with different degrees of renal function (classified according to the literature). The renal function groups will consist of eight individuals each and will be comparable to each other with respect to age, gender, and weight. The creatinine clearance (CL_{cr}) will be the measures of renal function.

Group 1 (normal renal function): $CL_{cr} > 80$ mL/min

Group 3 (moderate renal impairment): CL_{cr} 30 to 50 mL/min

Group 4 (severe renal impairment): $CL_{cr} < 30$ mL/min, but not requiring hemodialysis.

Individuals will be allocated to these renal function groups based on the weighted average of the two pre-treatment CL_{cr} values. Individuals will receive a single dose (0.15 IU/kg body weight) of HMR1964 (insulin glulisine) subcutaneously in the periumbilical abdomen. Blood for determinations of insulin in serum will be taken according to the sampling schedule.

Inclusion Criteria

Non-diabetic men or women, aged between 18 and 75 years. The renally compromised individuals will have to show a reasonably stable renal function in the previous three months (e.g. maximum change in CL_{cr} of approximately 15 mL/min, determined by urine and/or serum). Women either postmenopausal, surgically sterilized, or not pregnant and using adequate contraception.

EVALUATION

Statistical Procedures Pharmacokinetics

The relationship between renal impairment and the absorption and disposition of HMR1964 (insulin glulisine) will be assessed by regressing pharmacokinetic parameters onto CL_{cr}. Regression parameter estimates (± standard error) with confidence intervals and coefficients of correlation (Pearson) with p-values for test of difference from zero will be reported. Scatter plots of the concentration time profiles and pharmacokinetic parameters against creatinine clearance will be produced.

Statistical methods will be applied to the natural log (ln) transformed pharmacokinetic parameters, except for T_{max} on which statistical methods on raw data will be applied.

CRITICAL ASSESSMENT OF THE METHOD

HMR1964 (INN: insulin glulisine) is a human insulin analogue for the treatment of Type I and Type II patients with diabetes mellitus. Combinations of insulin preparations that differ both in their time of onset and duration of action are used to optimally control blood glucose in patients with diabetes mellitus. The most commonly used insulin regimens include a long-acting insulin to provide basal insulin requirements and control fasting and preprandial blood glucose in combination with a short-acting insulin to control prandial blood glucose excursions. HMR1964 (insulin glulisine) has a more rapid onset and shorter duration of action than regular insulin when administered subcutaneously.

Preclinical studies suggest HMR1964 (insulin glulisine) does not differ from the established elimination pathways of endogenous human insulin.

Study Rationale

The purpose of this study is to characterize the impact of renal impairment on the absorption and disposition of HMR1964 (insulin glulisine) in non-diabetic subjects with different degrees of renal function. A control group with normal renal function and similar demographics to the renally impaired subjects is included in this study, as suggested by the CPMP and FDA Guidance for Industry (CPMP/EWP/225/ 02 2004 and Guidance for Industry 1998). This study was conducted after the investigational phase in parallel to the confirmatory safety and efficacy trials.

Population

In order to cover a wide range of renal function, individuals from renal function groups 1, 3 and 4 were enrolled. Individuals with mild impairment (group 2) are often much more difficult to recruit for phase I studies, as they are mostly not yet diagnosed. Yet those patients can be found in phase II/III studies quite frequently, allowing a population PK approach for mild renal impairment. As described above, it is recommended that individuals of group 5 be studied separately.

For adequate representation of individuals with various degrees of renal impairment, an equal numbers of individuals from each renal function group was recruited, and the renally compromised individuals had to show a reasonably stable renal function in the previous three months (e.g. maximum change in CL_{cr} of approximately 15 mL/min, determined by urine and/or serum) before study start. This study followed an alternative and used a non-patient population with different degrees of renal function with comparable demographic factors such as weight, age, and gender. Individuals were matched for body mass index (BMI) and age between the three renal function groups (BMI matched ± 20 % between renal function groups, and with age, between 18 and 75 years, matched ± 20 years between renal function groups) and a similar number of men and women was enrolled in each renal function group.

Dosing Recommendation/Therapeutic Dose-Range

A single dose of 0.15 IU/kg HMR1964 (insulin glulisine) was chosen for the individuals with renal impairment as this is a well recognized average prandial insulin dose in Type I and Type II patients with diabetes mellitus. Based on the primary pharmacokinetics of HMR1964 (insulin glulisine) a single dose study was anticipated to be adequate.

MODIFICATIONS OF THE METHOD

Reduced/Staged Study Design

In a reduced design individuals at the extremes of renal function (normal and severely impaired individuals) are studied. The aim of such a study is to confirm that the pharmacokinetics is not altered to a clinically relevant extent. A prerequisite to this approach is a good reason to believe that renal impairment does not affect the PK to a degree sufficient to warrant dosage adjustment.

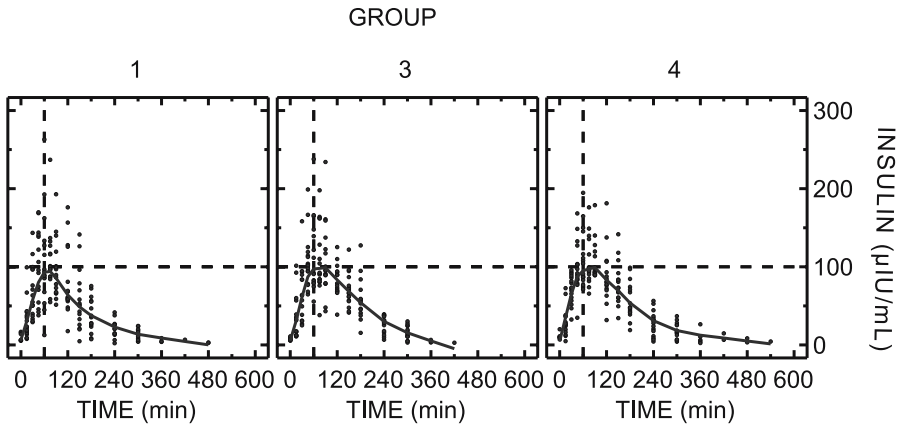

Fig. 6. Average insulin glulisine concentration time profiles of renal function groups 1, 3, 4.

Population PK

A population PK evaluation of patients from the safety and efficacy trials can be used to assess the impact of renal function on the disposition of a drug. Special care must be taken that patients with severe renal impairment are adequately represented in the population. The population PK approach assess the impact of various covariates on the disposition of a drug. Non linear mixed effects modeling may be used to model the relationship between various covariates and pharmacokinetic parameters. CL_{cr} as a measure of renal function may be one of the covariates. This type of approach has it advantageous as it involves assessment of the effect of renal impairment on the PK in the target population.

End-Stage Renal Diseased Individuals

Independently of a drug being eliminated by the renal route or not, the dialysis process can significantly alter the PK of a drug. Once significant fractions of a drug are removed by dialysis, the dosing regimen may need to be changed (e.g. supplementary dosing during/after dialysis). A study in end-stage renal diseased individuals also provide information about the value of dialysis in case of overdose.

Cross-Over Study Design

In case of two study medications, a sound alternative to a parallel design would be the (complete) cross-over study design.

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EXAMPLE

To illustrate the type of data that can be obtained using this study, a summary of the PK results obtained from the study described above under "Procedure" is presented below.

A population as described above completed the study according to the protocol.

There was no apparent relationship between PK parameters and degree of renal function, as there were no obvious differences in insulin concentration-time profiles (Figure 6) between the 3 renal function groups. The PK properties of HMR1964 (insulin glulisine) were similar in subjects with decreased renal function covering a wide range of renal impairment.

There were no statistically significant correlations between renal function as measured by CL_{cr} and the parameters characterizing the rapid-acting properties of HMR1964 (insulin glulisine), $AUC_{(0\text{-}2h)}$, C_{max} and T_{max} (Table 16). There were weak, though statistically significant correlations between renal function and pa-

Table 16 Pharmacokinetics of 0.15 IU/kg insulin glulisine in subjects with renal impairment).

	Geometric mean (CV%)		
	Normal (n = 8)	Moderate (n = 8)	Severe (n = 8)
$AUC_{(0-end)}$ [μIU.min.mL^{-1}]	13215 (31)	18473 (19)	17650 (19)
$AUC_{(0-5h)}$ [μIU.min.mL^{-1}]	13120 (29)	18412 (19)	16912 (16)
$AUC_{(0-2h)}$ [μIU.min.mL]	9005 (24)	11626 (26)	9622 (17)
CL_{tot}/F [mL/min]	852 (20)	637 (20)	680 (14)
C_{max} [μIU/mL]	108 (30)	131 (29)	108 (15)
T_{max} [min][a]	56 (29)	58 (18)	68 (22)

[a]: Median values.
Note: the apparent relative total clearance was calculated as $CL_{tot}/F = dose/AUC_{(0-5h)}$.

rameters characterizing total exposure ($AUC_{(0\text{-}end)}$ and $AUC_{(0\text{-}5h)}$) and relative total clearance of HMR1964 (insulin glulisine) (CL_{tot}/F).

The predicted changes, which are within the conventional equivalence bounds, are too small to suggest any meaningful clinical consequences.

In conclusion, the rapid acting insulin, HMR1964 (insulin glulisine) maintains its pharmacokinetic properties in subjects with decreased renal function covering a wide range of renal impairment.

As a consequence, no dose adjustments in patients with renal failure are warranted on the basis of intrinsic properties of HMR1964 (insulin glulisine).

ABBREVIATIONS

AUC: Area under the curve
C_{max}: Maximum concentration
CLtot/F: Relative total clearance
INN: International nonproprietary name
MRT: Mean residence time
T_{max}: Time to maximum concentration

II.S.11 Special Population: Subjects with Hepatic Impairment

PURPOSE AND RATIONALE

A pharmacokinetic study in individuals with impaired hepatic function is recommended when hepatic impairment is likely significantly to alter the pharmacokinetics of a drug or its metabolite(s) such that a dose adjustment may be required.

In liver disease, the likelihood of a pharmacokinetically relevant drug-disease interaction depends on the type of drug (extent of intra-/extra-hepatic elimination, hepatic extraction ratio, protein binding) and the nature (flow, enzymatic capacity/reserve) and extent of the liver dysfunction. The likelihood of a pharmacokinetic relevant interaction with the need for dosage adjustment in individuals with impaired hepatic function depends on the changes in systemic exposure.

The majority of drugs is cleared hepatically through a variety of oxidative and conjugative metabolic pathways and/or through biliary excretion of unchanged drug or metabolites. In the main, alterations of these excretory and metabolic activities by hepatic impairment can lead to drug accumulation, or, less often, to a failure to form metabolite(s). Liver disease may also alter kidney function, which can lead to accumulation of a drug and its metabolites even when the liver is not primarily responsible for elimination. Moreover, the specific impact of any disease on hepatic function is often poorly described and highly variable, particularly with regard to effects on the pharmacokinetics of a drug.

Hence, a study in individuals with impaired hepatic function may also be recommended if the liver is not the major organ for elimination, or if the drug has a narrow therapeutic window, or in case the metabolism is unknown and other information is lacking.

It is also central to distinguish between explorative studies (conducted during the investigational phase before the confirmatory safety and efficacy trials, aimed to give dosage recommendations for patients with reduced hepatic function which otherwise would be treated with the planned clinical dose for patients with normal hepatic function) and confirmative studies (usually conducted in parallel to the confirmatory safety and efficacy trials) when assessing the influence of hepatic impairment on the pharmacokinetics of a drug, as for instance study design and timing of study conduct may be different.

Measurements such as creatinine clearance have been used successfully to adjust dosing regimens for drugs eliminated primarily by the kidneys. Measures of hepatic function have been sought using endogenous

Table 17 The Child-Pugh classification.

Assessment	Degree of abnormality	Score
Encephalopathy	None	1
	Moderate	2
	Severe	3
Ascites	Absent	1
	Slight	2
	Moderate	3
Bilirubin (mg/dL)	< 2	1
	2.1–3	3
	> 3	3
Albumin (g/dL)	>3.5	1
	2.8–3.5	2
	< 2.83	
Prothrombin	0–3.9	1
Time (seconds $>$ control)	4–6	2
	> 63	
Total Score	Group	Serverity
5–6	A	Mild
7–9	B	Moderate
10–15	C	Severe

substances affected by the liver, such as bilirubin and albumin, or functional measures such as prothrombin time, or the ability of the liver to eliminate marker substrates such as indocyanine green or galactose (Figg et al. 1995; Tang and Hu 1992). Clinical variables such as ascites or encephalopathy, nutritional status, peripheral edema, histological evidence of fibrosis have also been used to categorize hepatic impairment. Despite extensive efforts, no single measure or group of measures has gained broad clinical use. The most widely used scheme system to classify the degree of hepatic impairment is the Child-Pugh System (Albers et al. 1989; Pugh et al. 1973).

More details about when such studies may or may not be important, and other aspects as study design and methods, can be found in CPMP/EWP/23339/02 2004 and Guidance for Industry 2003.

In summary, a pharmacokinetic study in individuals with impaired hepatic function is recommended if the extent of hepatic metabolism is unknown, the hepatic metabolism/excretion accounts for $> 20\,\%$ of the elimination of parent drug or metabolite(s), and for drugs with a narrow therapeutic window. The primary goal of such a study is to determine if the pharmacokinetic is altered to an extent that the dose and/or dosing regimen of a drug should be adjusted from that established in the confirmatory safety and efficacy trials.

The following presents a particular clinical study, together with results and conclusions, that was performed in two groups of individuals, namely hepatic impaired patients and healthy subjects.

For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic (PK) data, although clearly safety parameters are also studied.

PROCEDURE

The design of a typical hepatic impairment study is presented below.

Protocol Outline

To investigate the pharmacokinetics of XYZ 123 in individuals with hepatic impairment in comparison with those in healthy subjects after multiple oral administration of 800 MG XYZ 123 once a day for 7 days in an open, multi-center study.

Design, Population, Treatment

Open, non randomized, multi-center, repeated dose study in two groups of individuals. Twelve patients with hepatic impairment with a Child-Pugh score ≥ 5 and ≤ 14 (Table 17), and 12 healthy subjects matching for age, weight and sex. Individuals received a repeated oral administration of 800 mg XYZ123 once a day for 7 days after a standard meal. Samples were collected according to the sampling schedule for determinations of XYZ123 and of its main metabolite XYZ456 in plasma and XYZ123 in urine. The unbound fraction of XYZ123 in plasma was determined by ultra filtration.

Inclusion Criteria

Patients with hepatic impairment: 12 patients with hepatic impairment with a Child-Pugh score ≥ 5 and ≤ 14.

Healthy subjects: 12 healthy subjects with approximately similar range of age and weight, and sex matched, were

recruited in the same center as the patient he/she was matching. Age, weight and sex was matched to those of the patient (individual age within 5 years, weight within 20 % of the matching patient). Women either postmenopausal, surgically sterilized, or not pregnant and using adequate contraception.

EVALUATION

Statistical Procedures: Effect of Hepatic Impairment on PK Parameters

To compare PK parameters of XYZ123 and XYZ456 between the two groups a one way analysis of variance was used with group (patients with hepatic impairment and healthy subjects) as main effect in the model, after natural logarithmic transformation of the following pharmacokinetic parameters: C_{max}, $AUC_{(0–24h)}$ on day 1 and day 7 and $t_{1/2,\lambda z}$ (on day 7 only) for XYZ123 and XYZ456 $Ae_{(0–24h)}$, CL_R, $t_{1/2,\lambda 1}$ and fu for XYZ123 on day 1 and day 7, Rac for XYZ123. The ratio of the adjusted means (patients with hepatic impairment/healthy subjects) was obtained by calculating the exponential of the difference of the adjusted means of natural log transformed parameters. Ninety percent confidence intervals (CI 90 %) of the ratio was constructed using the mean square error (MSE) of the analysis of variance. For the discrete variable t_{max}, the effect of hepatic impairment was assessed using Kruskall-Wallis non-parametric test.

Plots of pharmacokinetic parameters (C_{max}, AUC of XYZ123 and XZY456, CL_R of XYZ123) versus Child-Pugh score and treatment day were drawn.

CRITICAL ASSESSMENT OF THE METHOD

Background to the Example

Drug XYZ123 is rapidly and almost completely absorbed after oral administration. There is a substantial first pass effect and the oral bioavailability after administration of the tablet is around 60 %. Food does not modify the bioavailability of XYZ123 tablet. In humans, XYZ123 is eliminated mainly by metabolism (70 % of the dose), the metabolites being mainly excreted in feces, and is also excreted unchanged in the urine (13 %) and feces (7 %). Some circulating metabolites have been identified: most of them are present at low concentrations with an AUC representing 2 to 13 % of that of XYZ123. The metabolites are not likely to contribute to the pharmacological effect. CYP3A4 is partially involved in the metabolism of XYZ123.

Study Rationale

The steady state pharmacokinetics of XYZ123 are not predicted from single dose data, since XYZ123 pharmacokinetics exhibit some moderate time dependency. In healthy subjects a 1.5-fold accumulation was observed which was not predicted from single dose data. The dosing regimen was based on the therapeutic dose (800 mg twice a day) with a 7 day duration being well within the duration of therapeutic treatment which varies from 5 to 10 days. Given the modest pharmacokinetic changes observed in Phase I studies after single dose administration, the exposure reached after repeated dose administration should not be much higher than those obtained in healthy subjects and should be in the range of the well tolerated exposure.

Thus, a multiple dose study was performed in which all healthy and hepatic impaired individuals, received the same dose. It was the aim to include 12 patients with various and well distributed degrees of hepatic impairment (according to the Child-Pugh score) and 12 pair-matched (based on demographic characteristics) healthy subjects, in order to have 10 patients and 10 subjects evaluable. The pharmacokinetics of XYZ123 in plasma (total and unbound) and in urine was assessed after the first dose and at steady state after the seventh dose. The pharmacokinetics in plasma of its main metabolite XYZ456 was also assessed.

MODIFICATIONS OF THE METHOD

Reduced Study Design

As individuals from the Child-Pugh category with mild impairment could have a normal hepatic function, and as for the majority of drugs clinically insignificant differences are more likely to be observed in individuals with moderate and severe impairment, in a reduced design just individuals with moderate hepatic impairment in combination with a control group are investigated. As a consequence of such a design, the findings in the moderate category would be applied to individuals with a mild Child-Pugh category, and dosing in the severe category would generally be a contraindication in the labeling document.

Population PK

A population PK evaluation of patients from the safety and efficacy trials can be used to assess the impact of altered hepatic function (as a covariate) on the PK of a drug. In each of the Child-Pugh category patients should adequately be represented, such that the population PK approach sound evaluates the impact of various covariates on the PK of the drug. Non linear mixed effects modeling may be used to model

the relationship between various covariates and PK parameters. This type of approach has its advantages as it involves assessment of the effect of hepatic impairment on the PK in the target population. However, this approach may prove difficult in hepatic impairment due to the low prevalence of hepatic disease in the general population.

Statistical procedures PK

Despite the fact that it is difficult to define a relationship between PK parameters and measures of hepatic function, the most appropriate statistical approach is to calculate geometric means and 95 % confidence intervals to compare the healthy and impaired groups (see example). Investigation of the relationships between hepatic functional abnormalities and selected PK parameters using linear and non linear models in order to derive dose recommendations are an appropriate alternative, yet, in spite of many constraints.

PK Results and Association with Measures of Hepatic Function

Past experience indicates that it has been difficult to develop a measure or group of measures of hepatic function that predict alterations in drug PK as the grounds for hepatic impairment are manifold (e.g. impaired hepatocellular function, impaired biliary excretion, decreased protein binding, consequences of shunting of blood passing the liver).

In contrast to renal impairment, no obvious marker exists for characterizing hepatic function with respect to predictions of drug elimination capacity. Therefore, dose recommendations may not be as accurate for hepatic impairment as for renal impairment.

Nonetheless, relationships between hepatic functional abnormalities (e.g. hepatic blood flow, serum albumin concentration, prothrombin time, or overall impairment scores such as Child-Pugh), and selected pharmacokinetic parameters (e.g. total body clearance, oral clearance, apparent volume of distribution, unbound clearance or dose-normalized area under the unbound concentration-time curve) can also be should using linear and nonlinear models. A regression approach for continuous variables describing hepatic impairment and pharmacokinetic parameters is appropriate, with the understanding that some correlations will rely on categorical variables (e.g. Child-Pugh). Typically, modeling results would include parameter estimates of the chosen model and measures of their precision (standard errors or confidence intervals). Prediction error estimates are also desirable to assess appropriateness of the model.

Classification of Hepatic Impairment

An alternative to ensure that the individuals have an impaired metabolic capacity, is to administer a metabolic probe (e.g. a CYP3A4 probe if the drug under investigation is a CYP3A4 substrate) to evaluate if the pharmacokinetics of the drug is altered.

Exogenous markers that have been used to assess different hepatic drug elimination mechanisms are for example antipyrine (Figg et al. 1995) and MEGX (lidocaine metabolite) (Testa et al. 1997) markers for the functional ability of the cytochrome P-450 oxidative pathway, or ICG (indocyane blue) (Figg et al. 1995), a marker for hepatic blood flow, and galactose (Tang and Hu 1992). Such markers may be used parallel to the Child-Pugh classification.

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Table 18 Demography.

Parameter	Statistics	Patients with hepatic impairment	Healthy subject
Number of subjects	N	13	13
Sex	Male	11	11
	Female	2	2
Age (years)	Mean (min–max)	52.2 (38–73)	51.8 (35–74)
Weight (kg)	Mean (min–max)	74.4 (54.5–104.7)	75.3 (62.2–101.0)
Creatine clearance (m/min)	Mean (min–max)	112 (71–152)	93 (68–133)
Child-Pugh score	Median (min–max)	7 (5–11)	NA

Table 19 Child Pugh score and grade of hepatic impairment.

Grade of hepatic impairment	A	B	C
Child Pugh Score	5–6	7–9	10–15
Number of subjects	4	6	3
Observed Child Pugh score [min–max]	5–6	7–9	10–11

Min = minimum, max = maximum

EXAMPLE

To illustrate the type of data that can be obtained using the discussed study, a summary of the PK results obtained from the study described above under "Procedure" is presented below.

A population as described above completed the study according to the protocol (Tables 18, 19).

The demographic characteristics were similar in both groups (Table 18). The median Child-Pugh score of patients with hepatic impairment was 7 with a range of 5–11 (Table 19).

Steady state was achieved after 2 days of dosing.

Figures 7 and 8 show the mean plasma concentration of XYZ123 (Figure 7) and of XYZ456 (Figure 8) after single and repeated doses of XYZ123 (800 mg) once a day. The PK parameters of XYZ123 (Table 20) and show that:

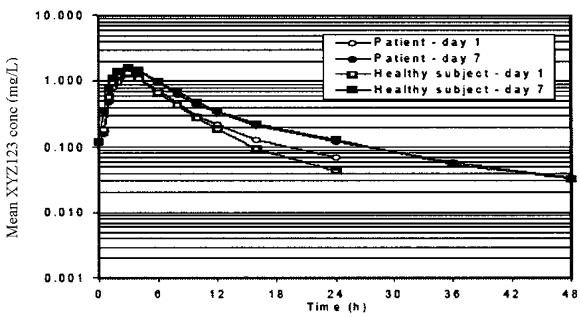


Fig. 7. Mean plasma concentration of XYZ456 after single and repeated doses of XYZ123 (800 mg) once a day.

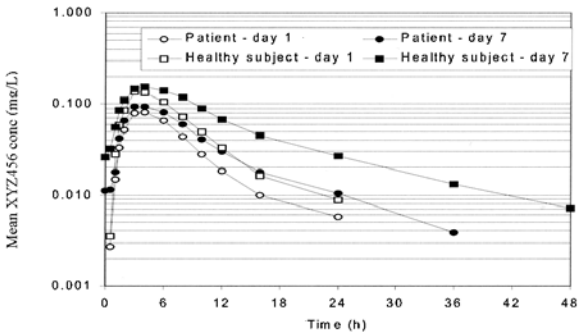


Fig. 8. Mean plasma concentration of XYZ123 after single and repeated doses of XYZ123 (800 mg) once a day.

After single and repeated doses of XYZ123 mean C_{max} and AUC were similar in both groups. Maximum values observed in patients were similar or smaller than those observed in healthy subjects. Between subject variability (CV) was around 30 % for both groups except for patients on day 7 where it was lower with 20 %.

After single and repeated doses of XYZ123 the main elimination half-life ($t_{1/2,\lambda 1}$) and the terminal elimination half-life ($t_{1/2,\lambda z}$) (after repeated doses) were similar in both groups, as well as the between subject variability which was around 20 %.

After single dose of XYZ123, there was a 27 % and 25 % increase in $Ae_{(0-24h)}$ and renal clearance respectively, in patients compared to healthy subjects. These increases were not statistically significant probably due to the high between subject variability (around 50 %) observed in patients.

Table 20 Pharmacokinetic parameters – XYZ123.

Parameter	Statistics	Patients with hepatic impairment (n = 13)		Healthy subjects (n = 13)		Ratio[a] (90 CI (%)) ANOVA	
		Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
C_{max} (mg/L)	Mean (CV%) (min–max)	1.54 (33) (0.48–2.52)	1.80 (23) (1.26–2.49)	1.73 (32) (0.78–2.66)	1.92 (30) (0.98–2.99)	87 (67–113) NS	96 (79–116) NS
t_{max} (h)	Median (min–max)	3.0 (0.5–4.0)	2.0 (1.5–6.0)	1.5 (0.5–4.0)	3.0 (0.5–6.0)	NS	NS
AUC (0–24 h) (mg.h/L)	Mean (CV%) (min–max)	9.11 (36) (4.18–15.60)	12.43 (20) (8.35–15.93)	8.79 (32) (5.44–14.96)	13.26 (27) (8.37–20.17)	102 (81–128) NS	95 (81–111) NS
C24 h (mg/L)	Mean (CV%) (min–max)	0.069 (82) (0.021–0.243)	0.122 (41) (0.050–0.220)	0.044 (43) (0.015–0.076)	0.127 (45) (0.055–0.254)	–	–
$t_{1/2}$, λ1 (h)	Mean (CV%) (min–max)	2.94 (23) (1.99–4.41)	3.89 (21) (2.56–5.33)	2.80 (21) (1.69–4.00)	3.84 (22) (2.51–5.51)	105 (90–122) NS	101 (88–117) NS
^{t}1/2, λz (h)	Mean (CV%) (min–max)	–	11.94 (21) (8.63–16.27)	–	11.04 (20) (8.80–15.75)	–	108 (123) (94–123)
fu[c] (%)	Mean (CV%) (min–max)	23.4 (21) (14.2–32.6)	24.5 (26) (15.1–34.3)	20.8 (19) (16.4–28.8)	21.0 (22) (15.6–32.2)	112 (97–128) NS	115 (98–135) NS
Ae (0–24 h) (% dose)	Mean (CV%) (min–max)	16.0 (55) (6.3–30.7)	22.6 (49) (9.6–50.4)	11.6 (35) (5.2–20.2)	19.2 (27) (11.5–31.2)	127 (93–173) NS	110 (86–142) NS
CL_R (L/h)	Mean (CV%) (min–max)	14.6 (48) (4.8–28.4)	14,8 (45) (5.9–27.8)	10.66 (20) (6.82–14.24)	11.69 (15) (9.20–14.45)	125 (96,161) NS	116 (92–147) NS
Rac[d]	GMean (CV%) (min–max)	1.43 (43) (0.85–3.25)		1.53 (16) (1.20–2.06)		93 (77–113) NS	

[a] Ratio of the adjusted means (patients with hepatic impairment/healthy subjects)
[b] Significance of p-value from ANOVA table: NS: non-significance. *0.01 < p ≤ 0.05. **0.001 < p ≤ 0.01, ***p ≤ 0.001
[c] fu = (fu C_{max} + fu C 12 h)/2
[d] Rac = AUC (0–24 h)=-day /7AUC (0–24 h)-day 1

Table 21 Pharmacokinetic parameters – XYZ456.

Parameter	Statistics	Patients with hepatic impairment (n = 12[a])		Healthy subjects (n = 13)		Ratio[a] (90 CI (%)) ANOVA	
		Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
C_{max} (mg/L)	Mean (CV%) (min–max)	0.089 (59) (0.018–0.214)	103 (53) (0.043–0.220)	0.148 (27) (0.087–0.220)	0.163 (18) (0.111–0.233)	53 (38–73) **	57 (44–73) ***
t_{max} (h)	Median (min–max)	3.0 (2.0–6.0)	3.5 (2.0–6.0)	4.0 (2.0–6.0)	4.0 (1.5–6.0)	NS	NS
AUC (0–24 h) (mg.h/L)	Mean (CV%) (min–max)	0.679 (51) (0.138–1.344)	0.93 (49) (0.41–1.85)	1.136 (30) (0.661–2.021)	1.83 (29) (1.16–3.21)	54 (39–74) **	47 (36–61) ***
^{t}1/2, λz (h)	Mean (CV%) (min–max)	–	9.5 (43) (5.3–18.0)	–	11.9 (48) (5.3–26.3)	–	80 (60–108) (NS)

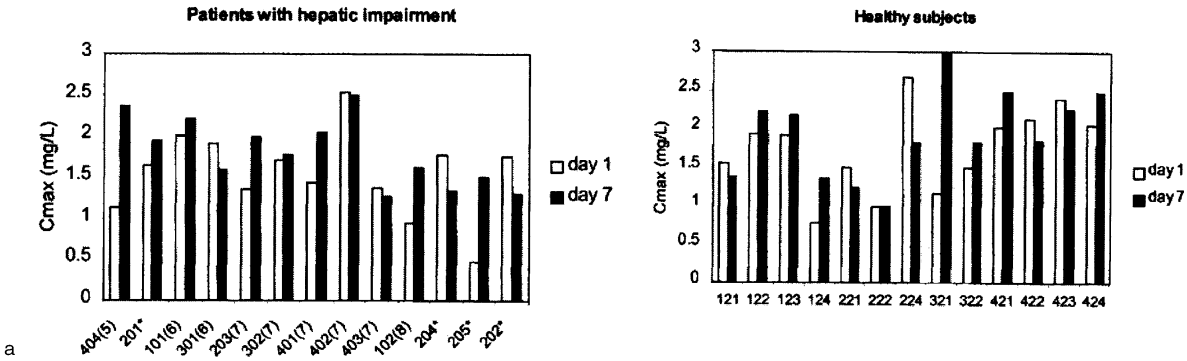
[a] Blood samples not withdrawn for (XYZ456) assay for subject No. 101 (for safety reason linked to hepatic impairment)
[b] Ratio of the adjusted means (patients with hepatic impairment/healthy subjects)
[c] Significance of p-value form ANOVA table. NS: non significant, *0.01 < p ≤ 0.05, **0.001 < p ≤ 0.01, ***p ≤ 0.001

At steady state the difference between both groups was less marked with a 10 and 16 % increases in $Ae_{0–24h}$ and renal clearance when comparing patients to healthy subjects. Again, with values not statistically different between groups and a between subject variability still around 50 % in patients.

The accumulation ratio (Rac) was similar in both groups with the between subject variability in patients (43 %) higher than in healthy subjects (16 %).

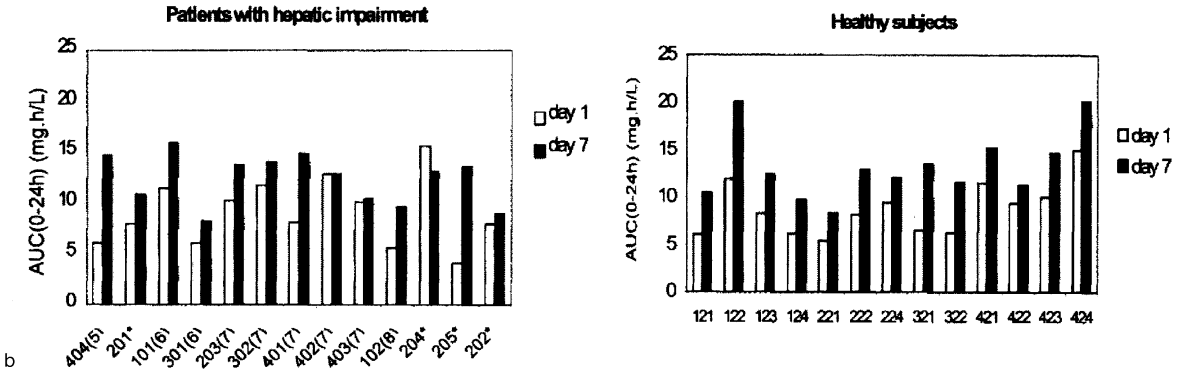
The results showed that whatever the group, there was no statistically significant difference in unbound fraction of XYZ123 whether it was measured at t_{max} or t_{12h}. Since there was no time effect, the mean unbound fraction (fu) was calculated from fu C_{max} and fu C_{12h} on day 1 and on day 7 for bothy groups.

Figure 9 indicates that in patients with hepatic impairment there was no trend towards any relationships



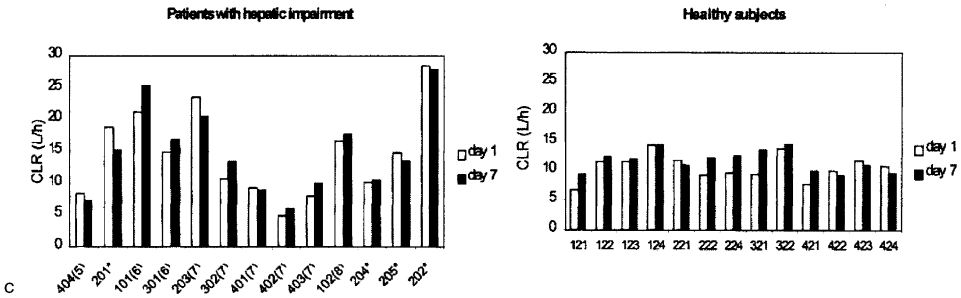
a

For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10



b

For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10



c

For patients with hepatic impairment, the subject number is followed by the Pugh score in brackets.
* Changes in Pugh score were observed for these subjects during the study with the following values on screening/day-1/day 8:
-patient 201: 5/5/6
-patient 204: 10/9/9
-patient 205: 10/10/9
-patient 202: 11/10/10

Fig. 9. Individual XYZ123 pharmacokinetic parameters after single and repeated oral doses of XYZ123 (800 mg) once a day.

between XYZ123 pharmacokinetic parameters and the Child-Pugh score.

The pharmacokinetic parameters of XYZ456 are summarized in Table 5 and show that:

After single and repeated doses of XYZ123 mean C_{max} and AUC of XYZ456 were approximately twice as low as in patients compared to healthy subjects. These differences were statistically significant. Be-

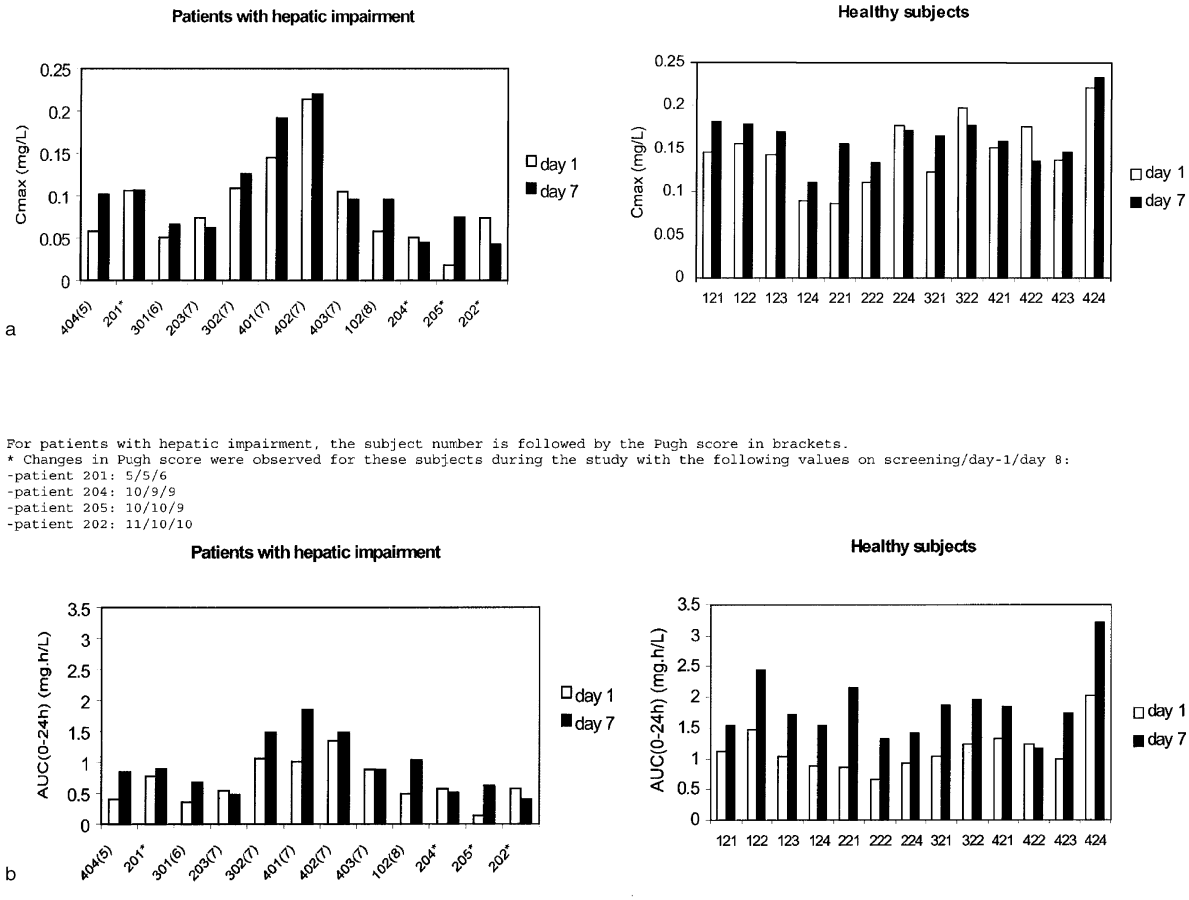


Fig. 10. Individual XYZ456 pharmacokinetic parameters after single and repeated oral dose of XYZ123 (800 mg) once a day.

tween subject variability ranged from 50 to 60 % in patients and from 20 to 30 % in healthy subjects.

The terminal elimination half-life was 20 % lower in patients compared to healthy subjects. Probably the elimination half-life was not accurately determined in patients as XYZ456 concentrations were close to the lower limit of quantification. Thus, as the elimination has a biphasic profile, for patients this elimination half-life probably corresponds to a mix of $t_{1/2,\lambda 1}$ and $t_{1/2,\lambda z}$.

Figure 10 indicates that in patients with hepatic impairment there was no trend towards any relationships between XYZ456 pharmacokinetic parameters and the Child-Pugh score.

In summary, the plasma pharmacokinetic profile of XYZ123 is similar to that achieved in healthy subjects with an alteration in one of the XYZ123 elimination pathways in patients with hepatic impairment. This was shown by a marked decrease in XYZ456 metabolite formation, though there was no clear relation between the extent of the decrease and the severity of hepatic impairment. There is a trend for a slight increase in XYZ123 renal clearance with no clear relation between the extent in renal clearance increase and severity of hepatic impairment. Furthermore, it is unlikely that the decrease in XYZ456 formation could modify the overall pharmacological activity as this metabolite is known not to contribute to a clinically relevant extent.

In conclusion, from a pharmacokinetic point of view the dosage regimen of 800 mg XYZ123 once daily should not be modified in patients with hepatic

impairment, provided that the renal function is not severely impaired.

Abbreviations/Glossary of Terms

Ae: Amount of drug excreted unchanged in urine
ANOVA: Analysis of variance
AUC: Area under the curve
C12, C24: Plasma concentration 12 and 24 h after administration
CI90 %: 90 % confidence Interval
CLR: Renal clearance
C_{max}: Maximum plasma concentration
CV: Coefficient of variation
CYP3A4: Cytochrome 3A4
day –1: Study day prior to day of study medication administration
fu: Unbound fraction of XYZ123 in plasma
h: Hour
L: Liter
mg: Milligram
Rac: Accumulation ratio
$t_{12,\ t24}$: Time 12 and 24 h after administration
$t_{1/2,\lambda1}$: Main elimination half-life
$t_{1/2,\lambda z}$: Terminal elimination half-life
t_{max}: Time to maximum plasma concentration

II.S.12
Special Populations: Profiling the Effect of Obesity on Drug Disposition

PURPOSE AND RATIONALE

Regulatory guidance requires that the pharmacokinetics and tolerability of a candidate drug is studied in the range of populations likely to receive the drug during the clinical development and later, once the drug is marketed (US FDA 1978). Over and above this regulatory need, in some cases an early switch to using special populations can be advantageous to clinical development.

Obesity is associated with physiological changes that can alter the pharmacokinetic parameters of many drugs (Blouin et al. 1987). For example, increases in the apparent volume of distribution and total body clearance have been reported for the antibacterial agents vancomycin and aminglycosides (Bearden and Rodvold 2000). For drugs with non-body weight adjusted dosing, obese individuals may be notably under-dosed, and thus show lower efficacy. Since many of these effects are not simply predictable, appropriate profiling of drug pharmacokinetics/pharmacodynamics in obese individuals is essential for drugs targeting indications where the obesity is a prevalent concomitant illness. European Agency for the Evaluation of Medicines Products (EMEA) and US Federal Drug Administration (FDA) guidance recommend that the pharmacokinetics of candidate weight control drugs are studied in obese (EMEA CPMP/EWP/281/96 1997, US FDA 1996).

The good availability of individuals who are obese but are otherwise healthy, makes the early profiling of drug pharmacokinetics/pharmacodynamics in healthy obese relatively easy. If no difference is observed between healthy obese and healthy lean individuals, then the recruitment of healthy lean subjects in many studies can be justified. In some cases, for example when developing drugs for metabolic diseases (e.g. obesity, diabetes), the advantages of an early switch to obese individuals – a population closer to the final patient group than the typical healthy lean subject – early in drug development can justify this limited complication of the recruitment process.

PROCEDURE

The design of a study comparing the steady-state pharmacokinetic/pharmacodynamic profile of Drug XYZ123 in obese and non-obese healthy adults, is presented below. Drug XYZ123 was developed for both subcutaneous (SC) and intravenous (IV) application routes. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data pertinent to the assessment of the impact of obesity, although other parameters were also studied.

Protocol Outline

A phase I, open-label study of the effect of obesity on the pharmacokinetic/pharmacodynamic profile of subcutaneous and intravenous drug XYZ123.

Objectives

Primary: The primary objective of the study was to compare steady-state pharmacokinetic/pharmacodynamic profile of SC Drug XYZ123 between obese and non-obese healthy adults.

Secondary: The secondary objective of the study was to compare steady-state pharmacokinetic parameters after IV administration and absolute bioavailability of Drug XYZ123 between obese and non-obese healthy adults.

Study Design

The study had an open-label, two-way crossover randomized design. Two groups of obese and non-obese healthy volunteers were administered multiple doses of SC Drug XYZ to reach steady-state (Treatment A), and single IV dose of Drug XYZ

(Treatment B). The washout period between trial periods was at least 10 days which approximated to > 10 Drug XYZ apparent terminal half-lives.

Number of Subjects

Twenty-five (25) obese adults and 25 adults age, height, and sex-matched non-obese. Estimations based on previous studies with non-obese showed that the inclusion of 24 subjects per strata (obese, non-obese) would give enough power to allow the detection of a > 20 % difference in AUC of Drug XYZ at steady-state with alpha = 0.05 and beta = 0.10. According to the draft guidance for industry by the US FDA 12 subjects is the typical minimum sample size for a bioavailability comparison. This requirement was fulfilled here for each of the sub-strata obese male, non-obese male, obese female, non-obese female.

Inclusion Criteria

The following inclusion criteria were met: Adults aged between 18 and 55 years; Obese with body mass index (BMI) of 30.0 $\leq$ 39.9 kg/m^2 ($\geq$40.0 kg/m^2 on a case-by-case basis) or non-obese volunteers with BMI of 18.5 $\leq$ 24.9 kg/m^2; men who are willing to use prescribed barrier contraceptive methods; women who are either post-menopausal or surgically sterile and willing to use prescribed barrier contraceptive methods; who – apart from being obese – are healthy for the purpose of the study and not receiving regular medication in the month preceding the study; with normal findings in the physical examination, unless the investigator considers an abnormality to be clinically irrelevant for the study; who are non-smoking or light smokers.

Treatments

Treatment A: Single (Day 1) 1.5 mg/kg Drug XYZ dose given as an IV infusion over 6 hours (h). Treatment B: Once daily 1.5 mg/kg Drug XYZ SC doses over 4 days.

Pharmacokinetic Data

Treatment A: Plasma concentrations of Drug XYZ, before and at pre-determined times post dose after IV dosing on Day 1. Treatment B: Plasma concentrations of Drug XYZ, before and at predetermined times after SC dosing on Days 1 and 4.

A 24 h urine sampling was performed during the IV dosing period of the study that started on Day 1 and ended on Day 2. The volume of urine collected and the concentration of creatinine were determined to allow the creatinine clearance to be calculated.

EVALUATION

The data pertinent to the assessment of the impact of obesity on the disposition of the developmental drug from study described above, was evaluated as follows:

Descriptive pharmacokinetic parameters (total clearance (CL or CL/F), mean residence time (MRT), volume of distribution (Vd or Vd/F), peak concentration (C_{max}), time of peak concentration (T_{max}), area-under-the curve between time 0 and the time of the last quantifiable concentration ($AUC_{0\text{-}t}$) and/or AUC for the dosing interval ($AUC_{0\text{-}\tau}$), apparent terminal half-life ($t_{1/2\_z}$) after Day 1, and $AUC_{0\text{-}\tau}$ after Day 4), for plasma Drug XYZ were calculated using a non-compartmental analysis employing a linear/log trapezoidal method as implemented in WinNonlin (Pharsight Corp.) protocols. The absolute bioavailability was calculated by comparison of Day 1 data for Treatment A and B.

Descriptive statistics (number of observations (n), mean, standard deviation, coefficient of variation in percent (CV %) or median and range) were calculated for each parameter. Statistical tests using SPSS software were as follows:

Homogeneity test of variance (Levene's test) for each dependent variable.

Independent t test on log transformed parameters: C_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}\tau}$, $t_{1/2\_z}$ for plasma concentrations of Drug XYZ.

CRITICAL ASSESSMENT OF THE METHOD

The type of study described in this section provides profiling of the impact of obesity on the disposition of the developmental drug after dosing via two dosing routes. Although for Drug XYZ this approach was justified given the intention to market the Drug XYZ for both SC and IV applications, typically, however, only the proposed therapeutic dosing route would be tested.

Although the impact of obesity on the disposition of the developmental drug can most comprehensively be studied based on steady-state data as described, the use of single-dose data could also suffice provided there is reason to believe that the pharmacokinetics of the drug studied are accurately predictable from single-dose data.

MODIFICATIONS OF THE METHOD

The described supportive study was specifically designed and suitably powered to identify differences in Drug XYZ pharmacokinetics between obese and non-obese. Typically this type of evaluation would be extended during the later clinical development by performing a population pharmacokinetic/pharmacodynamic assessment of the impact of obesity on the disposition of the developmental drug during the phase II/III studies.

If obesity is a not a prevalent concomitant illness in the targeted indication then this type of study is unlikely to be performed. Instead, a population pharmaco-

Table 22 Mean Drug XYZ pharmacokinetic parameters (CV%) on Day 1 and 4 following 1.5 mg/kg SC q24h dose of Drug XYZ in 24 non-obese and 24 obese healthy subjects.

	C_{max}(IU/ml)	T_{max}(h)	AUC_{0-t}(h.IU/ml)	$AUC_{0-\tau}$(h.IU/ml)	$t_{1/2\_z}$(h)
Day 1					
Non-obese	1.344 (10)	3.50* (1.50–4.00)	14.19 (13)	14.87 (14)	4.85 (11)
Obese	1.379 (14)	4.00* (3.00–6.00)	15.99 (14)	17.01 (16)	5.09 (18)
Obese/non-obese difference	+3% (NS)	+0.5 h (NS)	+13% (p = 0.007)	+14% (p = 0.006)	+14% (NS)
Obese PK/non-obese PK ratio difference	1.022	–	1.12	–	–
90% Confidence interval	0.961–1.086	–	1.05–1.21	–	–
Day 4					
Non-obese	1.488	3.00* (2.00–4.00)	16.43	17.52	5.45
Obese	1.563	4.00* (3.00–6.00)	19.12	20.78	5.76
Obese/non-obese (difference)	+5% (NS)	+1 h (p = 0.005)	+16% (p = 0.001)	+19% (p = 0.002)	+6% (NS)
Obese PK / non-obese PK ratio difference	1.049	–	1.16	–	–
90% Confidence interval	0.995–1.107	–	1.08–1.25	–	–

*Median (range); NS: not statistically significant; –: not reported

Table 23 Mean Drug XYZ pharmacokinetic parameters (CV %) following a 1.5 mg/kg IV dose of Drug XYZ by a 6 h infusion in 21 obese and 21 non-obese healthy subjects.

	C_{max} (IU/ml)		AUC_{0-t}(h.IU/ml)	$AUC_{0-\tau}$(h.IU/ml)	$t_{1/2\_z}$(h)
Non-obese	1.542 (12)		13.32 (14)	13.95 (15)	4.60 (11)
Obese	1.770 (8)		14.90 (13)	15.64 (14)	5.03 (15)
Obese/non-obese difference	+15% (p = < 0.0001)		+12% (p = 0.013)	+11% (p = 0.016)	+9% (p = 0.034)
Obese PK /non-obese PK ratio difference	1.151		1.12		
90% Confidence interval	1.090–1.216		1.04–1.20		
	CL [L/h/kg]	CL [L/h]	MRT [h]	Vd [L/kg]	Vd [L]
Non-obese	0.011 (15)	0.74 (17)	5.99 (12)	0.066 (12)	4.37 (17)
Obese	0.010 (15)	0.99 (23)	5.94 (13)	0.059 (11)	5.77 (18)
Obese/non-obese difference	–9% (p = 0.014)	+34% (p<0.0001)	–8% (NS)	–11% (p = 0.001)	+32% (p>0.0001)

*Median (range); NS: not statistically significant; –: not reported

kinetic/pharmacodynamic assessment of the impact of obesity on the disposition of the developmental drug would be performed during the phase II/III studies. A suitably powered clinical study would only be performed if such population pharmacokinetic/pharmacodynamic assessments raised significant questions.

EXAMPLE

To illustrate the type of data that can be obtained using this study, a high level summary of the pharmacokinetic results obtained from the study described above under "Procedure" is presented below.

Results – Pharmacokinetics

The data from study described above, as given in Tables 22 and 23 below, can be summarized as follows:

After 1.5 mg/kg SC dosing for four days, $AUC_{0\text{-}t}$ for Drug XYZ was 16 % higher in obese subjects compared to non-obese subjects. This is explained by:

- a similar, 100 % systemic absorption in both groups
- a lower weight-adjusted clearance in obese subjects (see below).

After SC dosing, absorption was slightly delayed in the obese (T_{max} 4 h vs. 3 h) and no difference in C_{max} values was observed after repeated SC dosing.

The difference was therefore mainly attributed to the elimination phase. Steady-state was reached on Day 4 in both obese and non obese subjects, with almost no unpredicted accumulation.

After a 6 h IV infusion, Drug XYZ clearance and volume of distribution estimates were over 30 % higher in obese than in controls. On a weight-adjusted basis these parameters were about 10 % lower in obese. MRT estimates were similar for both groups.

Exposure (AUC) to Drug XYZ was significantly higher in obese subjects. This observation was explained as follows: while the total clearance (L/h) is much higher in obese subjects (i.e. higher total elimination capacity: 121 mL/min creatinine clearance in obese versus 83 mL/min in non-obese), the clearance on a weight-adjusted basis was actually lower. Since the dose was weight adjusted, this led to higher AUC estimates.

The C_{max} is 15 % higher in obese, which can be explained by the fact that the distribution volume does not increase proportionally with weight either, similar to the argument for clearance.

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II.S.13 Special Population: Pediatric Population

PURPOSE AND RATIONALE

The number of medicinal products currently labeled for pediatric use is limited.

There is a general lack of pharmacokinetic (PK) and other clinical information to support the administration of many medicinal products to children which leads to a lack of appropriate dosage recommendations in this population. As a consequence, there is frequent off-label use of licensed medicines in children.

The disposition of a drug may differ considerably between adults and children because in the pediatric population the organs affecting the absorption, distribution metabolism and excretion of a drug are under continuous maturation, which is expected to lead at least to additional inter- and intra-individual variances in the PK of a drug in this population. To support the development of a pediatric formulation and especially in order to support the dosing recommendation in different age groups, PK studies should be performed. In case the systemic exposure of a drug is readily related to the pharmacological or therapeutic effect, PK results in the pediatric population can be used for extrapolation of efficacy data from studies performed in adults.

Current guidelines have recently been updated, proposing new pediatric rules and actually encouraging to investigate the safety and efficacy of a medicinal product in children, and also elucidate cases in which the drug blood concentration will become the basis of subsequent determination of the dosage schedule.

As the pediatric subgroup represents a vulnerable population, studies in children are endowed with specific challenges such as additional ethical (e.g., informed consent be obtained from the legal guardian) or clinical-technical (e.g., minimize the amount of blood drawn and the number of venipunctures) issues.

PK studies in the pediatric population are generally conducted in patients with the disease. This may lead to higher inter-subject variability than studies in healthy subjects, but the data better reflect clinical use.

More details about when such studies may or may not be important, and other aspects like study design and methods, can be found in the literature below.

In summary, PK studies in the pediatric population should determine if the dosage regimen in the pediatric population is to be adjusted to achieve approximately the same level of systemic exposure that is safe and effective in adults.

The following section present a particular study in a pediatric population (children and adolescents) with result illustration and conclusion. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of PK data although clearly safety and pharmacodynamic parameters were also studied.

PROCEDURE

The design of a typical pediatric study is presented below.

Protocol Outline

Pharmacokinetics of HMR1964 (insulin glulisine) and regular human insulin injected subcutaneously as a single dose in

pediatric subjects with type I diabetes in a single-center, double-blind, randomized, two-way crossover study.

Objective

To investigate the pharmacokinetics of insulin glulisine and regular human insulin in pediatric type I diabetic subjects.

Design, Treatment and Sample Size

Single-center, single-dose, double-blind, randomized, two-way crossover design; 20 pediatric type I diabetic subjects (10 per age class) of either gender. The 2 age classes were build by children aged between 5 and 11 years and adolescents aged between 12 and 17.

Pediatric patients received a single dose of 0.15 IU/kg body weight of HMR1964 (insulin glulisine) and regular human insulin administered subcutaneously in the periumbilical abdomen 2 minutes (min) before a standardized liquid meal. Volume and frequency of blood sampling for determinations of insulin in serum was minimized and was taken according to the sampling schedule.

Population Treated

20 type 1 diabetic pediatric subjects of both age classes children and adolescents: 10 children (5 male and 5 female), between 7 and 11 years of age, with body mass indices between 16.4 and 22.7 kg/m^2 and 10 adolescents (4 male and 6 female), between 12 and 16 years of age, with body mass indices between 17.7 and 26.3 kg/m^2.

EVALUATION

Statistical Procedures Pharmacokinetics

The relationship between age and pharmacokinetics were assessed by an analysis of variance (ANOVA) on AUCs, MRT and C_{max} with adjustments for treatment, period, sequence and subject within sequence effects by age class using the natural log transformed values to compare treatments within age class. Point estimates and 95 % confidence intervals were calculated for the treatment ratios per age class.

ANOVAs with adjustments for age class, period, sequence and subject within sequence effects were performed by treatment to compare age classes within treatment. Point estimates and 95 % confidence intervals were calculated for the age class ratios per treatment.

T_{max} was analyzed by non-parametric analyses. 95 % non-parametric confidence intervals for the respective median treatment and age class differences were calculated.

CRITICAL ASSESSMENT OF THE METHOD

Introduction

HMR1964 (INN: insulin glulisine) is a human insulin analogue for the treatment of Type I and Type II patients with diabetes mellitus. Combinations of insulin preparations that differ both in their time of onset and duration of action are used optimally to control blood glucose in patients with diabetes mellitus. The most commonly used insulin regimens include a long-acting insulin to provide basal insulin requirements and control fasting and pre-prandial blood glucose in combination with a short-acting insulin to control prandial blood glucose excursions. HMR1964 (insulin glulisine) has a more rapid onset and shorter duration of action than regular insulin when administered subcutaneously.

Preclinical studies suggest HMR1964 (insulin glulisine) does not differ from the established elimination pathways of endogenous human insulin.

Study Rational

HMR1964 (insulin glulisine) may be of particular use for children and adolescents who encounter more difficulties in adjusting their daily activities to fixed dosing meal intervals. Given immediately before a meal or thereafter, to balance the actual carbohydrate intake may be of significant advantage, provided the rapid and short acting nature of HMR1964 (insulin glulisine) also prevails in this patient population.

This study was conducted to investigate the pharmacokinetics of HMR1964 (insulin glulisine) and regular human insulin subcutaneously administered 2 min before a standard meal in pediatric type 1 diabetic patients from both age classes (10 children and 10 adolescent subjects) in order to verify the sameness of differences seen in adults.

Population

Twenty male and female pediatric type 1 diabetic patients from both age classes (10 children and 10 adolescents subjects) were treated.

Dosing Recommendation/Therapeutic Dose-Range

A single dose of 0.15 IU/kg HMR1964 (insulin glulisine) was chosen for the children following a dosing approach estimate on the basis of body weight. Based on the primary pharmacokinetics of HMR1964 (insulin glulisine) a single dose study was anticipated to be adequate.

MODIFICATIONS OF THE METHOD

Study Design Aspects

To study the plasma protein binding at least in newborns and infants is recommended, as the protein binding is reduced in the preterm and term infant at birth and in the first weeks of life. Also, the drug elimination is slowed down in this subgroup of children, due to immaturity of both metabolic pathways and renal function.

Initial titrating doses may be estimated on the basis of body weight or surface area from an extrapolation of adult data.

Classification of Age Classes

Any classification of the pediatric populations into age categories is to some extent arbitrary.

Suggestions for categorization are presented in the literature below and are as follows:

Neonate: birth to 1 month

Infant: 1 month to 2 years

Children: 2 to 12 years

Adolescent: 12 years to 16–18 years.

The pharmacokinetics of a drug in children 16–18 years and older is expected to be similar to that of adults.

Depending on the intended use of a drug in the pediatric population, studies should be performed in all pediatric age groups to allow dose adjustment within an individual over time.

Population Pharmacokinetics

An alternate approach is the population pharmacokinetic approach, or study. This approach relies on infrequent (sparse) sampling of blood from a larger population than would be used in a standard pharmacokinetic study to determine pharmacokinetic measures and/or parameters.

The population pharmacokinetic aproach assesses the impact of various covariates on the pharmacokinetic of a drug. Nonlinear mixed effects modeling may be used to model the relationship between various covariates and pharmacokinetic parameters. Age or age group may be one of the covariates. This type of approach has its advantages as it involves assessment of the effect of age on the pharmacokinetics in the target population.

Cross-Over Study Design

In case of two study medications, a sound alternative could be the (complete) cross-over study design (see example).

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Table 24 Demographic variables.

Demographic data	Arithmetic mean (range)		
	All subjects (n = 20)		
	all (n = 20)	male (n = 9)	female (n = 11)
Age (years)	12.4 (7–16)	12.6 (8–16)	12.3 (7–16)
Weight (kg)	52.1 (26.0–82.5)	54.1 (26.0–82.5)	50.5 (27.5–65.0)
BMI (kg/m^2)	20.9 (16.4–26.3)	20.9 (16.4–26.3)	20.9 (17.6–24.5)

	Children (n=10)			Adolescents (n=10)		
	all (n = 10)	male (n = 5)	female (n = 5)	all (n = 10)	male (n = 4)	female (n = 6)
Age (years)	10.1(7–11)	10.2(8–11)	10.0(7–11)	14.7(12–16)	15.5(14–16)	14.2(12–16)
Weight (kg)	40.0(26.0–50.0)	39.1(26.0–50.0)	40.8(27.5–49.0)	64.2(51.0–82.5)	72.7(53.0–82.5)	58.6(51.0–65.0)
BMI (kg/m^2)	19.4(16.4–22.7)	19.2(16.4–21.1)	19.7(17.6–22.7)	22.4(17.7–26.3)	23.1(17.7.–26.3)	22.0(20.2–24.5)

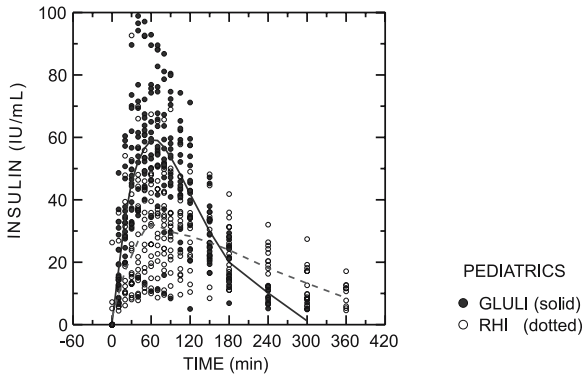

Fig. 11. Children and adolescents. Serum insulin concentrations – children and adolescents. Average insulin concentration (μIU/mL) after doses of 0.15 IU/kg insulin glulisine (GLULI) (n = 20) and regular human insulin (RHI) (n = 19) in pediatric type 1 diabetic subjects. SYSTAT Version 9.0 LOWESS function, tension factor 0.3

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EXAMPLE

To illustrate the type of data that can be obtained using this study, a summary of the PK results obtained from the study described above under "Procedure" is presented below.

Twenty pediatric type I diabetic patients as described above completed the study according to the protocol, 19 subjects were evaluable for PK evaluation of regular human insulin treatment. The demographic data are depicted in Table 24.

The concentration time profile of insulin glulisine differed considerably from regular human insulin (Figures 11, 12), with initial fractional AUCs being

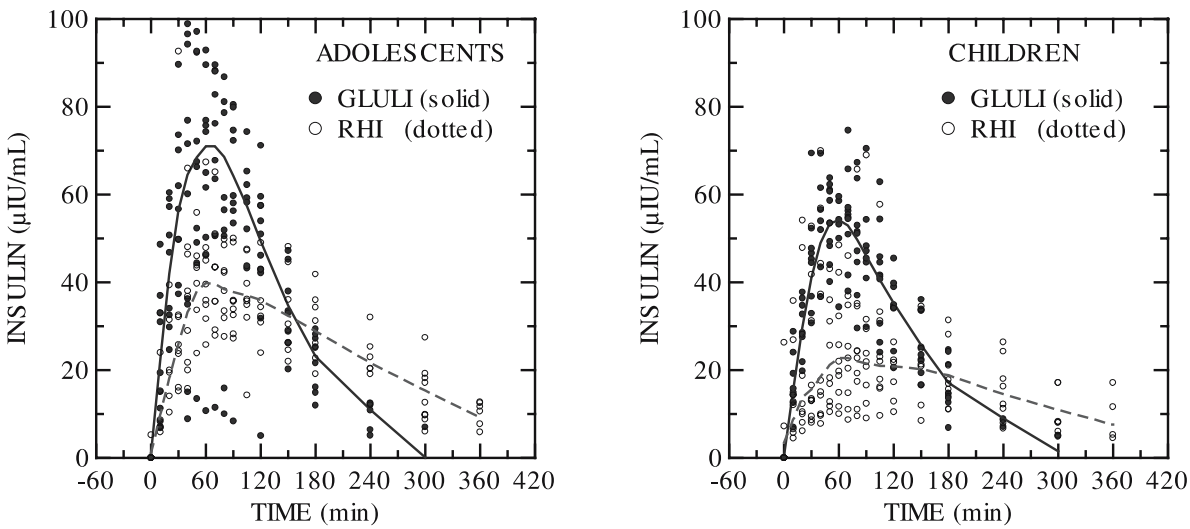

Fig. 12. Children vs. Adolescents. Serum insulin concentrations – children/adolescents. Average insulin concentration (μIU/mL) after doses of 0.15 IU/kg insulin glulisine (GLULI) (n = 10) and regular human insulin (RHI) (n = 9) in pediatric type 1 diabetic subjects. SYSTAT Version 9.0 LOWESS function, tension factor 0.3

Table 25 Pharmacokinetic results by treatment.

Variable	Glulisine (n = 20)	RHI (n = 19)	Point estimate(95% confidence interval)[1] Glulisine / RHI (n = 19)
$AUC_{(0-1h)}$ [μIU.min/mL]	2287	1246	176% (126.9; 243.8%)
$AUC_{(0-2h)}$ [μIU.min/mL]	5232	2994	169% (126.9; 224.3%)
$AUC_{(0-4h)}$ [μIU.min/mL]	7624	5703	130% (99.3; 170.3%)
$AUC_{(0-6h)}$ [μIU.min/mL]	8361	7052	116% (89.5; 149.8%)
C_{max} [μIU/mL]	58	33	171% (126.9; 229.4%)
T_{max} [min]	54**	66**	–8 min (–24; 7 min)[2]
MRT [min]	88	137	64% (59.0; 70.4%)

[1]Point estimates and 95% confidence intervals for the ratio of treatment means, based on (ln) transformed data
[2]Point estimates and 95% confidence intervals for the respective median differences from non-parametric data analysis
** Median

Table 26 Pharmacokinetic results by treatment and age class.

Variable	Geometric mean (*arithmetic mean*)					
	Glulisine			RHI		
	All subjects (n = 20)	Children (n = 10)	Adolescents (n = 10)	All subjects (n = 19)	Children (n = 10)	Adolescents (n = 9)
$AUC_{(0-1h)}$ [μIU.min/mL]	2287 (2491)	2170 (2212)	2410 (2769)	1246 (1440)	1023 (1246)	1552 (1656)
$AUC_{(0-2h)}$ [μIU.min/mL]	5232 (5637)	4948 (5030)	5534 (6244)	2994 (3335)	2383 (2747)	3860 (3988)
$AUC_{(0-4h)}$ [μIU.min/mL]	7624 (8190)	7193 (7314)	8081 (9067)	5703 (6231)	4530 (5068)	7367 (7523)
$AUC_{(0-6h)}$ [μIU.min/mL]	8361 (8922)	7934 (8055)	8811 (9789)	7052 (7673)	5581 (6214)	9145 (9294)
C_{max} [μIU/mL]	58(62)	55(55)	61(69)	33(37)	25(29)	44(46)
T_{max} [min]	54**	55**	52**	66**	59**	76**
MRT [min]	88(90)	87(88)	90(91)	137(139)	132(134)	144(146)

**Median values

higher after insulin glulisine than after regular human insulin, while the total AUC ($AUC_{(0-6h)}$) was only slightly larger. The C_{max} of insulin glulisine was higher by 71 % and reached earlier with a median T_{max} of 54 min compared to 66 min after regular human insulin. The MRT for insulin glulisine was distinctly shorter at 88 min compared to 137 min for regular human insulin, indicating the shorter residence of insulin glulisine in the systemic circulation (Table 25).

Contrasts between the concentration time profiles of insulin glulisine and regular human insulin obtained in the population as a whole were also present in both age classes, children and adolescents (Table 26).

The two age classes, children and adolescents, presented an almost equal PK profile of insulin glulisine, displayed by point estimates close to 100 % with a slight trend towards higher exposure in adolescents (Table 27). Nevertheless, T_{max} and MRT were the same in each age class.

In contrast to the findings with insulin glulisine, the comparison between age classes for regular human insulin revealed on average 60 % higher exposure (AUCs and C_{max}) in adolescents (Table 28). Nevertheless, there were no substantial differences in T_{max} and MRT.

In summary, in pediatric type I diabetic subjects, equally in each age class children and adolescents, insulin glulisine was more rapidly absorbed than regular human insulin. The fractional AUCs were larger, and C_{max} was higher with an earlier T_{max} for insulin glulisine. MRT was distinctly shorter, indicating the shorter residence of insulin glulisine in the systemic circulation compared to regular human insulin.

The two age classes, children and adolescents, presented an almost equal PK profile after insulin glulisine with a slight trend towards higher exposure in adolescents. In contrast, the comparison between age classes for regular insulin revealed on average 60 % higher exposure in adolescents.

Table 27 Pharmacokinetic results – children vs. adolescents – insulin glulisine. Comparison of pharmacokinetic results for insulin glulisine.

Variable	Geometric mean (*arithmetic mean*)		Point estimate (95% confidence interval)[1]
	Children (n = 10)	Adolescents (n = 10)	Adolescents / Children (n = 10)
$AUC_{(0-1h)}$ [μIU.min/mL]	2170 (2212)	2410 (2769)	111% (70.4; 175.4%)
$AUC_{(0-2h)}$ [μIU.min/mL]	4948 (5030)	5534 (6244)	112% (72.0; 173.7%)
$AUC_{(0-4h)}$[μIU.min/mL]	7193 (7314)	8081 (9067)	112% (72.1; 175.0%)
$AUC_{(0-6h)}$ [μIU.min/mL]	7934 (8055)	8811 (9789)	111% (73.0; 169.0%)
C_{max} [μIU/mL]	55 (55)	61 (69)	112% (73.0; 171.8%)
T_{max} [min]	55**	52**	-2 min (–9; 11 min)[2]
MRT [min]	87 (88)	90 (91)	103% (88.3; 120.9%)

[1]Point estimates and 95% confidence intervals for the ratio of treatment means, based on (ln) transformed data
[2]Point estimates and 95% confidence intervals for the respective median differences from non-parametric data analysis
**Median

Table 28 Pharmacokinetic results – children vs. adolescents – regular human insulin. Comparison of pharmacokinetic results for regular human insulin.

Variable	Geometric mean (*arithmetic mean*)		Point estimate (95% confidence interval)[1]
	Children (n = 10)	Adolescents (n = 9)	Adolescents/Children (n = 9)
$AUC_{(0-1h)}$ [μIU.min/mL]	1023 (1246)	1552 (1656)	152% (89.2; 258.1%)
$AUC_{(0-2h)}$ [μIU.min/mL]	2383 (2747)	3860 (3988)	162% (105.1; 249.5%)
$AUC_{(0-4h)}$ [μIU.min/mL]	4530 (5068)	7367 (7523)	163% (111.1; 238.0%)
$AUC_{(0-6h)}$ [μIU.min/mL]	5581 (6214)	9145 (9294)	164% (113.8; 235.9%)
C_{max} [μIU/mL]	25 (29)	44 (46)	177% (111.7; 280.5%)[2]
T_{max} [min]	59**	76**	9 min (−24; 49 min)
MRT [min]	132 (134)	144 (146)	110% (92.4; 130.1%)

[1]Point estimates and 95% confidence intervals for the ratio of treatment means, based on (ln) transformed data
[2]Point estimates and 95% confidence intervals for the respective median differences from non-parametric data analysis
**Median

In conclusion, in pediatric type I diabetic subjects, equally in each age class children and adolescents, insulin glulisine was more rapidly absorbed and had a shorter residence in the systemic circulation compared to regular human insulin. Insulin glulisine displays pharmacokinetic properties in pediatric type I diabetic subjects, which classify insulin glulisine as a rapid acting insulin analogue in this patient population with no specific dose adjustment warranted on the basis of intrinsic properties of HMR1964 (insulin glulisine).

ABBREVIATIONS

AUC: Area under the curve
BMI: Body mass index
C_{max}: Maximum concentration
INN: International nonproprietary name
MRT: Mean residence time
RHI: Regular human insulin
T_{max}: Time to maximum concentration

II.S.14
Special Populations: Assessment of Ethnic and/or Genetically Determined Differences

PURPOSE AND RATIONALE

Special populations are becoming more and more important for clinical trials. It is required to investigate the influence of organ dysfunctions (kidney, liver), of the underlying and concomitant illness, of gender/age, and also of ethnic differences. Increasing knowledge about population determined PD and PK, and especially about the genetic background of metabolic capacities, raises more and more complex questions during drug development. In this section the assessment of genetically determined (interethnic) differences will be discussed.

During recent years, genotyping of drug-metabolizing enzymes and transporters (DME+T), known to show a polymorph expression, has gained more and more importance in predicting phenotypes. This provides a helpful tool to predefine and select populations at risk, and to investigate them specifically in a dedi-

cated phase I study (for specific terminology see EU CPMP 2002). If one of the known polymorph DME+Ts is involved in the metabolism of the drug (or suspected to be), an explorative study – as described here – might be helpful during early development. Presently, the DME+T's with sufficiently established background knowledge to be considered 'validated' are: CYP 2C9, CYP 2C19, CYP 2D6, NAT2 and MDR1. Genetic phenotyping is increasingly recommended for other kinds of studies, and thus the investigation described here provides a tool to assess these influences.

PROCEDURE

The design of an exploratory profiling of the impact of polymorphism of CYP 2C19 on the pharmacokinetics of a candidate drug (XYZ1234) in a single clinical study is presented below. Two control groups (both genders) were required, since the poor CYP 2 C19 group consisted of males and females (due to the low prevalence in the European population).

For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although clearly safety parameters are also studied.

Protocol Outline

Explorative assessment of the pharmacokinetics of XYZ1234 following a single oral 100 mg dose, given in a special micronized formulation in poor and extensive CYP 2C19 metabolizing healthy male and female subjects.

Objective

Comparative assessment of the pharmacokinetics (PK) of XYZ1234 following a single oral 100 mg dose in healthy male and female subjects, which are poor or extensive metabolizers for CYP 2C19, respectively.

Study Design

The study was performed open labeled in a single center in three parallel groups.

Number of Subjects

18 subjects (6 poor CYP 2C19 metabolizers [2–4 of them males]; and 12 extensive CYP 2C19 metabolizers [6 males and 6 females], if possible with a matching Body Mass Indices (BMI) and age distribution).

Inclusion Criteria

Healthy men or women aged between 18 and 60 years, with BMI of 19.5–29.5 kg/m^2. Screening must have determined the CYP 2C19 metabolizer status beforehand.

Treatments

XYZ1234 100 mg per os (orally, p.o.) (in a special micronized formulation, under fasting conditions).

Pharmacokinetic Data

Concentrations of XYZ1234 in plasma and urine were measured before and at predetermined times up to 72 hours (h) post dosing and were used to determine the PK data.

If feasible and adequate, an explorative investigation on potential metabolites in plasma and urine was to be performed.

EVALUATION

Due to the small sample size, all variables were only presented descriptively for the different bioanalytical data and PK parameters calculated.

This descriptive statistic will be presented by CYP 2C19 metabolizer-status and where applicable by gender, including mean (arithmetic and/or geometric), standard deviation (usual and/or dispersion factor), standard error of the mean, coefficient of variation (in %), median, minimum, maximum, number of observations.

For pharmacokinetics in plasma: Individual concentrations of XYZ1234 will be tabulated together with descriptive statistics and plotted. Median profiles will be presented graphically by CYP 2C19 metabolizer status and gender. Pharmacokinetic parameters (at least C_{max}, t_{max}, $AUC_{(0\text{-}t)}$ [t = 24 h and 'last > LOQ'], AUC_{inf}, $t_{1/2z}$, MT, as well as CL/f and Vz/f) will be determined based on plasma concentrations of XYZ1234 using non-compartmental procedures.

For urine data, the individual and mean fractional and cumulative urinary excretion of XYZ1234 will be calculated if feasible and adequate ($Ae_{(t2\text{-}t1)}$; $Ae_{(0\text{-}72h)}$), urinary recovery [% of administered dose], and renal clearance CL_R (fractional and total) .

CRITICAL ASSESSMENT OF THE METHOD

In the actual development program, this study was needed for explorative elucidation of the influence of the CYP 2C19 phenotype before starting the first multiple-dose study, due to the observation of a deviating pharmacokinetics in a poor CYP 2C19 metabolizing subject included in an earlier single-dose study (the first-in-man study). The study described had to clarify if this deviating PK for this subject is based on the status of being a 'poor CYP 2C19 metabolizer' or was based on a further individuality of that specific subject (e.g. a different degree of resorption).

Since the prevalence of poor metabolizers for CYP 2C19 in a Caucasian population is only 2–5 %, the recruiting of those subjects became cumbersome, and the consequence for the design of the study was that some study details were more adjusted to the needs of those subjects than in a 'normal' phase I study.

The formulation used in this study had proven to be superior to other formulations tested; as dose 100 mg is selected (instead of the targeted 400 mg), in order to generate an additional safety margin for the case that the metabolizer-status consistently influences the extent of exposure.

The impact of selected phenotypes on candidate drug pharmacokinetics and safety can be conveniently investigated in an explorative study with the design described in this section. Typically, this study would run shortly after key data are available, indicating the influence of polymorph DME+T. The dose should be adjusted (i.e. reduced, compared to the clinical dose) based on the expected degree of interaction.

But especially since a predicted phenotype based on genotyping data was used to define the study-groups, this investigation cannot consider all the extrinsic and intrinsic factors influencing the real activity of enzymes and transporters. During the further clinical development additional (population) analyses and studies might be required to elucidate the respective influence of the DME+T polymorphism and its consequences further.

MODIFICATIONS OF THE METHOD

The design of study described here is relatively simple, but nevertheless provides explorative profiling of issues related to DME+T polymorphism, and the same principle could be applied to address further influencing factors or populations. The more complicated part is to define clearly both relatively small test and control groups based on the DME+T properties or other characteristics.

Typically, this type of evaluation would be extended during later clinical development by performing a population pharmacokinetic/pharmacodynamic assessment of the impact of genetically determined (inter-ethnic) differences on the disposition of the developmental drug during the phase II/III studies.

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US FDA (2003) Draft Guideline for Industry: Pharmacogenomic Data Submissions. November 2003

EXAMPLE

To illustrate the type of data that can be obtained using this study, a high level summary of the data from the study described above under "Procedure" is presented below.

Results

Compared to the extensive metabolizers for CYP 2C19, the plasma profiles clearly differ for the poor CYP 2C19 metabolizers: the initial peak is higher and especially the elimination phase takes much longer. There is in addition an indication of a difference between the two control groups (females vs. males). Some profiles for the females show peaks similar to those seen for the poor metabolizers, and at least in one case a similar long elimination phase was to be observed. Nevertheless in average the two control groups of extensive CYP 2C19 metabolizers are highly overlapping, and clearly below the profiles for the poor CYP 2C19 metabolizers. See Figure 13.

The peak values (C_{max} as median) are 0.95 μg/ml, 0.35 μg/ml and 0.61 μg/ml for the poor metabolizers, the male extensive metabolizers and the female extensive metabolizers respectively. The differences in the overall exposure (AUC, extrapolated to infinity) are much more prominent, because of the intensive differences in the elimination phase. The median values were 8.83 μg*h/ml, 0.83 μg*h/ml and 2.49 μg*h/ml for the poor metabolizers, the male extensive metabolizers and the female extensive metabolizers respectively. This difference is already obvious when comparing the exposure only over the first 24 h: the median values for $AUC_{(0\text{-}24h)}$ were 4.21 μg*h/ml, 0.83 μg*h/ml and 1.38 μg*h/ml for the poor metabolizers, the male extensive metabolizers and the female extensive metabolizers respectively.

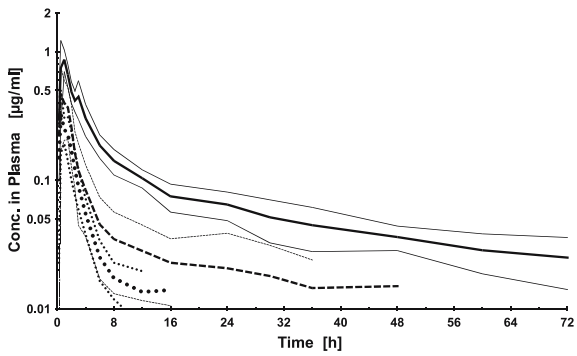

Fig. 13. Plasma concentration vs. time profiles of XYZ1234 following a single oral 100 mg dose given to poor CYP 2C19 metabolizing male and female subjects (*straight lines*), to extensive CYP 2C19 metabolizing females (*dashed lines*), and to extensive CYP 2C19 metabolizing males (*dotted lines*) respectively: *Mean values (bold) and 90% Confidence Interval*

Also the half-life of the terminal phase strongly differed. The median values were 43.6 h, 4.6 h and 18.5 h for the poor metabolizers, the male extensive metabolizers and the female extensive metabolizers respectively.

The PK parameters showed a significant variability in these small (n = 6) groups. In addition the extrapolated portion of the exposure (AUC: %Extrap) is quite high for the poor metabolizers and also the extensive metabolizing females, indicating that (at least for the poor metabolizers) even the 72 h blood collection period was not sufficient, which in addition contributes to the variability of the PK data.

II.S.15 Profiling of the Gastrointestinal Site of Absorption

PURPOSE AND RATIONALE

For several reasons it may be helpful to investigate the extent of absorption by determining the window for absorption in the gastrointestinal (GI) tract, for example:

- If the drug is intended to reach the colon, a drug loss in the small intestine is not desirable as this portion does not contribute to efficacy, but possibly to intolerance
- The drug has a (too) short systemic half-life and the feasibility for the development of an extended release formulation has to be investigated.

In the present study example the feasibility of the development of a slow-release formulation was evaluated. Guidelines on the design and conduct of such studies have not yet been published.

PROCEDURE

The design of an exploratory assessment of the absorption window for a high-clearance drug candidate named XYZ1234 is presented below. The aim of the present study is to compare the regional drug absorption from the proximal small bowel, the distal small bowel and ascending colon with an immediate release reference formulation, using pharmaco-scintigraphy and remote release via the Enterion capsule to evaluate the feasibility of the development of an extended-release formulation.

Protocol Outline

Pharmacoscintigraphic evaluation of the regional drug absorption of XYZ1234 in healthy men.

Primary Objective

To investigate the pharmacokinetics (PK) of an XYZ1234 formulation (200 mg) following topical release in the proximal small bowel, distal small bowel and colon via the Enterion capsule in healthy subjects and to compare to the PK of an XYZ1234 immediate release reference formulation (200 mg).

Study Design

Randomized four way cross-over design.

Inclusion Criteria

Healthy male subjects aged between 18 and 65 years.

Treatments

Regimen A: XYZ1234 immediate release formulation in tablet form (200 mg, reference).

Regimen B: XYZ1234 formulation (200 mg) delivered to the proximal small bowel via the Enterion capsule.

Regimen C: XYZ1234 formulation (200 mg) delivered to the distal small bowel via the Enterion capsule.

Regimen D: XYZ1234 formulation (200 mg) delivered to the ascending colon via the Enterion capsule.

Pharmacokinetic Data

Derived from concentrations of the parent drug XYZ1234 in whole blood and of the active metabolite ABC4321 in plasma before and at predefined times after dosing (Regimen A) and following activation of the Enterion capsule (Regimens B, C and D).

EVALUATION

PK data: The PK parameters of ABC4321 in plasma were determined by individual PK analyses. The individual and mean concentrations of ABC4321 in plasma were tabulated and plotted. PK variables were listed and summarized by treatment with descriptive statistics. An analysis of variance (ANOVA) including sequence, subject nested within sequence, period, and treatment effects, was performed on the ln-transformed parameters (except t_{max}). The mean square error was used to construct the 90 % confidence interval for treatment ratios. The point estimates were calculated as a ratio of the antilog of the least square means. Pairwise comparisons to treatment A were made. Whole blood concentrations of XYZ1234 were not used to perform PK analyses.

Scintigraphic data: The activation time post-dose and the arrival, residence and transit times of the capsule in different regions of the GI tract (i.e. dosing time subtracted from the actual clock time for each transit event) were derived from the scintigraphic images and tabulated for each treatment condition and

for subjects. Descriptive statistics were performed where appropriate.

CRITICAL ASSESSMENT OF THE METHOD

The pharmacoscintigraphy of a drug vehicle from which the contents can be released via remote signal, has been applied to early drug development in recent years with increasing frequency.

Via remote control of its current location, this capsule represents a reliable tool for immediate release at the GI location where the permeation capability of the drug substance should be assessed.

As the capsule can be 'loaded' with the drug as powder, as suspension or solution, the physico-chemical situations in the different parts of the GI tract can be mimicked.

An in vitro compatibility study ('leakage test') had been performed beforehand:

1. Three capsules were filled with powdered tablet formulation of XYZ1234, containing 200 mg of the drug each.
2. Each capsule was transferred to simulated gastric fluid (SGF) and placed in a shaking water bath maintained at 37 °C for 2 hours.
3. After this 2 h incubation, each capsule was transferred to simulated intestinal fluid (SIF) and placed in a shaking water bath maintained at 37 °C for 24 hours.
4. Aliquots of SGF were taken at 1 and 2 h, aliquots of SIF were taken at 1 and 24 h, and analyzed for XYZ1234.
5. The content of the capsules was analyzed for XYZ1234.

The leakage of powdered drug formulation (0.01 %) under simulated in vivo conditions was considered acceptable. The recovery of drug from the capsules was within the specification limits for the film-coated tablets.

In the study presented here, absorption from the colon was not reliable, thus making it non-feasible to develop a formulation with a 24 hour (h) release profile reducing the administration frequency to QD.

MODIFICATIONS OF THE METHOD

Other attempts to define the site of absorption were other kinds of vehicles as well as catheters. Three main perfusion methods have been employed in the small intestine: (i) a triple lumen tube including a mixing segment, (ii) a multilumen tube with a proximal occluding balloon, (iii) a multilumen tube with two balloons occluding a 10 cm long intestinal segment. A critical assessment of these perfusion techniques can be found in Lennernäs 1998. Whereas vehicles inherit the advantage of minimal invasiveness, their transit through the GI tract depends on the individual physiology of the study subject. On the other hand, this is exactly the fate of a solid dosage form.

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EXAMPLE

To illustrate the type of data that can be obtained using this study, a summary of the pharmacokinetic and scintigraphy results obtained from the study described above under "Procedure" is presented below.

Results

PK data: PK parameters were calculated for all subjects for Regimens A (immediate release), B (proximal small bowel activation) and C (distal small bowel activation).

However, for Regimen D (ascending colon activation), out of the 6 subjects available, 2 subjects had plasma concentrations which were low and $AUC_{(0-\infty)}$ could not be calculated and a further 2 subjects had $AUC_{(0-\infty)}$ of $> 20\,\%$ extrapolated from the last observation. Hence for this parameter contrasts for Regimen D should be interpreted cautiously. Mean $AUC_{(0-\infty)}$ values for regimens A, B, C and D were 4.236 μg/mL*h, 4.175 μg/mL*h, 2.36 μg/mL*h and 0.965 μg/mL*h, respectively. For the contrast Regimen B vs. Regimen A the ratio was 100.3 %, for the contrast Regimen C vs. Regimen A the ra-

Table 29 Summary of pharmacokinetic parameters in plasma.

Treatment/ Location		$AUC_{(0-\infty)}$ (μg/mL.h)	$AUC_{(0-t)}$ (μg/mL.h)	AUC ext (%)	C_{max} (μg/mL)	t_{max} (h)	$t_{1/2}$ (h)
Immediate release (reference, A)	N	8	8	8	8	8	8
	mean	4.236	4.168	1.96	2.0226	0.63	2.129
Proximal small bowel (B)	N	8	8	8	8	8	8
	mean	4.175	4.111	1.68	1.3791	1.31	2.119
Distal small bowel (C)	N	8	8	8	8	8	8
	mean	2.360	2.298	3.31	0.4563	2.38	2.686
Ascending colon (D)	N	4	5	4	5	5	4
	mean	0.965	0.722	18.25	0.1020	3.50	7.22

tio was 55.9 % and for the contrast Regimen D vs. Regimen A the ratio was 27.8 %.

Mean C_{max} values for regimens A, B, C, and D were 2.02 μg/mL, 1.38 μg/mL, 0.46 μg/mL and 0.10 μg/mL, respectively. For C_{max} for the contrast Regimen B vs. Regimen A the ratio was 64.5 %, for the contrast Regimen C vs. Regimen A the ratio was 20.2 % and for the contrast Regimen D vs. Regimen A the ratio was 4.0 %.

The t_{max} tended to increase through the regimen order of A (0.63 h) to B (1.31 h) to C (2.38 h) to D (3.50 h).

The $t_{1/2}$was similar for Regimens A and B (2.1 h), and slightly longer for Regimen C (2.7 h). Half-life was longer for Regimen D (7.2 h) based on the four subjects for which this was calculated.

Scintigraphic data: Gastric emptying of the Enterion capsule occurred on average at 1.15 h post-dose for Regimen B, at 1.28 h post-dose for Regimen C and at 2.39 h post-dose for Regimen D.

Small intestinal transit times for the Enterion capsule were on average 4.19 h for Regimen B, 4.69 h for Regimen C and 4.11 h for Regimen D.

Colon arrival of the Enterion capsule occurred on average at 5.34 h post-dose for Regimen B, at 6.25 h post-dose for Regimen C and at 6.49 h post-dose for Regimen D.

Capsule recovery occurred on average at 23.86 h post-dose for Regimen B, at 34.23 h post-dose for Regimen C and at 30.59 h post-dose for Regimen D. This resulted in colonic transit times of 18.52 h for Regimen B, 25.11 h for Regimen C and 24.53 h for Regimen D.

Enterion capsules were successfully activated in 8 subjects for Regimen B, 8 subjects for Regimen C and 7 subjects for Regimen D.

Gastrointestinal transit data: Two main parameters influence the gastric emptying of pharmaceutical formulations, the physical size of the dosage form, and whether it is administered in the fasted or fed state. When the stomach is empty of food, i.e. in the fasted or interdigestive state, indigestible solids such as large single unit dosage forms will be emptied by the action of the migrating myoelectric complex (MMC). The MMC is usually divided into four phases of activity, which recur approximately every 2 h. Phase III of the MMC is known as the 'housekeeper wave', and acts to empty the stomach of swallowed saliva, cellular debris and indigestible solids. If the formulation is administered just before a 'housekeeper wave', then it may be rapidly emptied from the stomach. However, if the preparation is administered just after phase III activity then it is probable that the Enterion capsule would not be emptied until the next 'housekeeper wave' occurred some 2 h later.

The Enterion capsules demonstrated typical gastric emptying for the majority of subjects for preparations administered in the fasted state. The capsules left the stomach within 3 h of dosing in 7 of the 8 subjects for Regimen B (proximal small bowel activation), in 7 of the 8 subjects (8 subjects dosed on 9 occasions) for Regimen C (distal small bowel activation) and in 5 of the 7 subjects for Regimen D (ascending colon activation). This suggests that in the majority of individuals, the first cycle of phase III MMC activity was 100 % efficient. Gastric emptying occurred within 5 h of dosing in the majority of the remaining subjects suggesting that the capsule was removed from the stomach by the second cycle of phase III MMC activity. An extended gastric residence time of 19.08 h was recorded for subject 3 (Regimen C). Extended gastric residence times have been observed previously with a radiotelemetry capsule (RTC), which is comparable in size to an Enterion capsule. The RTC

did not empty from the fasted stomach within a 12 h study period, even though phase III contractions were noted at 4.5, 6.4 and 8.5 h post-dose, therefore indicating a 2 hourly recurring MMC [US FDA Guidance for Industry (1997) Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations. September 1997]. The study demonstrated that the phase III contractions of the MMC were not always efficient at removing large non-disintegrating dosage forms from the stomach.

Mean small intestinal transit times for the Enterion capsule of 4.19 ± 1.19, 4.69 ± 1.71 and 4.11 ± 1.45 h for Regimens B, C and D, respectively, are not unusual. There is always a high degree of intra- and inter-subject variability observed in gastrointestinal tract data. These intestinal transit times are in general agreement with the 3 h (± 1 h, range 1.3 to 6 h) previously reported for solutions, pellets and tablets.

Colon arrival of the Enterion capsule occurred on average at 5.34 ± 1.13, 6.25 ± 1.88 and 6.49 ± 2.50 h post-dose for Regimens B, C and D, respectively. For many of the subjects colon arrival occurred after the ingestion of food at 5 h post-dose (lunch). The ingestion of a meal is known to stimulate colonic activity and this is termed the gastrocolonic response (GCR). The GCR has been shown to play an important role in determining colon arrival of pharmaceutical preparations following an overnight fast or a light meal.

Capsule recovery occurred on average at 23.86 ± 6.39, 34.23 ± 28.85 and 30.59 ± 26.60 post-dose for Regimens B, C and D, respectively. This resulted in average colonic transit times of 18.52 ± 5.65, 25.11 ± 24.00 and 24.53 ± 25.12 h for Regimens B, C and D, respectively. Colonic transit times of dosage forms ranging from 1 h to 60 h have been reported. The results for colonic transit for this study are therefore in good agreement with those previously reported.

The pharmacokinetics of XYZ1234 formulations after release in different regions of the gastrointestinal tract reveal similar exposure for the proximal small bowel as compared to the immediate release formulation, halved exposure for the distal small bowel and only poor absorption from the ascending colon. Thus colonic resorption cannot be relied on for the development of an extended release formulation. Analysis of the scintigraphic data has confirmed release of the formulation at the target locations in the required number of subjects.

II.S.16
Special Situations for Drug Delivery: Modified Release Formulations

PURPOSE AND RATIONALE

One of the special situations for drug delivery is the assessment of the pharmacokinetic (PK) properties of a modified release formulation. Modified release products always gain importance if the PK/PD profile of a drug is not close to optimal for its target indication, mostly because the (short) PK or efficacy half-life does not match the intended dosing frequency.

Details on the design, objectives and interpretation of bioavailability studies on modified release products can be found in the literature.

PROCEDURE

The design of an exploratory bioavailability study on modified release drug products is presented below. For the design of the study information from a recent BA study with other modified release products, from a site-of-absorption study and from a modeling & simulation experiment was used.

Protocol Outline

Comparison of pharmacokinetics and safety of extended release formulations of 600 mg XYZ1234 with that of an immediate release formulation – a single-center, open-label, cross-over study in healthy men.

Primary Objective

To compare the PK characteristics of extended release (ER) formulations of XYZ1234 with the PK of an immediate release (IR) formulation of XYZ1234.

Secondary objective

To assess the influence of food on the PK of ER formulations of XYZ1234.

Study Design

The study was carried out in a single center, open-label, single-dose, four period cross-over study design with two independent treatment groups.

Single oral doses of 600 mg XYZ1234 were given under fasting and under non-fasting conditions. The order of treatments and of fasting and non-fasting conditions was randomized in a 4-way cross-over incomplete block design (Treatment Groups I and II). The washout periods between the administrations of study medication were at least 48 hours (h) each.

Inclusion Criteria

Healthy men aged 18 to 55 years and assessed as healthy based on findings in medical history, physical examination, blood pressure, pulse rate, and electrocardiogram (ECG) during screening.

Treatments

Treatment Group I

Treatment A: 600 mg XYZ1234 (1 film-coated tablet containing 200 mg + 1 film-coated tablet containing 400 mg given together) as IR formulation under non-fasting (NF) conditions (reference).

Treatment B: 600 mg XYZ1234 in ER formulation (LLL matrix tablet) under fasting (F) and NF conditions.

Treatment C: 600 mg XYZ1234 in ER formulation (LLL bilayer tablet) under F and NF conditions.

Treatment Group II

Treatment A 600 mg XYZ1234 (1 film-coated tablet containing 200 mg + 1 film-coated tablet containing 400 mg given together) as IR formulation under non-fasting (NF) conditions (reference).

Treatment D: 600 mg XYZ1234 in ER formulation (KKK matrix tablet) under F and NF conditions.

Treatment E: 600 mg XYZ1234 in ER formulation (KKK bilayer tablet) under F and NF conditions.

Pharmacokinetic Data

Concentration of XYZ1234 in plasma before and at predefined times after dosing.

EVALUATION

Bioanalytical data: Individual plasma concentrations of XYZ1234 were tabulated together with standard descriptive statistics for each treatment. Individual and median profiles were presented graphically.

In vivo dissolution data: The individual hypothetical in vivo dissolutions for the four extended release formulations administered under fasting and non-fasting conditions relating to the metabolite XYZ1234 were estimated by numerical deconvolution using the individual response to the immediate release formulation given under non-fasting conditions as the weighting-(impulse) function using a hidden function of the validated HOEREP-PC software.

Plateau time data: The additional pharmacokinetic characteristics i.e., plateau times of XYZ1234 (time above 200, 500, 800 and 1,000 ng/mL [h]) were calculated in the interval from administration (t = 0) to exactly 12 h thereafter from the plasma concentration-time data pairs and subjected to ANOVA. Points of intersection with a specific plateau concentration were obtained by linear interpolation.

PK data: PK parameters were determined based on plasma concentrations of XYZ1234 using non-compartmental procedures.

Primary PK measure: $AUC_{0\text{-inf}}$

Secondary PK measures: $C_{12\ h}$, $AUC_{0\text{-}12\ h}$, $AUC_{0\text{-}t}$, AUC_{ext} [%], C_{max}, t_{max}, MRT, $t_{1/2\lambda z}$, t_{lag}.

The primary measure was subject to an analysis of variance (ANOVA) including sequence, subject nested within sequence (subject (sequence)), period and treatment effects. According to the treatment groups and fasting conditions there were five realizations of the variable treatment. The sequence effect was tested using the subject (sequence) mean square from the ANOVA as an error term. All other main effects were tested against the residual error (error mean square) from the ANOVA. The ANOVA was performed on ln-transformed data. The mean square error was used to construct 90 % confidence intervals for treatment ratios. The point estimates were calculated as ratio of the antilogs of the least square means and were expressed as percentages. The ANOVA was performed separately for subjects in Treatment Group I and subjects in Treatment Group II. Point estimates and confidence intervals were primarily calculated for the ratio of each ER formulation to the IR reference formulation. Additionally, the ratios fasting/non-fasting for each ER formulation were calculated.

The secondary pharmacokinetic measures were evaluated descriptively.

For t_{max} frequency, distribution tables were given for each group, formulation and fasting/non-fasting condition.

CRITICAL ASSESSMENT OF THE METHOD

The study described here has a very complex design for its exploratory approach. It combines four different extended release formulations, each tested under fasting and non-fasting conditions, and compares the results to the immediate release drug product as the reference formulation. The bilayer tablets combines an immediate release component and an extended release component in one vehicle. In this project a close cooperation between the galenics department and the clinical pharmacokinetic function was mandatory. The in vitro/in vivo correlation was done by means of the deconvolution which is an appropriate surrogate to describe the in vivo dissolution.

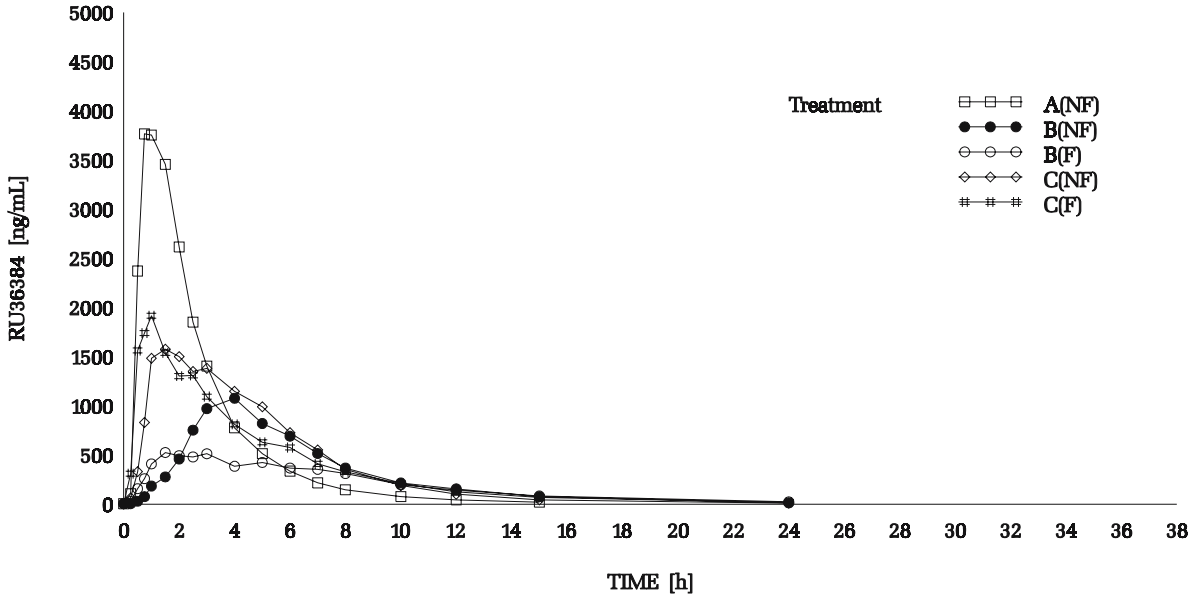

Fig. 14. XYZ1234 concentration in plasma vs. time following Treatments A(NF), B(NF), B(F), C(NF) and C(F) Group I. Median plot-lin-lin scale.

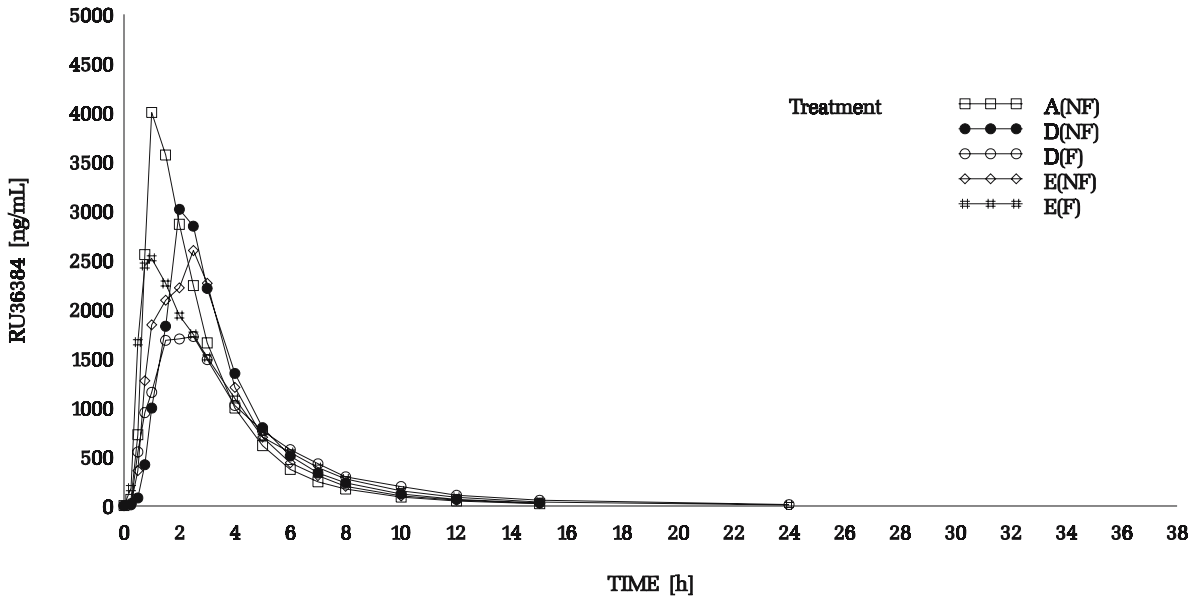

Fig. 15. XYZ1234 concentration in plasma vs. time following Treatments A(NF), D(NF), D(F), E(NF) and E(F) Group II. Median plot-lin-lin scale.

MODIFICATIONS OF THE METHOD

Recommendations exist to conduct in vivo studies first in an animal species (preferably the pig) before going to man. This approach has limited validity because modified release is primarily defined by the absorption properties of a drug. As absorption is influenced by the general composition, the sums of the physicochemical properties, the length and residence times of each section of the GI tract, no animal species is similar to man in this respect. Repeated dosing studies are recommended if the drug product is intended for subchronic or chronic use.

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US FDA Guidance for Industry: Exposure-Response Relationships – Study Design, Data Analysis, and Regulatory Applications. April 2003
US FDA Guidance for Industry: SUPAC-MR: Modified Release Solid Oral Dosage Forms. September 1997

EXAMPLE

To illustrate the amount of data that can be obtained using the study type under discussion, an overview of the pharmacokinetic and the deconvolution results obtained from the study described above under "Procedure" is presented below.

Results – Pharmacokinetics

The primary objective of the present study was to investigate the pharmacokinetic characteristics of four ER formulations of XYZ1234. Two LLL formulations (matrix and bilayer tablets, Treatments B & C respectively), and two KKK formulations (matrix and bilayer tablets, Treatments D & E) were compared with the pharmacokinetic characteristics of an IR formulation (Treatment A) in the non-fasting state. The secondary PK objective was to assess the influence of food on the pharmacokinetics of these ER formulations of XYZ1234.

Extended-Release Characteristics (Primary Objective)

LLL tablets (Treatments B and C) provided lower C_{max} values and lower relative bioavailability compared to the IR formulation (Treatment A). A longer half-life was observed for Treatment B (matrix tablets), under both fasting and non-fasting conditions, but for Treatment C (bilayer tablets) the lower limit of the 90 % confidence was above 100 % only under fasting conditions.

KKK tablets (Treatments D and E), provided lower C_{max} values with similar (though not higher) AUC values for Treatment E (bilayer tablets), and similar AUC for Treatment D – only under non-fasting conditions. Increased half-lives were recorded for Treatments D and E, only under fasting conditions.

Higher C_{12h} values and longer MRTs, both features important for ER formulations, were obtained for all LLL tablets, compared to Treatment A. A similar but less pronounced effect was observed for KKK formulations, notably under fasting conditions. Only Treatment C (LLL, bilayer) had significantly greater time values than Treatment A for the time above 800 ng/mL.

The effect of the IR component within the bilayer tablets on the absorption profile was most markedly observed in the figures describing the hypothetical in vivo dissolution. The increase in amount absorbed was steeper for Treatments C and E, especially under fasting conditions.

Effects of Food (Secondary PK Objective)

No effect of food was recorded on $AUC_{0-\infty}$ and MRT values for the bilayer tablets (Treatments C and E), with marginal effect on their rate of absorption and on C_{max} values. The absorption rate of matrix tablets, as well as their C_{max} values, were more affected. C_{12h} values presented food consumption effects for all formulations, however to a larger extent for KKK than for LLL tablets.

The following figures show the plots of the median concentration-time-profiles of XYZ1234 following administration of the several treatments synoptically for the two groups in lin-lin-scaling.

Pharmacokinetic Measures and Parameters

The model independent pharmacokinetic characteristics for XYZ1234 following single dose administration of the different treatments were calculated using non-compartmental procedures. The following table gives the arithmetic means, standard deviations, and coefficients of variation as well as the medians and ranges of the primary pharmacokinetic measure $AUC_{0-\infty}$, and of the secondary measures C_{12h} ,C_{max} and MRT.

Table 30 gives further arithmetic mean PK measures and parameters of XYZ1234 following oral single dose administration of XYZ1234.

Statistical/Analytical Issues

The extrapolated part $AUC_{t-\infty}$ of the total areas $AUC_{0-\infty}$ following the four different treatments for XYZ1234 did not exceed 9 % in maximum.

The primary parameter $AUC_{0-\infty}$ was subjected to an analysis of variance (ANOVA) including sequence, subject nested within sequence (subject (sequence)), period and treatment (non-fasting/fasting) effects. The sequence effect was tested using the subject (sequence) mean square from the ANOVA as an error term. All other main effects were tested against the residual error (error mean square) from the ANOVA. The ANOVA was performed on ln-transformed data. For ratios 90 % confidence intervals were constructed. The point estimates and confidence limits were calculated as antilogs and were expressed as percentages. The

Table 30 Arithmetic means and standard deviations (SD) of the primary pharmacokinetic measure $AUC_{0-\infty}$, and of the secondary measures C_{12h}, C_{max} and MRT following single dose administration of the different treatments.

Treatment		$AUC_{0-\infty}$ [ng*h/mL]	C_{12h} [ng/mL]	C_{max} [ng/mL]	MRT [h]
Group I					
A(NF)	Arithmetic mean	11076.98	47.66	4879.50	2.87
	SD	2357.94	20.93	1723.06	0.68
B(NF)	Arithmetic mean	7126.00	149.81	1343.34	6.65
	SD	2220.73	61.62	650.08	1.31
B(F)	Arithmetic mean	5396.57	167.41	660.95	8.27
	SD	1469.50	95.17	205.40	1.99
C(NF)	Arithmetic mean	9264.27	110.70	1955.25	4.80
	SD	2147.19	53.02	472.25	0.79
C(F)	Arithmetic mean	9195.10	148.35	2319.81	5.24
	SD	1930.40	81.09	598.63	1.13
Group II					
A(NF)	Arithmetic mean	11079.02	56.68	4460.81	3.12
	SD	1988.99	19.80	1212.04	0.66
D(NF)	Arithmetic mean	10194.24	68.02	3226.19	3.90
	SD	1681.87	18.34	794.93	0.64
D(F)	Arithmetic mean	9020.77	113.47	1858.81	5.10
	SD	1963.15	44.71	355.81	1.36
E(NF)	Arithmetic mean	10244.97	62.47	3340.25	3.55
	SD	2021.91	23.64	902.63	0.48
E(F)	Arithmetic mean	10634.66	106.72	2747.50	4.10
	SD	1506.00	67.24	315.39	0.77

Table 31 Mean pharmacokinetic measures and parameters of XYZ1234 following oral single dose administration of XYZ1234.

Treatment	AUC_{0-12h} [ng*h/L]	AUC_{ext}[%]	t_{max}[h]	$t_{1/2\lambda z}$[h]	t_{lag}[h]
Treatment Group I					
A(NF)	10881.36	0.83	1.27	2.56	0.02
B(NF)	6309.06	2.02	3.31	3.66	0.20
B(F)	4182.51	3.16	2.72	4.86	0.11
C(NF)	8743.51	0.96	2.34	3.01	0.06
C(F)	8303.25	1.84	0.98	4.24	0.02
Treatment Group II					
A(NF)	10857.58	0.90	1.52	2.53	0.09
D(NF)	9908.92	0.95	2.20	2.73	0.11
D(F)	8282.20	1.44	2.06	4.11	0.08
E(NF)	9991.45	0.90	2.09	2.76	0.09
E(F)	10052.60	1.01	1.08	3.25	0.02

ANOVA was performed separately for subjects in Group I and subjects in Group II.

Point estimates and confidence intervals were primarily calculated for the ratio of each ER formulation and the IR reference formulation. Additionally, the ratios fasting/non-fasting for each ER formulation were computed.

No significant effect of either period or sequence was found for the primary parameter $AUC_{0-\infty}$, and for the secondary parameters C_{max} and $t_{1/2\lambda z}$. Treatment effect was highly significant for all three PK parameters and subject effect was significant for $AUC_{0-\infty}$, and $t_{1/2\lambda z}$ but not for C_{max}.

In addition, the times for which plasma concentrations of XYZ1234 were above certain values (200, 500, 800 and 1000 ng/mL), within the 12 h dosing interval, were calculated for the various formulations and treatment modalities. These results were subjected to ANOVA to assess the statistical significance of the differences. The control Treatment A was tested under non-fasting conditions only. In order to get the statistical significance of the differences for the various formulations, without interference of food effects, ANOVA was carried out again comparing Treatment A with B, C, D, and E non-fasting. This analysis revealed highly significant treatment effect for

Table 32 Hypothetical dissolution data for XYZ1234 obtained by deconvolution using Treatment A(NF) as impulse function. Medians.

Measures	B(NF)	B(F)	C(NF)	C(F)	D(NF)	D(F)	E(NF)	E(F)
Maximum amount absorbed [mg]	358.97	290.50	485.97	561.49	582.56	460.12	629.25	652.95
Maximum amount absorbed [% of dose]	63.22	51.16	85.58	98.88	102.59	81.03	110.82	114.99
Time to reach maximum amount [h]	15.00	24.00	15.00	15.00	9.00	15.00	2.50	15.00
Time to reach 50% of maximum amount [h]	2.80	3.34	0.91	0.40	1.04	0.69	0.81	0.37
Time to reach 80% of maximum amount [h]	5.27	6.95	2.95	1.64	1.79	2.54	1.44	0.48

both Treatment Groups I (LLL) and II (KKK) for the time above 200 ng/mL and the time above 500 ng/mL. However, for the time above 800 and 1000 ng/mL, only Group I (LLL) showed statistical significance. Further examination of the results revealed that in fact only Treatment C (LLL, bilayer) had greater time values than Treatment A (Treatment B had lower values).

The additional pharmacokinetic characteristics i.e., plateau times of XYZ1234 (time above 200, 500, 800 and 1000 ng/mL [h]) were calculated in the interval from administration (t = 0) to exactly 12 h thereafter from the plasma concentration-time data pairs. Points of intersection with a specific plateau concentration were obtained by linear interpolation.

Hypothetical in vivo Dissolution

The individual hypothetical in vivo dissolutions for the four extended release formulations administered under fasting and non-fasting conditions relating to the metabolite XYZ1234 were estimated by numerical deconvolution using the individual response to the immediate release formulation given under non-fasting conditions as the weighting- (impulse-) function using a hidden function of a validated software.

Deconvolution is used to evaluate in vivo drug release and drug absorption from orally administered drug formulations (i.e., extended release) when data from a known drug input are available. The applied deconvolution method requires data from a formulation with zero order absorption as known input, e.g., an oral solution (oral bolus input); the immediate release formulation used as known input only provides an approximation to the required properties.

The medians and ranges of the hypothetical dissolution data for the metabolite XYZ1234 obtained by deconvolution are listed in Table 32.

Treatments C and E (the bilayer tablets that contain the IR component) had a steeper amount absorbed profile as compared to the parallel matrix tablets (Treatments B and D). This effect was more pronounced under fasting conditions. Only with Treatment E (KKK bilayer tablets) did the hypothetical in vivo dissolution profiles surpass the 100 % absorption, both under fasting and non-fasting conditions. For Treatment C, this occurred only under fasting conditions and for Treatment D only under non-fasting conditions.

The mismatch of surpassing 100 % absorption of the active metabolite is probably due to method constraints in combination with the immediate release data, as the deconvolution method requires data from a formulation with zero order absorption for the impulse function, e.g. an oral solution (oral bolus input); the immediate release formulation only provides an approximation to the required properties.

Chapter II.T
Pharmacogenomics in DME

Guy Montay
Jochen Maas

The understanding of the role of pharmacogenetics in drug metabolism expanded greatly in the 1990s. This is mainly due to technological improvements in gene scanning and gene variant identification. The number of variant alleles identified for genes coding for Drug Metabolizing Enzymes (DME) considerably increased in the early 2000s, and continues to increasing. The clinical consequences – or at least genotyping-phenotyping relationships – of DME polymorphisms have not been demonstrated for all variants. In the text below, we will only indicate the DME allele variants for which significant changes in enzyme activity have been found using probe drugs. Complete information on CYPs alleles can be found at www.imm.ki.se/CYPalleles and Phase I and Phase II DMEs at www.pharmgkb.org/index.jsp.

II.T.1 Phase I Enzymes

II.T.1.1 CYP1A2

PURPOSE AND RATIONALE

CYP1A2 is involved significantly in the metabolism of several drugs (imipramine, clozapine, fluvoxamine, olanzapine, theophylline, acetaminophen, propranolol, tacrine) as well as in the metabolism of diet components (methylxanthines), endogenous substrates (oestrogens), numerous aryl, aromatic and heterocyclic amines, and polycyclic aromatic hydrocarbons. It is inducible, notably by cigarette smoking, diet habits such as consumption of cruciferous vegetables and of charbroiled meats, some drugs (omeprazole, phenytoin, rifampicin) and is a target enzyme for the development of some cancers. Probe drugs for CYP1A2 phenotyping are caffeine and theophylline. For safety concerns and drug availability, the preferred probe is caffeine. Caffeine 3- demethylation is mediated by CYP1A2, and accounts for 80 % of caffeine clearance. Caffeine is also a probe drug for N-Acetyltransferase and xanthine oxidase (Kalow and Tang 1993).

PROCEDURE

Phenotyping: A fixed or weight-adjusted dose of caffeine (solution, tablet, coffee) ranging from 1 to 3 mg/kg is administered. Diet requirements are needed (stable xanthine-free diet avoiding beverages like coffee, tea, cola, chocolate, no food component with CYP1A2-inducing properties) during the test period, as well as control of stable smoking status.

There are two commonly used and robust methods for phenotyping. The first one measures caffeine (1,3,7-methylxanthine) and its N-demethylated metabolite 1,7-dimethylxanthine (paraxanthine) in a plasma or saliva sample collected within 5 to 7 hours post caffeine dosing (Fuhr and Rost 1996). The second one uses the assay of the metabolites 1-methylurate

(1U), 1-methylxanthine (1X) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1,7-dimethylurate (17U) levels in urine collected at least for 8 hours post dosing (Campbell et al. 1987; Rostami-Hodjegan et al. 1996).

Commonly used methods for caffeine and metabolite(s) assay in plasma or urine involve an extraction step followed by HPLC with UV detection (Krul and Hageman 1998a; Rasmussen and Bosen 1996; Schreiber-Deturmeny and Bruguerolle 1996). Urine needs to be acidified (pH 3.0-3.5) before sample freezing.

Genotyping: Reduced activity has been reported for CYP1A2*1C and CYP1A2*F alleles in smoking subjects. Induction of CYP1A2 activity has been associated with these alleles, but the effect of CYP1A21F mutation on CYP1A2 activity has not been confirmed (Nordmak et al. 2002). In Caucasians, frequency of the CYP1A2*1C and CYP1A2*1F variants is about 1 and 33 % respectively (Sachse et al. 2003). The 1*C allele frequency has been found higher in Japanese as compared to Caucasian subjects (Todesco et al. 2003).

EVALUATION

Metabolic ratios (MR) used are plasma 17X/137X and urinary (1U + 1X + AFMU)/17U.

In controlled conditions, in non-smoking young and elderly subjects, intra-individual and interindividual variability in 17X/137X MR was about 17 and 47 % respectively, with no effect of age (Simon et al.). A 70-fold range in MR has been observed in smoking and non-smoking female Caucasian subjects using the urinary MR (Nordmark et al. 1999). Up to 200-fold differences were found using the urinary test. Lower variability is expected using the plasma caffeine test.

Higher CYP1A2 activity in men versus women has been reported, though inconsistently, and in children. Higher MR is usually observed in smokers versus non-smokers, when population sample size is large. Pregnancy and oral contraceptives intake were found to decrease CYP1A2 activity (Abernathy and Todd 1985; Caubet et al. 2004; Kalow and Tang 1993). CYP1A2 activity was found lower in colorectal patients versus controls (Sachse et al. 2003).

Large variability in CYP1A2 activity explains that its distribution has been described unimodal, bimodal or trimodal. Poor metabolizers (characterized with a MR < 0.12) have been identified in a Chinese population) and represented about 5 % of the population tested, whereas PMs could represent 5–10 % of Caucasian populations and 14 % in Japanese population (Ou-yang et al. 2000).

CRITICAL ASSESSMENT OF THE METHOD

Numerous studies have shown good correlation between the 17X/137X plasma MR and caffeine systemic clearance, and plasma MR is considered more robust than the urinary one, since this last one can be affected by the effect of urinary flow on metabolite renal clearances.

Currently, no relationship between CYP1A2 genotype characteristics and CP1A2 activity, as assessed by the caffeine test, has been usually found. Some associations have been found in specific genetic and environmental conditions (Han et al. 2001). Non-well controlled conditions for urine sample collection, the effects (induction) linked to environmental factors may overcome the role of CYP1A2 polymorphism, which can explain the paucity of clear associations between CYP1A2 genotyping and phenotyping.

Further investigations are needed to characterize the effect of variants (SNPs, haplotypes) on CYP1A2 activity.

MODIFICATIONS OF THE METHOD

Recent drug assay development involved LC-MS methods (Caubet et al. 2004; Kanazawa et al. 2000). A less practical breath test, using ^{13}C or ^{14}C labeled caffeine, can also be used (Kalow and Tang 1991).

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II.T.1.2 CYP2C9

PURPOSE AND RATIONALE

CYP2C9 is involved significantly in the hydroxylation of about 16 % of drugs (Schwarz 2003), including drugs with narrow therapeutic index such as anticoagulants (warfarin, acenocoumarol, phenprocoumon active S-enantiomers), and anticonvulsivants (phenytoin, hexobarbital), as well as numerous antidiabetic agents (i.e. tolbutamide, glibenclamide, glipizide), antihypertensive drugs (losartan, irbesartan), non steroidal anti-inflammatory agents (i.e. diclofenac, ibuprofen, celecoxib), diuretic (torsemide) and anti-rhumatoid agents (leflunomide).

Up to now, two main CYP2C9 variants – CYP2C9*2 and CYP2C9*3 – coding for in vivo decreased activity, and one – CYP2C9*6 – resulting in none activity have been identified. In Caucasians, CYP2C9*2 and CYP2C9*3 are encountered in about 35 % subjects (Lee 2002).

Probe drugs used for CYP2C9 phenotyping are: tolbutamide, S-warfarin, phenytoin and losartan. Diclofenac, flurbiprofen, phenprocoumone and torasemide have also been used. For safety concerns, the current preferred probe is tolbutamide, despite some risk of hypoglycemia.

PROCEDURE

Phenotyping: The method measures tolbutamide, its CYP2C9-formed 4′-hydroxylated metabolite and the subsequent carboxytolbutamide metabolite of hydroxytolbutamide, formed by dehydrogenase enzymes. The urinary excretion of these two metabolites represented more than 85 % dose of administered tolbutamide (Veronese 1990, 1993).

Subjects receive a single oral 500 mg tolbutamide tablet in usual Phase I standard controlled conditions, with care to be paid to blood glucose. Urine is collected from drug intake to 8 or 24 h post-dosing.

The assay of tolbutamide and its metabolite is usually performed using HPLC with UV or fluorescence detection (Csillag 1989; Veronese 1990; Kircheiner 2002; Hansen 1999).

Genotyping: About two third of Caucasian subjects express the wild genotype C9*1/*1. C9*1/*2 and C9*1/*3 heterozygote variants are expressed in 15–25 and 7–16 % of Caucasian subjects, whereas the frequency of other variants is lower: 0.5–2.5, 1–3 and < 1–1.5 % for C9*2/*2, C9*2/*3 and C9*3/*3 variants respectively (Scordo 2001; Lee 2002; Schwarz 2003). More than 95 % of Afro-American subjects express the wild genotype C9*1/*1 (Lee 2002). In Asian populations, CYP2C9*1/*3 is expressed in 2–8 % subjects, but CYP2C9*2 is absent or extremely rare (Schwarz 2003; Xie 2002). Overall, it has been estimated that 0.2–1 and 2–3 % of Caucasian and Asian population could be qualified as poor metabolizers (PMs), respectively (Meyer 2000).

EVALUATION

The urinary metabolic ratio (hydroxytolbutamide plus carboxytolbutamide)/tolbutamide is generally used. There is a large interindividual variability in

MRs in subjects with the same genotype. Different studies performed with different probe drugs (Yasar 2002; Kirchheiner 2002, 2003; Lee 2002; Miners 1998; Morin 2004), highlighted that a PM status could be given to subjects which are homozygous for CYP2C9*3, or expressing CYP2C9*2/*3 variant, but intermediate situations – from extensive to slow metabolizer status – may vary not only among different allele combinations but also with the probe drug used.

Oral contraceptives were found to inhibit CYP2C9 activity using losartan for phenotyping (Sandberg 2004).

CRITICAL ASSESSMENT OF THE METHOD

The tolbutamide test has the most convincing ability to discriminate between genotype variants and pharmacokinetics. There could be an analytical issue linked to the urine assay precision, as the urinary concentrations of the parent drug are very low in comparison with those of its metabolites.

To date, the CYP2C9*3 variant has been the only one found influencing significantly drug pharmacodynamics for warfarin, acenocoumarol (Sandberg 2003; Morin 2004; Versuyft 2003), glipizide and glyburide (Kirchheiner 2002), or drug side-effects (Sevilla-Mantilla 2004). Unconstant results were found regarding tolbutamide effects (Kirchheiner 2002; Shong 2002). For anticoagulants, the possession of CYP2C9*2 and CYP2C9*3 variants was associated with decreased warfarin dose requirement in patients, and an increased risk of adverse events such as bleeding (Daly 2003). An African-American subject with only the CYP2C9*6 variant exhibited serious phenytoin side-effects associated with a marked impaired elimination of the drug (Kidd 2001).

The variability of CYP2C9 activity observed among ethnic groups cannot be explained with our current knowledge on CYP2C9 variant alleles distribution (Xie 2002).

MODIFICATIONS OF THE METHOD

Losartan (25 mg dose) has been proposed as a safer alternative to tolbutamide. The determination of losartan/E3174(oxidized metabolite) ratio in 0–8 hour urine or in plasma at 6 hours post dosing have been proposed (Yasar 2002; Sekino 2003). However, in a comparative study in sixteen subjects, a better correlation between genotyping and phenotyping was found with tolbutamide, as compared to losartan or flurbiprofen, though there was no subject with the C9*2/*3 or C9*3/*3 variants (Lee 2003).

Recently a 125 mg tolbutamide dose has been validated, with proposal of the use of just one blood sample collected 24 hour post-dosing. Its safer use needs the drug to be assayed using LC-MS/MS methodology (Jetter 2004).

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II.T.1.3 CYP2C19

PURPOSE AND RATIONALE

CYP2C19 contributes to the metabolism of about 8 % of drugs (Rogers 2002), including S-mephenytoin, proton pump inhibitors (omeprazole, lansoprazole, pantoprazole), tricyclic antidepressants (amitriptyline, imipramine, clomipramine, citalopram), benzodiazepines (diazepam, flunitrazepam), torsemide, fluvastatin and proguanil. Two main variants – CYP2C19*2 and CYP2C19*3 – are coding for in vivo nil activity, as well as for CYP2C19*4, *5, *6, *7 and *8 variants. About 15–20 % Asians, 4–7 % Black Africans and 3 % Caucasians are PMs (Scordo 2004).

Probe drugs used for CYP2C19 phenotyping are: mephenytoin, omeprazole and proguanil. The most currently used probe drug is omeprazole.

PROCEDURE

Phenotyping: The method measures omeprazole, and its CYP2C19-formed 5-hydroxylated metabolite in plasma.

Subjects receive a single oral 20 or 40 mg omeprazole capsule in usual Phase I standard controlled conditions. Plasma can be collected from drug intake up to 24 h post-dosing, or only one plasma sample is collected at 2 or 3 h post-dosing.

The assay of omeprazole and its metabolite is usually performed using HPLC and UV detection (Lagerstrom 1984; Ieri 1996; Yim 2001; Tybring 1997) or LC-MS/MS assay (Kanazawa 2002).

Genotyping: The two alleles CYP2C19*2 and CYP2C19*3 account for quite all PMs in Asians (> 99 %) and Black Africans, but defective alleles have not been fully characterized in 10–15 % Caucasians. The CYP2C19*2 allele is the most frequent in Asian populations (30 % in Chinese), as well as in Black Africans (about 17 %) and in Caucasians (about 15 %) (Xie 2001). The CYP2C19*3 accounts for about 25 % of inactive forms in Orientals, and is extremely rare in Caucasians (Scordo 2004).

About two third of Caucasian subjects express the wild genotype C9*1/*1. C9*1/*2 and C9*1/*3 heterozygote variants are expressed in 15–25 and 7–16 % of Caucasian subjects, whereas the frequency of other variants is lower: 0.5–2.5, 1–3 and < 1–1.5 % for C9*2/*2, C9*2/*3 and C9*3/*3 variants respectively (Scordo 2001).

EVALUATION

The AUC or plasma ratio of omeprazole to 5-hydroxyomeprazole is used.

As expected, homozygous PM subjects have lower metabolic activity as compared to heterozygous PM subjects, and potential interethnic difference has been noticed within a genotype (Yin 2004).

Decreased CYP2C19 activity has been observed with oral contraceptives containing ethinyloestradiol (Tamminga 1999; Laine 2000).

CRITICAL ASSESSMENT OF THE METHOD

Omeprazole hydroxylation rate correlates with S-mephenytoin hydroxylation rate, which was initially the CYP2C19 probe drug (Andersson 1990; Chang 1995; Balian 1995). The alternate pathway – conversion of omeprazole to its sulfone derivative –, which is mediated via CYP3A4, does not influence the CYP2C19 pathway of omeprazole (Balian 1995).

Time-dependent kinetics of omeprazole limits its use for phenotyping during chronic therapy (Gafni 2001). CYP2C19 phenotyping with omeprazole may be affected by age, liver disease and omeprazole therapy (Kimura 1999).

Inter-ethnicity differences observed with different CYP2C19 substrates for subjects with same genotype have been attributed to differences in substrate specificity or enzyme isoforms (Bertilsson 1992). The clearance of omeprazole is higher in Caucasian EMs than in Oriental EMs, due to an higher proportion of heterozygous EMs in this latter population (Ishizaki 1994).

MODIFICATIONS OF THE METHOD

It has been proposed to use omeprazole for both CYP2C19 and CYP3A4 phenotyping (Gonzalez 2003).

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II.T.1.4 CYP2D6

PURPOSE AND RATIONALE

CYP2D6 is involved significantly in the metabolism of drugs mainly used in CNS (antidepressants, i.e. imipramine, paroxetine, citalopram, neuroleptics, i.e. haloperidol, risperidone) or cardio-vascular (β-adrenoceptor blockers, i.e. metoprolol, antiarrhythmics, i.e. propafenone, flecainide) disorders. Significant interethnic and interindividual intraethnic differences in CYP2D6 activity has been found. 5–10 % Caucasians, 6–8 % Afro-americans and only 1 % Asians have reduced CYP2D6 activity, and exhibit the Poor Metabolizer (PM) phenotype. Expression of CYP2D6 has been shown to be polymorphic with up to now more than 80 genetic variants detected for the encoding gene, with more than 15 encoding for inactive enzyme. Probe drugs for CYP2D6 phenotyping are: dextromethorphan, debrisoquin, sparteine and metoprolol. For safety concerns and drug availability, the preferred probe is dextromethorphan (Schmid et al. 1985).

PROCEDURE

Phenotyping: The method measures dextromethorphan DM and its O-demethylated metabolite, dextrorphan DX, which is formed by CYP2D6. DM and DX, and other metabolites, are excreted in urine, mainly as glucuronide conjugates.

Subjects receive a single oral 10 to 30 mg dextromethorphan (generally hydrobromide salt syrup)

dose. Urine is collected from drug intake to 8 hours post-dosing. Other collection times (0–6, 0–10, 0–12 or 0–24 h) can be used, but short collection intervals might lead to increased intra-subject variability.

Urine is first hydrolyzed with β-glucuronidase. Then, different methods can be used involving DM and DX extraction, followed either by HPLC and fluorescence detection (Chladek et al. 1999; Hoskins et al. 1997) or capillary gas-chromatography (Wu et al. 2003).

Genotyping: The importance of alleles involved metabolism vary between populations: three "population specific" alleles are CYP2D6*4 in Caucasians, *10 in Asians and *17 in Africans (Bertilsson et al. 2002). CYP2D6*3, *4, *5, *6 are the main inactive alleles producing the PM phenotype in Caucasians, with CYP2D6*4 most commonly associated with the PM phenotype. Lower CYP2D6 activity in Asian extensive metabolizers (EM) as compared Caucasians EM is related to more frequency (50 % vs 2 %) of the *10 allele which encodes for a decreased enzyme-activity, as compared to the *1 wild type allele (Johansson et al.; Zanger et al.). The frequency of the "less efficient" *17 allele is high in black Africans and in black Americans (Bapiro et al. 2002; Gaedigk et al. 2002). Four potential subgroups (UMs, EMs, IMs and PMs) have been defined based on the genotype–phenotype relationships.

In Caucasian subjects, it has been recommended for "routine test" to genotype for alleles *1, *3, *4, *5, *6 which allow to detect 86–100 % of poor metabolizers (Sachse et al. 1997). To assign correct phenotype in nearly 100 % subjects, *9 and *10 variants should also be determined.

EVALUATION

Subjects with a DM/DX metabolic ratio (MR) > 0.3 are poor metabolizers. Subjects with DM/DX < 0.03 are extensive metabolizers. Those with 0.03 < MR < 0.3 are intermediate metabolizers.

No difference or slightly higher CYP2D6 activity in females have been found between male and female subjects (Hägg et al. 2001; McCune et al. 2001).

Relationship between phenotyping and genotyping is investigated by plotting log MRs versus CYP2D6 allele combinations (Chou et al. 2003).

CRITICAL ASSESSMENT OF THE METHOD

Widely used due to easy and safe administration. High intrasubject variability limits the test for discriminating between extensive metabolizers (EMs) and ultra-rapid metabolizers (UMs) (Zanger et al. 2004).

Not appropriate in renal failure, due to impairment in renal excretion of DM glucuronide metabolites. Sparteine test has been recommended for this population and for discriminate for the four phenotypes UM, EM, IM and PM. The DM/DX MR does not allow for distinguish consistently between CYP2D6 extensive metabolizers with one or two active alleles.

MODIFICATIONS OF THE METHOD

Assays have been developed to determine DM and DX in plasma or saliva (Bolden et al. 2002; Hu et al 1998; Chladek et al. 2000; Härtter et al. 1996). The use of saliva or plasma for CYP2D6 phenotyping has been developed for subject convenience, or for the development of single point methods to be easily incorporated in the "cocktail methods". Good correlation between metabolic ratios calculated from plasma, saliva samples and those obtained from urine have been observed.

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II.T.1.5 CYP3A

PURPOSE AND RATIONALE

CYP3A is the predominant P450 subfamily (CYP3A4, CYP3A5, CYP3A7, CYPA43) in the human liver, and contributes significantly to the metabolism of many (at least 50 %) drugs in numerous therapeutic classes. CYP3A4 is the major P450 present notably and predominantly in the liver and small intestine, and inter-individual variability in the level of its expression is very high – 20-fold or more – (Shimada 1994). CYP3A5 shares rather similar tissue distribution with CYP3A4, is preferentially expressed in the lung, but represents generally a few % of total CYP3A as compared to CYP3A4 (exceptions are oesophagus and prostate, specific for CYP3A5, and kidney in which CYP3A5 is predominantly expressed). CYP3A4 and CYP3A5 exhibit overlapping substrate specificity, and there is currently no specific CYP3A5 specific probe drug. CYP3A7 is primarily the major fetal CYP3A enzyme.

Most of drugs biotransformed with CYP3A are also P-glycoprotein substrates (noticeable exceptions are midazolam and nifedipine). CYP3A and P-glycoprotein contribute substantially to the firs-pass elimination of highly cleared CYP3A substrates when orally administered. However, CYP3A4 and P-glycoprotein activities are not coordinately regulated in the liver and in the intestine (von Richter 2004).

Currently, 40 and 24 alleles have been identified for CYP3A4 and CYP3A5, respectively. Expression of CYP3A5 varies greatly among individuals (Lamba 2002).

Due to multiple confounding factors, such as those involved in endogenous expression of CYP3A regulatory factors, numerous exogenous factors (environment, diet), the interplay between CYP3A and transporters in regulating drug disposition, the establishment of consistent relationships between CYP3A genotype and phenotype is actually a challenge (Wilkinson 2004). Currently, the value of CYP3A genotyping in drug development is far from being clinically useful.

The most used and validated probe drugs for CYP3A phenotyping are midazolam and ^{14}C-erythromycin (Watkins 1994). Alfentanyl, alprazolam, dapsone, dextromethorphan, lidocaine ,nifedipine, omeprazole, quinine, verapamil have also been used but less frequently, and CYP3A specificity for some of them has been questioned. The "endogenous" 6β-hydroxycortisol test (measurement of 6β-hydroxycortisol: cortisol ratio in urine) is only useful for detecting CYP3A induction, and may be influenced by renal CYP3A activity.

Due to intra-individual differences in liver and intestinal CYP3A activity, phenotyping test results are related to the probe drug route of administration.

PROCEDURE

Phenotyping: Midazolam test: Midazolam is primarily metabolized to 1′-hydroxymidazolam by CYP3A. It is rapidly and completely absorbed after oral administration (Gorski 1998). It is the practical probe drug to assess intestine and liver or liver CYP3A activities after oral (Thummel 1996) or intravenous administration respectively.

Oral test doses are 2, 5 or 7.5 mg (as a solution). IV doses are 0.015, 0.025 or 0.05 mg/kg, or 1 or 2 mg per subject, as a 2 to 30-minute infusion.

Blood samples are collected over a 6-hour period. Numerous GC, GC/MS, HPLC/UV, or LC/MS methods have been developed for plasma midazolam assay (Lepper 2004; Frison 2001).

^{14}C- erythromycin breath test or ERMBT: CYP3A4 catalyzes the N-desmethylation of [^{14}C N-methyl] erythromycin. The test consists in the measurement of a single breath expired $^{14}CO_2$ collection obtained at 20 minutes following the iv administration of a 0.03 mg

dose of ^{14}C- erythromycin (2–4 μCi administered) (Watkins 1994). This test is used for assessing hepatic CYP3A activity.

Genotyping: Allelic CYP3A4 gene variants are rare. No impact of the presence of the most common CYP3A4*1B mutation (with a frequency ranging from 0 % in Chinese and Japanese to 45 in Afro-American) on midazolam, erythromycin or nifedipine clearance has been evidenced. Most significant mutations are observed for CYP3A5 and CYP3A7. Further information on polymorphic expression of CYP3A5 and CYP3A7 can be found in Lamba publication.

EVALUATION

Complete pharmacokinetic profile is required to assess midazolam clearance, and is therefore more invasive than the ERMBT, but this last one required specific logistics for radiolabeled material use. The midazolam or ERMBT phenotype tests are used for dose individualizing of narrow therapeutic index CYP3A-metabolized drugs such as anticancer agents. The ratio 1′-hydroxymidazolam: midazolam has generally been found not useful for phenotyping.

Within a population of similar demographic and health characteristics, a 4 to 6-fold range in the metabolic clearance a CYP3A-drug substrate is usual, with common individual outliers exhibiting high or low activity (Lamba 2002).

CRITICAL ASSESSMENT OF THE METHOD

Midazolam clearance has been found to correlate with hepatic CYP3A levels (Thummel 1994) as well as ERMBT results (Lown 1992). However weak, inconstant or lack of correlations between midazolam and ERBT test results have been observed, which could be explained by binding to different CYP3A active sites. In addition, contrary to the midazolam test, the ERMBT does not measure CYP3A5 activity.

An ethnic difference – that could be drug-specific – in CYP3A4 activity has been observed for few CYP3A4 substrates (alprazolam, nifedipine), with a lower clearance in Asians than in Caucasians (Xie et al. 2001).

CYP3A4 and CYP3A5 genotyping tests could not explain sufficiently the inter-individual variability observed in midazolam pharmacokinetics (Eap 2004).

MODIFICATIONS OF THE METHOD

The combined use of iv midazolam and oral ^{15}N-midazolam or of the EMBT and oral midazolam tests have been proposed to assess simultaneously the contributions of liver and intestine in CYP3A activity (Gorski 1998; McCrea 1999). The administration of orally given midazolam followed by an intravenous administration has also been validated (Lee 2002). A low oral 75 μg oral dose has recently been proposed, but needs validation on a large-scale (Eap 2004b).

Modifications of the EMBT have been described to improve its predictability in drug clearance estimations in cancer patients (Rivory 2000).

A single 4-hour single blood sampling for midazolam assay has been retrospectively found an excellent estimator for iv or oral midazolam clearance determination (Lin 2001).

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II.T.1.6 Other CYPs

This section summarizes succinctly the current knowledge on some other CYPs, the role of which in drug metabolism and the impact of genetics on it have been more recently investigated as compared to other CYPs.

II.T.1.6.1 CYP2A6

CYP2A6, primarily expressed in the liver, is the major (the sole at usual low concentrations) involved in nicotine oxidation, and is also involved in the metabolism of carcinogen or procarcinogen compounds (such as nitrosamines and aflatoxins). Few drugs are metabolized by CYP2A6: coumarin, halothane, chlormethiazole. CYP2A6 PMs are less than 1 % in Caucasians but up to 20 % in Orientals (Oscarson 2001; Raunio et al. 2001; Xu et al. 2002). The most "in vivo deficient" alleles are CYPA6*2 and CYP2A6*4, rather common in Orientals (15 % in Chinese, 20 % in Japanese).

Phenotyping has been performed in some countries with coumarine (not available in all countries), despite some limitations with data accuracy obtained with the analytical methods used (Pelkonen et al. 2000; Cok et al. 2001). The test assesses the amount of 7-hydroxycoumarine (free and conjugated) in urine after ingestion of 2–5 mg coumarine by the subjects. Nicotine has also been used as the probe drug for CYP2A6 in vivo activity testing.

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II.T.1.6.2 CYP2B6

CYP2B6 is present at low level (< 1 %) in the liver. It catalyzes bupropion hydroxylation, S-mephenytoin N-demethylation, is involved in the metabolism of cyclophosphamide, ifosfamide, mianserin and propofol. CYP2B6*6 has been associated with reduced bupropion clearance in vitro (Hesse 2004), but not in vivo whereas a moderate clearance increase was observed with CYP2B6*4 (Kirchheiner 2003). Bupropion (150 mg dose) has been proposed for phenotyping, but it is recommended to adjust dose based on subject weight (Faucette 2000).

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II.T.1.6.3 CYP2C8

CYP2C8 is involved in the metabolism of arachidonic acid, all-trans retinoic acid, paclitaxel, amodiarone, amodiaquine, repaglinide, roziglitazone, torsemide, troglitazone and zopiclone. Most of these drugs are also metabolized by CYP3A4. Recently, the potential contribution of CYP2C8 to the metabolism of NSAIDs

in addition to the well-known CYP2C9 role has been highlighted for ibuprofen (Garcia-Martin 2004). The CYP2C8*3 allele (present in 13 % and 2 % of Caucasians and Afro-American subjects respectively) has been shown in vitro deficient for paclitaxel and arachidonic acid metabolism (Dai et al. 2001; Bahadur et al. 2002). For the antidiabetic repaglinide, unexpected in vivo lower exposure was observed in subjects with CYP2C8*1/*3 genotype, without any pharmacological consequences (Niemi et al. 2003). For ibuprofen, reduced clearance of the R(–) enantiomer was related to CYP2C8*3 allele, and reduced clearance of the S(+) enantiomer was influenced by CYP2C8*3 and CYP2C9*3 alleles. In subjects homozygous or double-heterozygous for these variants (8 % of 130 subject evaluated), the clearances of ibuprofen were only 7–27 % of the clearances observed in subjects with no CYP mutations. A strong association between CYP2C8*3 and CYP2C9*2 occurrence has been characterized in a large Swedish population, highlighting linkage between CYP2C8 and CYP2C9 polymorphisms (Yasar et al. 2002).

Further in vitro/in vivo investigations are needed to assess the relationship between CYP2C8 (and CYP2C9) polymorphisms and drug metabolic clearance, in order to address the clinical relevance of CYP2C8 genotyping.

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II.T.1.6.4 CYP2E1

CYP2E1, an ethanol-inducible P450, is involved in the metabolism of endogenous substrates (steroids, bile acids, acetone...), alcohols, xanthines, chemicals (toluene, styrene, halocarbons, nitrosamines) but of few drugs (chlorzoxazone, etoposide, dapsone, high dose acetominophen) (Lieber 1997). Seven alleles, 13 genetic mutations have been described, but no genotyping-phenotyping relationships have been well established to date. Based on safe use and CYP selectivity (though CYP1A1, CYP1A2 have been found involved in its biotransformation in vitro), chlorzoxazone is the only in vivo probe drug to phenotype CYP2E1 activity, towards assessment of its 6-hydroxylation (Ono et al. 1995; Lucas et al. 1995; Ernstgard et al. 2004). Due to dose-dependent metabolism, the dose should be preferably administered on a mg/kg basis (10 mg/kg rather than the common 250 or 500 mg doses). Relatively low intra-individual variability in chlorzoxazone metabolism has been observed. Measurement can be done in urine or in plasma, after enzymatic hydrolysis of 6-chlorzoxazone glucuronide, using HPLC and UV detection or LC/MS/MS methods (Frye and Stiff 1996; Frye et al. 1998; Scoot et al. 1999). The use of plasma metabolite ratio determined with only one plasma sample – at 2 h post-dosing – has been recently validated.

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II.T.2 Phase II Enzymes

With the exception of N-acetyltransferases (detailed below), there are few deficiencies in Phase II drug metabolism enzymes that have resulted in clinically significant effects. Each Phase II enzyme class is most often a superfamily of enzymes, and usually there are large inter-individual and interethnic variability in drug conjugations, and overlapping substrate specificity exists for numerous isoenzymes. Despite the crucial role of conjugation enzymes in xenobiotic metabolism, the functional significance of enzyme polymorphism is only known for few substrates. Therefore, with the exception of the caffeine and TPMT tests (see below), no probe test drug has been yet investigated for in vivo phenotyping and validated to assess phenotyping-genotyping relationships. Nevertheless, some important aspects of enzyme polymorphism on the pharmacokinetics of drugs with narrow therapeutic index are summarized below.

II.T.2.1 N-Acetyltransferases

PURPOSE AND RATIONALE

N-acetyl transferases Type I (NAT1) and Type II (NAT2) catalyze N- and O-acetylation reactions involved in the metabolism of drugs containing arylamino, hydroxyl, sulphydryl groups & hydrazine structure, and also in environmental carcinogens (such as those present in tobacco smoke, or in diet such as charcoal-broiled food) (Weber 1985). Pending on the drug, and on the interplays between P450s and N-acetylases (and other Phase II conjugation enzymes) in xenobiotic metabolism, the impact of subject status "poor acetylator" or "rapid acetylator" on drug activity and/or toxicity may vary, and then is drug specific. NAT1 and NAT2 exhibit a high degree (81 %) of amino-acid sequence homology, and also share common substrates (Meisel 2002) but coding genes loci are regulated independently. Main NAT2 drug substrates are isoniazid, sulfonamides, procainamide, hydralazine, acebutolol, aminogluthetimide, and dapsone.

Para-aminosalicylic and para-minobenzoic acids are considered specific substrates for human NAT1, and sulfamethazine, isoniazid, procainamide and dapsone are considered specific substrates for human NAT2 (Butcher 2002). NAT1 is considered as ubiquitously distributed in the body, whereas NAT2 is expressed in liver and intestinal mucosa.

Polymorphic N-acetylation was first described for isoniazid in 1950s and is the first example of interindividual pharmacogenetic variability. Until 2002, 26 and 29 variant alleles have been described for NAT1 and NAT2 respectively. The presence of some NAT1 variants, as well as NAT2 variants, has been linked to increased susceptibility to some cancers (notably bladder and colon cancers), and NAT2 polymorphism associated with some drug-induced diseases such as lupus erythematosus (hydralazine, procainamide), Stevens-Johnson or Lyell syndromes (sulfonamides).

Significant interethnic and geographic differences in NAT2 activity have been found. Slow acetylators represent 40–70 % Caucasians and 10–20 % Asians. High acetylation capacity has been reported in 5 % Caucasians (Meyer 1997).

Probe drugs for NAT-1 phenotyping is PAS, and for NAT-2 phenotyping are caffeine, sulfamethazine, procainamide and isoniazid or dapsone. In vivo testing for NAT2 has been proved to be useful for drug monitoring to avoid potential side effects generally observed in slow metabolizers (the exception was the anticancer agent amonafide, with myelotoxicity observed in rapid acetylators). The most used test to identify rapid and slow acetylators is the caffeine test, which is described thereafter, though the N-acetylation step takes place after the N-desmethylation of caffeine by CYP1A2 followed by the biotransformation into an unstable intermediate.

PROCEDURE

Phenotyping: Caffeine is metabolized by CYP1A2, NAT2 and xanthine oxidases. The methods could involve the measurement of 5-acetyl-formylamino-3-methyluracil (AFMU), 5-acetyl-amino-3-methyluracil (AAMU, degradation product of AFMU), 1-methylxanthine (1MX) and 1-methyluric acid (1MU) in 0–8, 0–12, 0–24 h urine of subjects orally given 200 mg or 2–3 mg/kg caffeine after a xanthine-free regimen.. The common metabolic ratio used is AFMU/1MX, but the AFMU/(AFMU+1MX+1MU) is more discriminating (Relling 1992; Rostami 1995) and has to be used when intake of xanthine-oxidase inhibitors (Fuchs 1999). Other ratios such as AFMU/(1MX+1MU), or AAMU/1MX, AAMU/(AAMU+1MX+1MU) have been validated (Tang 1991; Nyeki 2002).

The most common methods to assay caffeine and its metabolite in urine used HPLC with UV detection (Grant 1984; Krul 1998b) or mass spectrometry (Baud-Camus 2001).

Genotyping: Mutations of NAT2*5, NAT2*6, NAT2*7, NAT2*14 and NAT2*17 alleles are associ-

ated with a slow acetylation phenotype for homozygous subjects (Butcher 2002).

There are large differences among ethnic groups regarding alleles frequency. High frequency (> 28 %) of NAT2*5 alleles has been observed in Caucasians and Africans, and of NAT2*7 in Asians (> 10 %) and of NAT2*14 in Africans (> 8 %), this last one being <1 % in Caucasians and Asians (Meyer 1997).

EVALUATION

Caffeine test: subjects with a AFMU/1MX ratio < 0.55 or a AFMU/(AFMU+1MX+1MU) ratio < 0.26 are slow acetylators (Fuchs 1999). Higher activity has been observed in black as compared to white subjects (Relling 1992), and a gender effect has generally not been observed (Kashuba 1998).

CRITICAL ASSESSMENT OF THE METHOD

Depending on the probe drug used and on the experimental method, 2 or 3 acetylator types can be described: slow, intermediate and rapid, the intermediate one being not always distinguished from the rapid one. Phenotype distribution has been considered as a continuous variable (Meisel 2002). Due to slow post-natal maturation of the acetylation enzymatic systems, the acetylation status is evolving in newborns and infants, and depends on the probe drug used (Rane 1999).

Good relationships between genotyping and phenotyping tests have been reported (Meisel 1997; Kita 2001).

The urinary caffeine test is not based on assays of specific substrates and products of NAT2 ("including" other metabolism pathways involving at least xanthine-oxidases), and is affected by diet habits, xanthine-oxidase inhibitors such as allopurinol (Fuchs 1999), or other drugs (Klebovitch 1995). NAT activities are affected by anti-inflammatory drugs. Of note, acetominophen is an inhibitor of NAT2 in vivo (Rothen 1998).

Discordances between caffeine and dapsone phenotyping data, and between NAT2 phenotyping status and genotyping have been observed in acutely ill patients infected with HIV (O'Neil 2000), which may be due partly to non-detection of rare NAT2 alleles (Alfirevic 2003).

MODIFICATIONS OF THE METHOD

Some recent references for other used NAT2 phenotyping tests can be found for dapsone in Alfirevic (2003), O'Neil (2000), Queiroz (1997), for sulphamethazine in Hadasova (1996) and Meisel (1997), and for procainamide in Okumura (1997) and Mongey (1999).

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II.T.2.2 Uridine Diphosphate Glucuronosyltransferases

Glucuronidation is a potent detoxification pathway. The uridine diphosphate glucuronosyltransferases (UGTs) are involved in the biotransformation of endogenous substances (bilrirubin, biliary acids, steroid hormones) and numerous drugs and carcinogens. Currently 20 functional UGTs have been characterized with activity mainly expressed in the liver and GI tract. There are 3 subfamilies: UGT1A, UGT2A and UGT2B, with distint but broad overlapping substrate specificity existing for the different isoforms of each family. UGT1A1 is the most abundant UGT in the liver. Human diseases related to deficient UGT1A1 alleles are the well characterized inherited unconjugated hyperbilirubinemias, including the Gilbert's syndrome which affects 6–12 % of Caucasian subjects. Exhaustive reviews on roles, tissue patterns of expression and pharmacogenomics of UGTs can be found in papers from Tukey & Strassburg (2000), Fischer et al. (2001), Guillemette (2003) and Wells et al. (2004).

A decreased clearance has been observed for some drugs metabolized by glucuronidaion in patients with Gilbert's syndrome. A clinically significant impact of UGT polymorphism has to date be only demonstrated for some anticancer agents: clearly for irinotecan, and with contradictory results for flavopiridol (Zhai 2003). UGT1A1 and UGT1A9 are involved in the glucuronidation of the active metabolite (SN-38) of irinotecan. The presence of the deficient UGT1A1*28 variant (most frequent variant as compared to UGT1A9 variants) has been clinically linked to a decrease in SN-38 glucuronidation rate and to an increased occurrence of serious side effects, mainly severe diarrhea and neutropenia (Ando 1998; Innocenti 2004; Iyer 2002; Paoluzzi 2004). Variants of UGT1A7 were reported to affect SN-38 glucuronidation but only in vitro (Villeneuve et al. 2003). Other factors, such as polymorphism in drug transporter P-glycoprotein and renal excretion-may play a role in the complex disposition pattern of irinotecan.

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II.T.2.3 Methyltransferases

There are at least 4 enzymes catalyzing S-, N- and O-methylation using S-adenosylmethionine, but only thiopurine methyltransferase (TPMT) polymorphism has been found to have important clinical consequences. TPMT is involved in the metabolism of mercaptopurine, azathioprine and thioguanine. About 0.3 % of Caucasian subjects have no detectable enzyme activity and 10 % intermediate activity (McLeod 2001). Three alleles TPMT*2, TPMT*3A and TPMT*3C account for 95 % of intermediate or low enzyme activities, resulting in high risk for severe potentially life-threatening hematopoietic toxicity with treatment by the above mentioned drugs. Patients with 2 nonfunctional variant TPMT alleles should receive 5–10 % of drug standard doses. TPMT genotyping has proved its usefulness in individualizing mercaptopurine dose in patients, and can replace the phenotyping test: measurement of the erythrocyte enzyme activity, based on the in vitro conversion of 6-mercaptopurine to 6-methylmercaptopurine or 6-thioguanine to 6-methylthioguanine (Innocenti 2002; Evans 2004). A cut-off concentration of 45.5 thioguanine nmol/gHb.h^{-1} for this TPMT phenotyping test has been proposed for assessing the need of the genotyping test (Wusk 2004).

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II.T.2.4 Glutathione S-Transferases and Sulfotransferases

Glutathione and sulfatation conjugations are important pathways for generally detoxifying endogenous substrates and xenobiotics (Commanndeur et al. 1995). However, some produced metabolites (ie mercapturic acids, O-sulfo conjugates) are toxic by different mechanisms, often by reaction with DNA and other cellular nucleophiles.

Eight classes of glutathione-S-transferases (GST) have been described. The role of the glutathione pathway and the impact of enzyme polymorphism have been highlighted for detoxification and some disease susceptibility, and routine phenotyping of some GSTs exists for clinical safety measurement, but currently there is not yet evidence of genotyping or phenotyping usefulness for drug dosage adjustment (Hayes and Strange 2000; Tetlow et al. 2004).

Soluble sulfotransferases are involved in the sulfonation of endogenous substrates (notably steroids, neurotransmitters, eicosanoids) and numerous xenobiotics (ie acetaminophen, organic-platin anticancer agents). The presence of some sulfotransferases variants could be associated with some cancer risk. Phenotyping tests have been developed for some forms (SULT1A, SULT1A3) by measuring platelet sulfotransferase activity (Glatt and Meinl 2004).

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Chapter II.U
Typical PK/PD Approaches

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INTRODUCTION

Pharmacokinetics (PK) is generally thought as what the body does to the drug, whereas pharmacodynamics (PD) is thought of as what the drug does to the body. When used independently of each other, valuable information can be derived. For example, pharmacokinetic characterization of a drug can quantitatively describe the drug concentration versus time profile and modeling of this profile can result in determination of key pharmacokinetic parameters such as half-life, volume of distribution, clearance, etc. Similarly, pharmacodynamic characterization of a drug quantitatively describes the modulation of a given effect as a function of time. When PK and PD are used in concert, descriptive information on concentration versus effect relationships can be obtained (Levy 1964, 1966, 1994a, b). Knowledge derived using PK/PD relationships or exposure/response relationships can be used objectively to support many drug discovery and development decisions. Development of a PK/PD model can enable the conduct of deterministic or stochastic simulations to be performed to support these decisions. Some of these include, but not necessarily limited to, dose selection, effect of exposure alteration on desired pharmacological response, defining target effect concentration margins, and prediction of response for a given concentration/exposure. Given the tremendous significance of PK/PD in drug development, it is not surprising that the US Food and Drug Administration issued a regulatory guidance on the application of exposure/response relationships in 2003.

In simple terms, there are four primary pharmacodynamic models that have been widely used. These are as follows:

(1) Linear model

The linear model is defined as $E = E0 + SC$, where E is the effect at concentration C, E0 is the baseline effect, and S is the slope of the line that defines E versus C.

(2) Log-linear model

This model is defined as $E = S \text{ Log } C + I$, where E is the effect at Log C concentration, S is the slope, I the intercept of the line that defines E versus C.

(3) E_{max} model

The E_{max} model is defined as $E = (E_{max}.C)/EC_{50} + C$, where E_{max} is maximum effect, C the concentration, and EC_{50} the drug concentration that results in 50 % of the maximum response.

(4) Sigmodial E_{max} model

This model is defined as $E = (E_{max} * C^{\gamma}) / (C^{\gamma} + EC_{50}{}^{\gamma})$, where E_{max} = E when concentration, C, approaches infinity. EC_{50} is drug concentration that results in 50 % of the maximum response. This model is characterized by a sigmoidal shape and γ is the degree of sigmoidicity in effect versus concentration. When $\gamma = 1$, the model reduces itself to the E_{max} model. The values of γ may vary depending upon receptor/affector attributes.

The E_{max} model and the sigmoidal E_{max} model and their variations are typically used PK/PD approaches in drug discovery and development in cases where the equilibration between plasma and biophase is not problematic. As a variation of this approach, drugs which exhibit delays in response can be modeled using a hypothetical effect site compartment as an intermediate between time course of plasma concentrations and drug effects (Sheiner 1979). In particular, the EC_{50} provides invaluable guidance to drug discovery programs in characterizing the target therapeutic range for new drug entities. Consequently, this chapter will primarily focus on these two models. Apart from these

elementary models, there are several new and complex PK/PD models that are available to the drug discovery scientist that have specific applications in complex pharmacological cases (Rohatagi et al. 2004).

In order to illustrate the practical applications of PK/PD modeling, two case studies are presented in the following sections. In the first case study, the application of PK/PD modeling as applied in preclinical development is presented and in the second case study, the application of PK/PD modeling as applied in clinical development is presented.

II.U.1 Case Study #1: Preclinical PK/PD Example

PURPOSE AND RATIONALE

Aggregation of platelets is an important phenomenon in the pathogenesis of arterial thrombosis. Plaque rupture with subsequent platelet activation and thrombosis is a hallmark of acute ischemic syndromes of unstable angina and acute myocardial infarction. Platelet aggregation can be inhibited by several pharmacological mechanisms. For example, aspirin inhibits the cyclooxygenase-dependent formation of proaggregatory prostaglandins. Ticlopidine and clopidogrel block the adenosine diphosphate (ADP)-mediated activation of platelets. However, various pathways of platelet activation converge in a final common event, which is the surface expression of glycoprotein (GP) IIb/IIIa receptors and their cross-linking with soluble fibrinogen. During aggregation, platelets form a complex with soluble fibrinogen by their integrin receptors. The platelet GPIIb/IIIa complex is the recognized receptor that constitutes the final pathway in aggregation. Pharmacologic blockade of the GPIIb/IIIa receptors, therefore, offers an effective means of achieving anticoagulation, irrespective of the mode of platelet activation. In search of a safe and effective GPIIb/IIIa receptor antagonist it is important to evaluate the pharmacokinetics of preclinical compounds in animals followed by the assessment of their pharmacodynamics. The following example with a GPIIb/IIIa receptor antagonist (UM-203) demonstrates how one can use animal PK and PD information to design dosing regimens for efficacy evaluation in animals prior to selecting a candidate for first-in-man studies.

PROCEDURE

Pharmacokinetic Evaluation

Based on the physicochemical properties, UM-203 was intended for development as a parenteral formulation with a moderate PK half-life such that in the event of increased bleeding, the risk could be managed clinically by simply altering the infusion rate. To examine the intravenous pharmacokinetic profile, male, beagle dogs were chosen because this species was also used for the pharmacological models. UM-203 was administered as an intravenous bolus in varying doses of 0.1, 0.3 and 1 mg/kg. For pharmacokinetic assessment, blood samples were obtained at 0 (predrug), 2, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min after UM-203 administration.

Table 1 Mean plasma concentration – time profile of UM-203 after intravenous administration to beagle dogs.

Time (min)	Plasma concentration (ng/ml) 0.1 mg/kg, iv	0.3 mg/kg, iv	1 mg/kg, iv
2	457.9	1919.6	5251.5
15	136.8	627.0	1404.7
30	86.0	455.7	871.9
45	77.5	418.6	790.8
60	73.6	393.4	752.1
90	67.2	348.8	687.0
120	61.4	309.4	627.9
180	51.3	243.4	524.5
240	42.8	191.4	438.1
300	35.8	150.6	365.9
360	29.9	118.5	305.6
420	25.0	93.2	255.3
480	20.8	73.3	213.2

The first step in performing PK modeling is to graph the plasma concentration versus time profile to examine the shape of the curve and to get some preliminary ideas whether the data would fit a one-, two- or a three-compartment PK model. From the semi-logarithmic plot (Figure 1), it was obvious that the compound exhibited either two- or three-compartment kinetics.

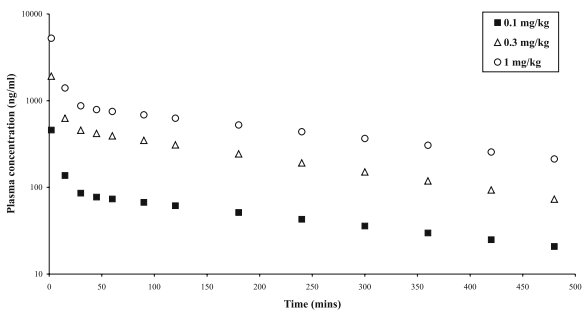

Fig. 1. Semi-logarithmic plot of observed Concentration versus Time

Based on this information, WinNonlin™ (version 3.3, Pharsight Corporation.) was used to generate a PK model. The use of compiled PK models offers good flexibility to perform re-analysis with different

Table 2 PK model diagnostics.

Model 7 Diagnostics							
Dose	CSS	SSR	S	DF	R2	AIC	SBC
0.1	158607	0.006	0.027	9	1.00	−57.82	−55.56
0.3	2780000	0.008	0.030	9	1.00	−54.30	−52.04
1	21200000	0.006	0.027	9	1.00	−57.80	−55.54
Model 18 Diagnostics							
Dose	CSS	SSR	S	DF	R2	AIC	SBC
0.1	158607	0.006	0.028	8	1.00	−53.82	−50.43
0.3	2780000	0.006	0.025	9	1.00	−55.11	−51.72
1	21200000	0.006	0.026	9	1.00	−53.95	−50.56

Table 3 PK parameters for PK Model 7.

Primary parameters								
Dose (mg/kg)	V1 (ml/kg)	K10 (1/min)	K12 (1/min)	K21 (1/min)				
0.1	170.04	0.018	0.110	0.025				
%CV	0.01	0.04	0.03	0.04				
0.3	119.99	0.018	0.119	0.037				
%CV	0.00	0.01	0.01	0.01				
1	144.93	0.020	0.119	0.023				
%CV	0.00	0.00	0.00	0.0				
Secondary parameters								
Dose (mg/kg)	AUC (ng.min/ml)	T1/2α (min)	T1/2β (min)	A (ng/ml)	B (ng/ml)	C0 (ng/ml)	CL (ml/min/kg)	Vss (ml/kg)
0.1	32665	4.6	230.9	500.1	88.0	588.1	3.06	918.0
%CV	0.03	0.03	0.05	0.01	0.02	0.01	0.03	0.02
0.3	136764	4.1	173.3	2000.1	500.0	2500.2	2.19	502.3
%CV	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.00
1	337495	4.3	231.1	6000.0	900.0	6900.0	2.96	880.0
%CV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

models and to compare the fits. For this example, Model 7 (IV bolus, 2-compartments, first-order elimination and micro-constants as initial estimates) and Model 18 (IV bolus, 3-compartments, first-order elimination and macro-constants as initial estimates) were used and are depicted in Figure 2.

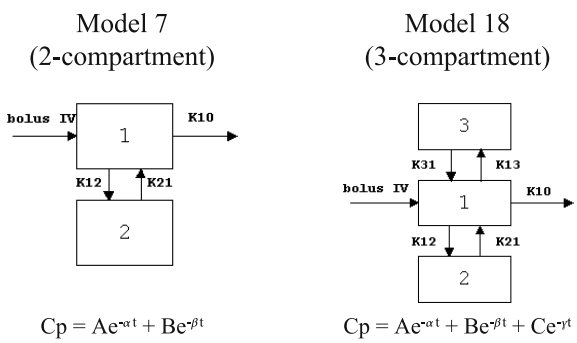

Fig. 2. PK Models. 1 = Central or plasma compartment. 2 & 3 = Second and/or third compartment where the compound distributes and is in equilibrium with the central compartment. K12, K21, K13 and K31 = inter-compartmental transfer rate constants. K10 = elimination rate constant.

All data was fitted to both models using uniform weighting and standard algorithms specified in WinNonlin for compartmental analyses. Both models fit the data well and this is evident from the model diagnostics (Table 2).

Although both models could be valid theoretically, Model 18 does not constitute a significant benefit over Model 7 in terms of AIC (Akaike Criteria) and SBC (Schwarz's Bayesian Criteria). In principle, any model should be practical and as simple as possible. UM-203 has its target within the central compartment, i.e., the platelets. Therefore, one could visualize the compound distributing between two compartments within the blood, i.e., plasma and platelets. For this reason, Model 7 was selected for further analyses. The final parameters from Model 7 are listed in Table 3.

Model 7 fitted data are depicted in Figure 3 for all 3 doses.

Pharmacodynamic Evaluation

For pharmacodynamic evaluation, blood samples were obtained at 0 (predrug), 30, 60, 120, 240, 360, and

Table 4 Time course data for platelet aggregation and plasma concentration.

Time (min)	0.1 mg/kg, iv Cp (ng/ml)	% Inhibition	0.3 mg/kg, iv Cp (ng/ml)	% Inhibition	1 mg/kg, iv Cp (ng/ml)	% Inhibition
0	0	0	0	0	0	0
30	86.0	50	455.7	97	871.9	100
60	73.6	45	393.4	96	752.1	99
120	61.4	30	309.4	94	627.9	98
240	42.8	20	191.4	85	438.1	98
360	29.9	13	118.5	69	305.6	94
480	20.8	6	73.3	55	213.2	92

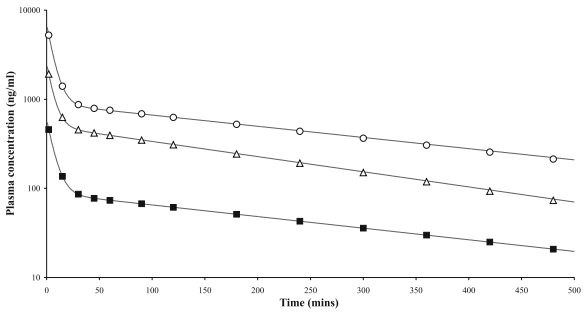

Fig. 3. Semi-logarithmic plot of fitted concentrations

480 min after UM-203 administration. The pharmacodynamis of platelet inhibition was assessed by ex vivo measurement of ADP-induced platelet aggregation and the data obtained is shown in Table 4.

To investigate the pharmacodynamics of the antiplatelet effects of UM-203, the observed platelet-inhibition and measured plasma concentration data from all the dose groups were pooled and graphed as Concentration versus Effect (% inhibition of platelet aggregation). The shape of the graph indicated that a E_{max} model could be used to describe the data. Since platelet inhibition is zero at time zero (when concentration is zero), a simple E_{max} model without baseline effect was used. Similar approach as PK modeling was followed. Two compiled models for E_{max} were used from WinNonlin^{TM} (version 3.3, Pharsight Corporation). Data was fitted to a Simple E_{max} model (Model 101, Effect C = 0 at 0, C = infinity at E_{max}) and a Sigmoid E_{max} model (Model 105, Effect C = 0 at 0, C = infinity at E_{max}).

Model 101 equation (Simple E_{max}): $E = (E_{max} * C)/(C + EC_{50})$

Model 105 equation (Sigmoid E_{max}): $E = (E_{max} * C^{\gamma})/(C^{\gamma} + EC_{50}{}^{\gamma})$

where E is the effect (% platelet inhibition) at plasma concentration C, E_{max} is the maximal effect (% platelet inhibition), IC50 is the plasma concentration of UM-203 that produces 50 % of the maximal inhibition, and γ is the Hill coefficient, which measures the steepness of curve. Uniform weighting and standard algorithms specified in WinNonlin were used to obtain least-squares estimates of E_{max}, IC50 and γ.

Table 5 PD model diagnostics.

Parameter	Model 101 Value	%CV	Model 105 Value	%CV
E_{max}	123	4.92	100	1.49
EC_{50}	119.6	15.57	80.23	3.19
γ	–	–	2.1	7
CSS	31473		31473	
SSR	1180		167	
AIC	152		113	
SBC	152		116	

Model diagnostics (Table 5) indicated that Model 105 was better than Model 101 in terms of goodness-of-fit because the error associated with estimation of parameters was small and the AIC/SBC values were also relatively smaller. Based on Model 105 the observed data were fitted as shown in Figure 4.

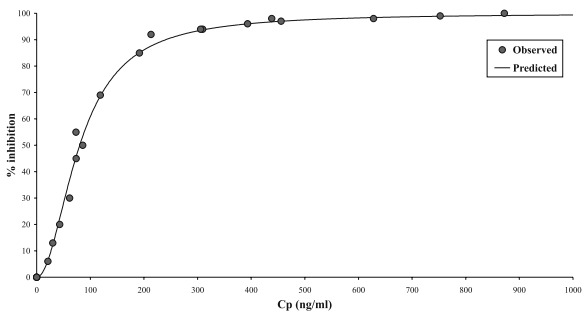

Fig. 4. Plot of observed versus predicted effect

Establishing Relationship between PK and PD Models
Given the mechanism of action of UM-203, it was evident that the effect compartment for the compound is in

the central compartment (platelets), which was in direct equilibrium with the plasma compartment. It was also known that UM-203 bound to the platelet GPIIb/IIIa receptors in a reversible and time-independent manner. Therefore, at any given time the Effect could be directly correlated with the plasma concentration and no time lag in effect could be anticipated. Based on these assumptions, the PK and PD could be directly linked as shown in Figure 5.

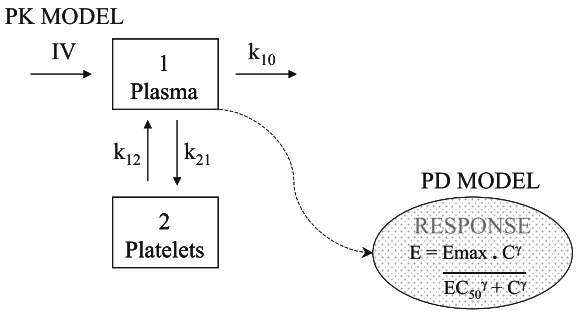

Fig. 5. Combined of PK/PD model

EVALUATION

Since there is a direct link between the Effect and plasma concentration, such PK/PD model could be used for generating duration of effect curves after either bolus dosing, infusion or a combination of bolus plus infusion. To do this, the plasma concentrations from the PK profile were used as input values to arrive at the corresponding Effect values such that in the end, one would have a Effect versus Time profile. For example, a bolus dose of 0.1 mg/kg could result in a brief period of platelet inhibition followed by rapid decline of the effect below the threshold level that is required for pharmacological efficacy. On the other extreme, a bolus dose of 1 mg/kg could completely inhibit platelets for ~ 6 hours, but such effect could cause excessive bleeding (Figure 6).

Alternatively, one could use such modeling results to also predict duration of effect following therapeutic dosing regimens. For UM-203, the goal was to design dosing regimens for efficacy evaluation such that the starting dose would provide an immediate and sustained level of the compound in plasma (80 ng/ml, EC_{50}), which would maintain 50 % platelet inhibition for a period of 4–8 hours. For this trial, using the 2-compartment kinetic parameters and standard equations, the plasma concentrations following a bolus, infusion and bolus plus infusion were calculated using Microsoft Excel. The corresponding Effect for these concentrations was then predicted using the PD model in WinNonlin. This could also be done in Microsoft Excel because the PD equation and parameter values are known. The results of this trial are depicted in Figure 7. Only bolus administration would provide a minimum of 50 % platelet inhibition for ~ 25 min. A continuous infusion would need considerable time to reach the 50 % level of platelet inhibition. In contrast, a combination of bolus plus infusion was predicted to provide a sustained and target level of platelet inhibition for up to 8 hours.

Based on the above bolus plus infusion regimen, multiples were estimated and tested for efficacy in

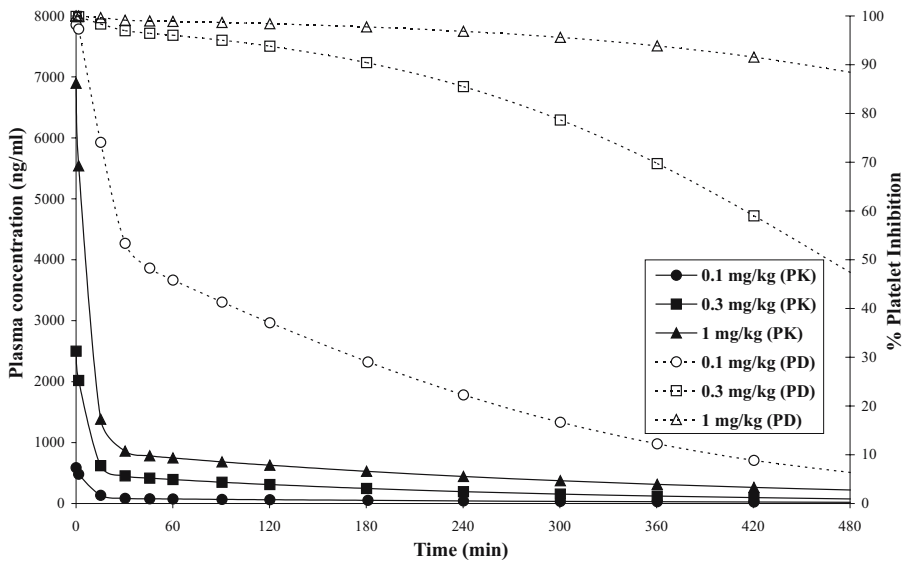

Fig. 6. Predicted concentration and effect profiles after bolus dosing

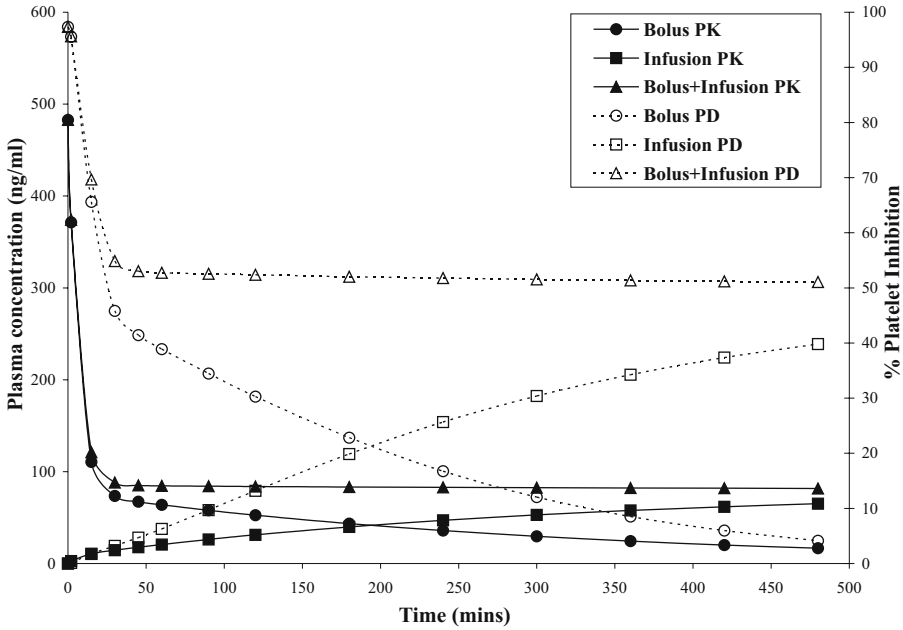

Fig. 7. Predicted duration of effect for various dosing regimens. Bolus = 70 μg/kg and Infusion = 0.2 μg/kg/min

a canine model of arterial thrombosis. In this model thrombus formation was induced by electrolytic injury to the endothelium of the arterial wall (carotid artery) and continuous monitoring of blood flow assessed occlusion of the blood vessel. The results indicated that the 70 μg/kg + 0.2 μg/kg/min regimen was sub-therapeutic (50 % platelet inhibition) and the higher multiples of this regimen was pharmacologically effective in preventing occlusive thrombus formation for the duration of the infusion. Once the infusion was terminated, the compound decayed in a bi-exponential fashion and the effect paralled the plasma concentrations.

CRITICAL ASSESSMENT OF THE METHOD

Such E_{max} model has been applied to examine decrease in heart rate by propranolol (Lalonde et al. 1987) and anticonvulsant effect of phenobartibal (Dingemanse et al. 1989). It could also be used to relate other effects such as blood pressure, protein phosphorylation, blood flow variations, etc. to the plasma concentrations. In conclusion, early PK/PD approach offers better understanding of the drug target and could be used to predict effective and safe dosing regimens for preclinical evaluation. Moreover, early knowledge of PK/PD relationships could serve as a basis for first-in-man dose selection and the PK/PD relationship could be further validated during the development phase of the compound. This could not only reduce the cycle time for drug development but also reduce the costs associated with clinical trials.

II.U.2 Case Study #2: Clinical PK/PD Example

PURPOSE AND RATIONALE

Pharmacokinetic/pharmacodynamic modeling and subsequent simulations may be applied to answer key questions during the drug development process. This example shows that a PK/PD approach could be used to determine whether reduction in exposure of a given drug by 25 % due to an interacting variable results in any clinically relevant effect.

Drug X when administered with food resulted in an approximately 25 % reduction in exposure. The approach used involves the development of a population PK/PD model and use of clinical trial simulations to predict an outcome of a virtual trial.

PROCEDURE

Creation of a Dataset

A data structure was created by combining PK and PD data from three clinical studies. In double-blind, randomized, placebo-controlled, parallel-group, 2 week Phase III studies performed in the target patient population, sparse blood samples were collected for population pharmacokinetic analysis (Table 6).

Table 6 Summary of pharmacokinetic data in the presence of an interacting variable at different doses.

Study Number	Sample Size	Dose (mg)	% Coadministered Condition / Single Treatment Ratio	
			AUC (90% CI)	Cmax (90% CI)
1	24	×	83 (75, 92)	89 (79, 100)
2	24	1.2×	76 (69, 84)	75 (67, 85)
3	22	1.5×	85 (75, 96)	86 (73, 102)
4	21	2.5×	79 (68, 91)	80 (64, 100)
5	23	1.2×	59 (51, 70)	57 (47, 69)

These studies evaluated the safety and efficacy of five dose strengths, three BID regimens and one QD regimen (including BID and QD for one dose level). Individual recorded 24-hour scores for baseline and for 14 day post-baseline determinations. For BID doses, 24-hour scores were calculated as an average of two 12-hour score responses between PM doses. The clinical efficacy measure was symptom score: 0 (absent, symptom not present), 1 (mild, symptom was present but not annoying/troublesome), 2 (moderate, symptom was frequently troublesome but did not interfere with either normal daily activity or sleep), 3 (severe, symptom was sufficiently troublesome to interfere with normal daily activity or sleep), 4 (very severe, symptom was so severe as to warrant an immediate visit to physician). Scores were calculated for four such measures and combined with score ranging from 0 to 16. The observed total symptom score (TSS) data are presented in the Figure 8.

Technical Details

In the modeling process, data points with weighted residuals greater than 5 or less than –3 were inspected for possible data errors. The parameter estimates and model goodness-of-fit were compared in the absence and presence of outlier data. Covariate modeling was performed using a stepwise backward-elimination technique. The software used for analyses was NONMEM, version V, level 1.1 with NM-TRAN version III, level 1.0 and PREDPP version IV, level 1.0. A first-order conditional estimation method with eta-epsilon interaction was used. The likelihood ratio test for nested model used an a priori defined significance level of $p < 0.005$ at one degree of freedom. Goodness-of-fit

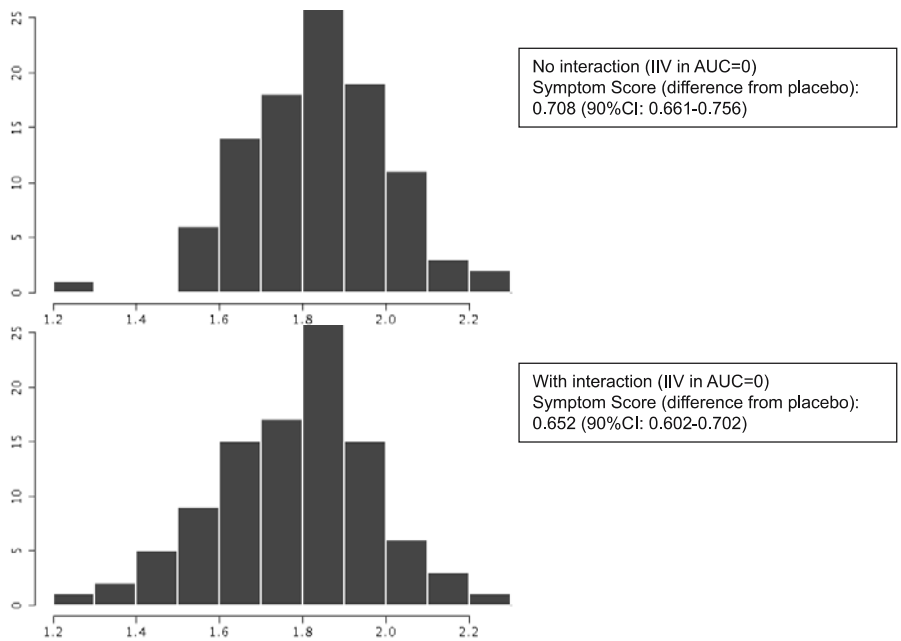

Fig. 8. Observed symptom score vs. time (days) data across various treatments (placebo [PLB] and doses, ranging from x to 6x mg)

Table 7 Summary of models evaluated to examine the relationship between Drug X exposure (AUC) and response (Total symptom score).

Model	Description	No. of Parameters		Delta Objective function*
		Fixed	Random	
1. Placebo effect	$TSS = [E_0 - (PL_1 + PL_2 \cdot DAY)]$	3	4 (3 IIV) (1 RV)	Not applicable as this is the base model
2. E_{max} model	$TSS = [E_0 - (PL_1 + PL_2 \cdot DAY)] \cdot \left(1 - \frac{E_{MAX} \cdot AUC}{E_{50} + AUC}\right)$	4	4 (3 IIV) (1 RV)	**34** Significantly better than model 1 at $p < 0.001$ level
3. E_{max} model with time effect	$TSS = [E_0 - (PL_1 + PL_2 \cdot DAY)] \cdot \left(1 - \frac{(D_1 \cdot AUC + PL_2 \cdot D_2 \cdot DAY)}{E_{50} + (D_1 \cdot AUC + PL_2 \cdot D_2 \cdot DAY)}\right)$	6	4 (3 IIV) (1 RV)	**39** Significantly better than model 1 at $p < 0.001$ level; good stability but poor sensitivity
4. Linear model	$TSS = [E_0 - (PL_1 + PL_2 \cdot DAY)] - D_1 \cdot AUC$	4	4 (3 IIV) (1 RV)	**45** Significantly better than models 1, 2 and 3 at $p < 0.001$ level
5. Linear model with covariance between PL_2 and D_1	$TSS = [E_0 - (PL_1 + PL_2 \cdot DAY)] - D_1 \cdot AUC$	4	5 (4 IIV) (1 RV)	**51** Significantly better than models 1, 2 and 3 at $p < 0.001$; better than model 4 at $p < 0.05$ plus increased parameter stability; normality assumption for IIV passed

* For each model, the Delta Objective function was calculated by subtracting the value for Model 1 from the respective model.

and Akaike Information Criteron (AIC) were used to compare non-nested models.

Modeling

Population pharmacokinetic data were pooled and a structural model fitted to pooled data using NONMEM. Individual pharmacokinetic parameter estimates were obtained from the base structural model. Individual steady state AUCs for the 24-hour efficacy score interval were then determined.

Population pharmacodynamic data, i.e., observed 24-hour efficacy scores were modeled as a function of individual predicted 24-hour steady state AUCs. Various pharmacodynamic models were explored including linear, E_{max}, and sigmoidal E_{max} models. Fixed and random-effect parameters were used to describe the PK/PD relationship. The results of the model development are presented in Table 7.

The final PK/PD model developed was defined by the following equation which includes three components, namely, baseline, placebo effect and drug effect:

$$TSS = [E_0 - (PL_1 + PL_2 \cdot DAY)] \cdot \left(1 - \frac{(D_1 \cdot AUC + PL_2 \cdot D_2 \cdot DAY)}{E_{50} + (D_1 \cdot AUC + PL_2 \cdot D_2 \cdot DAY)}\right)$$

where TSS – total symptom score, E_0 – baseline total symptom score, PL_1 – intercept of placebo effect, PL_2 – slope of placebo effect as a function of time (in days), D_1 – slope of drug effect as a function of AUC (24 h steady state), D_2 – slope of drug effect as a fraction of the slope of placebo effect on time, E_{50} – drug effect that results in a 50 % reduction in TSS, AUC – area under the plasma concentration versus time curve over 24 h at steady state calculated from individual predicted CL in the analysis.

Simulation

The expected response data were then simulated using NONMEM (see Appendix for the control files used for simulation). The study design used for this simulation exercise was as follows. Parallel-group, placebo-controlled, efficacy trial consisting of three treatment arms, i.e., placebo, drug X plus food, and drug X alone), n = 900 per trial (N = 300/arm). A hundred trials were simulated. The average of each individual's simulated baseline and 14-day post-baseline 24 hour scores were determined for each trial. For the primary efficacy variable, change from baseline in 7 pm scores were calculated. An ANCOVA model with baseline 7 pm 24-hour score as covariate and treatment as predictor variable was used assuming an interindividual variability of 0 to 40 % CV.

Exposure simulations used the following key considerations: typical values of AUCss, typical value

Table 8 Counts and proportions of simulated trials with p values $\leq 5\,\%$.

Treatment	P-value Group	Total Simulated Trials	Number (%) of Trials
Alone	p-value $\leq$ 0.05	100	97 (97.0 %)
	p-value $>$ 0.05	100	3 (3.0 %)
With IV	p-value $\leq$ 0.05	100	88 (88.0 %)
	p-value $>$ 0.05	100	12 (12.0 %)

Treatment	N	Average P-value (Mean)	Average P-value (SD)	Average P-value (SE)	95 % CI Lower Limit	95 % CI Upper Limit
Alone	100	0.0056	0.0201	0.0020	0.0016	0.0096
With IV	100	0.0302	0.0785	0.0078	0.0146	0.0457

where IV refers to the interacting variable.

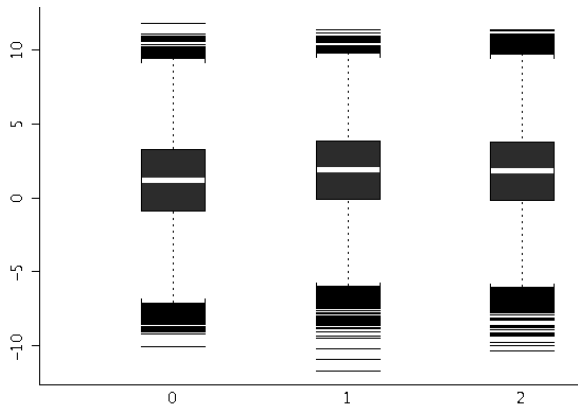

Fig. 9. Change in TSS from baseline for placebo (0), fasted (1), and interacting variable (2) groups

of the ratio of AUC (with/without food), uncertainty in typical value of AUC ratio, and interindividual variability. The typical value of AUC was obtained from a Phase I study and the value for the ratio was defined as 75 %. A prior distribution range of 68 to 96 % was used to describe the parameter uncertainty. These values reflect the 90 % confidence intervals defined in prior clinical studies. Efficacy score simulations used 24-hour symptom scores using a final population PK/PD model and parameter estimates for fixed and random effects. Responses for baseline and 14-day post-baseline assessment periods were simulated.

The results of the simulation are presented in Figure 9, which illustrates the change in TSS from baseline for placebo (0), fasted (1), and interacting variable (2) groups.

The statistical analysis of simulated data is presented in Table 8.

EVALUATION

Symptom score responses were similar whether drug X was administered alone or in combination with interacting variable. A 25 % (± 8 %) mean decrease in overall exposure in the presence of food had no statistically significant effect on symptom scores. A sample size of at least 13 000 subjects/arm would have been needed to reach statistical significance using currently simulated treatment difference of 0.057.

CRITICAL ASSESSMENT OF THE METHOD

The PK/PD model developed here is in accordance to the fundamental principles of pharmacodynamic E_{max} model, wherein effective concentration is dependent on the interval between baseline, E0, and the maximal response. The E_{max} model allows for a baseline effect to be captured by assigning a concentration at 0 time. The method used here highlights the value of modeling and simulation in understanding the relationship between plasma concentrations and symptom scores of the disease. Once the model was developed, it was useful in investigating the magnitude of reduction in efficacy that results when an interacting variable causes a reduction in exposure. The potential impact of the uncertainty in the interacting variable on efficacy was also determined.

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Chapter II.V
Population Pharmacokinetics in Drug Development

Willi Weber
Diether Rüppel

LIST OF ACRONYMS

- CL_{tot} total clearance
- CL_{CR} creatinine clearance
- CONC observed concentration
- C_{ss} concentration at steady-state
- η random inter-individual variability $\eta \sim N(0, \omega^2)$
- HT height in cm
- IPRE model predictions for the individual subject with random η_i
- LBM lean body mass in kg
- MIC minimum inhibitory concentration
- NAD naive averaging data method
- NONMEM nonlinear mixed-effects modeling
- NPD naive pooled data
- OF objective function: negative log of probability, – 2ln(Prob), OFV calculated by NONMEM
- ω^2 covariance matrix describing the between subject variability $\eta_i \sim N(0, \omega^2)$
- PK pharmacokinetics
- PPK population pharmacokinetic
- PRED model predictions for the population with $\eta = 0$
- SEX 1 = male and 2 = female
- σ^2 covariance matrix describing the within subject or residual variability $\epsilon_i \sim N(0, \sigma^2)$
- STS standard two stage method
- θ Vector of parameter, describing the fixed effect model
- V volume of distribution
- WT body weight [kg]

PURPOSE AND RATIONALE

Variability in exposure to a drug leads to variability in the clinical response across a patient population (Rowland et al. 1985). Estimating the variability of the PK (pharmacokinetics) across a patient population requires data obtained from a large study, typically including more than 100 patients. For ethical and practical reasons, pharmacokinetic properties of a drug are difficult to study in large numbers of patients using the traditional approach.

The PPK (population pharmacokinetic) approach was suggested by Sheiner et. al. (1977) for investigating the typical PK of a drug in a large target population using sparse and unbalanced data obtained without any additional cost during routine care of patients. The PPK approach aims to quantitate the effect of various physiologic factors on drug PK with the overall goal of explaining as much variability as possible.

Using the PPK approach in the development of a new drug has the advantage that the relevant pharmacokinetic parameters for a reasonably large population can be obtained from only a few blood samples per subject. The PPK approach is the method of choice in all situations when only sparse and unbalanced data can be obtained. This situation exists when the PK needs to be studied in elderly, critically ill and pediatric patients, but also very often in preclinical studies investigating the effects of the drug in animals.

Once such a mathematical model is available, the concentration time courses for various scenarios of administration can be predicted. The dosage can be adjusted to achieve a specific clinical goal like drug exposure within the therapeutic concentration window in the whole population or, if necessary, for special sub-populations characterized by their individual physiology. Following the learning and confirming approach (Sheiner 1997), the predicted clinical success for these optimized dose regimens needs to be confirmed in the next clinical study.

The PPK approach estimates the joint distribution of population specific pharmacokinetic model parameters for a given drug. Fixed effect parameters quantify the relationship e.g. of clearance to individual physiology like function of liver, kidney, or heart. The volume of distribution is typically related to body size. Random effect parameters quantify the inter-subject variability which remains after the fixed effects have been taken into account. Then the observed concentrations will still be randomly distributed around the concentration time course predicted by the model for an individual subject. This last error term called residual variability

needs to be estimated. As fixed and random effects are included, this method is called mixed effects modeling.

The essential features of a population pharmacokinetic study are summarized in a guideline (FDA 1999).

PROCEDURE

The NONMEM (nonlinear mixed-effects modeling) software (Beal et al. 1992), mostly used in population pharmacokinetics, was developed at the University of California and is presently distributed by Globomax. For data management, post processing and diagnostic plots, the software S-plus (Mathsoft) is frequently used.

Before starting model fitting, all available information obtained in previous studies should be assembled (prior knowledge). The analysis starts with an exploration of the data to generate hypotheses for the model: a statistical summary of demography, plots of the logarithm of the concentration versus time indicate the number of pharmacokinetic compartments involved. With the help of plots of individual time courses in a common coordinate system, subgroups in the population may be identified. Normalising the curves to unit doses should indicate dose linearity or non linearity.

Prior knowledge and various hypotheses are condensed into models. NONMEM determines the parameter vector including fixed and random effects of each model using the maximum likelihood algorithm. NONMEM uses each model to predict the observed data set and selects the best PPK parameter vector minimising the deviation between model prediction and observed data. Comparing model fits by the criteria discussed in the section "Evaluation" should decide which hypothesis is the most likely. As a general rule, the model should be as simple as possible and the number of parameters should be at a minimum.

The situation after an iv bolus for a system described by a one compartment model with first order elimination can serve to illustrate the procedure. The observed concentration $c_{i,j}$ of an individual i at the time t_j can be modeled as

$$c_{i,j} = \left(\frac{\text{Dose}}{V_i}\right) e^{-k_i t_j} + \epsilon_{i,j} \; ; \quad \epsilon_{i,j} \sim N(0, \sigma^2) \qquad (1)$$

with $k_i = \frac{CL_i}{V_i}$ where CL_i and V_i are the individual clearance and the individual volume of subject i. $\epsilon_{i,j}$ is the residual error drawn from a normal distribution with zero mean and variance σ^2 (covariance matrix describing the within subject or residual variability), the intra individual variability.

The pharmacokinetic parameters themselves are modeled like

$$CL_i = \theta_{CL} e^{\eta_{CL_i}} \; ; \quad \eta_{CL_i} \sim N(0, \omega^2) \; , \qquad (2)$$

where θ_{CL} is a population mean clearance and η_{CL_i} is again a random variable representing the deviation from the population mean of the clearance for the i-th subject. η_{CL_i} is normally distributed with zero mean and variance ω^2 (covariance matrix describing the between subject variability). The unexplained inter subject variability acts as random effect η_{CL_i} on the clearance.

It is important to emphasize that all pharmacokinetic, fixed effect and random parameters, i.e. θ, ω^2, and σ^2, are fitted in one step as mean values with standard error by NONMEM. A covariance matrix of the random effects can be calculated. For a detailed description of the procedure see Grasela and Sheiner (1991) and Sheiner and Grasela (1991).

In a subsequent step the modeler tries to explain part of the unexplained inter individual variability. Fitted individual parameters (or the variable part expressed by η) are plotted against physiological parameters like weight or indicators of renal or metabolic functionality. Identified dependencies should enter into the model. For example clearance is very often modeled as depending on the covariate CL_{CR} (creatinine clearance):

$$CL_i = \theta_{CL} \left(\frac{CL_{CR_i}}{4\text{L/h}}\right)^{\theta_{CL_{CR}}} e^{\eta_{CL_i}} \; . \qquad (3)$$

In this equation CL_{CR_i} is the actual creatinine clearance of subject i. The fixed effect parameters are now θ_{CL} corresponding to the clearance of a person with a CL_{CR} of 4 L/h the and $\theta_{CL_{CR}}$ as an exponent describing the increase of CL_i with CL_{CR}.

The relevance of CL_{CR} for clearance is tested using the likelihood ratio test (Beal et al. 1992). The so called full model (alternative hypothesis) given in equation 3 is tested against the reduced model with $\theta_{CL_{CR}} = 0$ (null hypothesis) characterised by equation 2.

The more complex full model is accepted only if the objective function obtained with the full model is more favourable than the objective function obtained with the reduced model (see Evaluation).

Concentration time courses can be simulated by the model and the demographic parameters for different dose regiments. The final administration of the drug has to be adjusted so that e.g. 95% of the target population falls into the therapeutic window. If sub-populations differ too much, adjusted administration regiments have to be considered.

EVALUATION

The following criteria determine about the best model:

1. The OF (objective function: negative log of probability, –2 ln(Prob)), calculated by NONMEM, is a measure for the deviation between the model prediction and the observed data. It enters into the likelihood ratio test as follows: if the OF of the full model minus the OF of the reduced model is smaller than –3.84, than the full model can be accepted at a significance level of $p < 0.05$ (Beal et al. 1992).
2. the observed concentrations plotted against the predicted concentrations had to be more randomly distributed around the line of unity
3. the weighted residuals, and the individual residuals plotted against the predicted concentrations had to show the most symmetric distribution around zero.

In order to validate the final model, the data set can be split randomly into two parts. The model is developed with one part, the index data set. With this model and the demographic data of the second part, the validation data set, observations for the validation data set can be predicted. The difference of predicted data and observations is a measure of the accuracy of the model. An alternative is the bootstrap method (Efron 1981).

MODIFICATION OF THE METHOD

Data from individuals drawn from a target population are not completely independent. Concentration time curves (longitudinal data) of a subject are considered to be driven by a functionality depending on individual parameter values. But what is the connection between the same parameters in different persons? Parts of it may be described by a functionality depending on demographic variables. In any case, unexplained intra and inter individual random effects remain. Mixed effect modeling clearly distinguishes between these two sources of randomness.

Modifications of the method differ in the way they deal with these different levels of random effects, i.e. how they distribute or confound them. It should be noted that the different handling of random effects has also consequences for the fixed effects.

1. In a situation with many data from each individual drawn in an inter subject balanced manner, a two stage method is very often used: each individual is fitted individually without considering the inter individual dependencies. In a second step, the parameters are resumed as population mean and standard deviation, often considered as inter individual variability. (STS (standard two stage method), (Steimer et al. 1985)).
2. If only a few data per subject is available, they are sometimes pooled and considered as coming from one hyperanimal. If several observations are available at the same time they are averaged and means and standard deviations can be calculated. In a second step the mean values are fitted to a pharmacokinetic model. (NAD (naive averaging data method) (Steimer et al. 1985)). A different naive technique is the NPD (naive pooled data) method proposed by Sheiner (Sheiner and Beal 1980). Again all data are pooled, but fitted in one step to a pharmacokinetic model. In both cases intra and inter individual random effects are confounded. An influence of covariates cannot be determined by this approach
3. Mixed effect modeling deals with the situation in between. Inter and intra individual variability are separated and calculated within the same step. Inter individual random effects are calculated for those parameters for which this information can be drawn from the data set. In general only one residual error is calculated. The method is very well suited for sparse and unbalanced data situations.

Population pharmacokinetics can be extended to pharmacodynamics and PK/PD modeling using a link model like an effect compartment (Sheiner et al. 1979). In huge clinical trials only a limited number of patients can be included in a pharmacokinetic satellite study. The model is developed in this satellite. Knowing the demographic covariates of the patients in the whole study, concentration time curves and even effect time curves can be predicted.

Alternative software like NPEM use nonparametric procedures for the statistical part of the models (Jellife et al. 1990).

CRITICAL ASSESSMENT OF THE METHOD

The NAD and NPD methods confound several sources of variability and very often give biased estimates of the mean values of the pharmacokinetic parameters (Steimer et al. 1985). But when the population is very homogeneous, the naive approaches already give reasonable results. The widespread STS method requires the estimation of a large number of parameters, reducing the degree of freedom and leading to over parametrization (large SEMs).

Mixed effect modeling is a very flexible one step method. It can cope with many situations. It is the only method which can deal with sparse data and

unbalanced data situations. The method can start in preclinical phases with animal data. In phase I with a homogeneous population and many observations per individual, the structural model, dose linearity and bioavailability are determined. In phase II and phase III patients are investigated and the demographic parameters should spread over a large range in order to determine the variability in the target population. The method is well suited to perform meta analysis of several studies.

It should be emphasized that models are not the truth and that different models can describe the same data with the same accuracy. Whereas interpolation for doses or covariates is in general possible, extrapolations should be considered with care. Extrapolation with different models, if available, can give a feeling about the range for the observations to be expected.

Simulations should be used for the design of the next experiment (trial). The new observations should be compared to the prediction allowing improvement of the model in an iterative manner (Sheiner 1997).

EXAMPLE

Introduction

Levofloxacin is the l-isomer of the racemate ofloxacin, a quinolone antibacterial agent used worldwide to treat a wide range of infections. The PK profile of levofloxacin was first characterized in healthy volunteers. The following prior knowledge was obtained before the clinical study presented below. Levofloxacin is primarily excreted renally. Increasing doses of levofloxacin showed linear PK over the investigated dose range between 50 mg and 600 mg. The PPK of levofloxacin used in patients with respiratory tract infections was investigated by Tanigawara et al. (1995).

Objective

Can 500 mg levofloxacin given twice daily achieve the therapeutic goal of plasma levels above 2 mg/L, the MIC (minimum inhibitory concentration) in male and in female patients?

Materials and Methods

The PPK were analysed in a subpopulation of 44 out of 314 patients with pneumonia being treated with levofloxacin. Patients received two daily doses of 500 mg for 10 to 15 days. Initially the drug was given intravenously as an infusion for approximately 60 min. The switch from i.v. to oral treatment was suggested after a minimum of 4 i.v. doses. 3–5 blood samples were taken from each patient, 199 blood samples in total.

The available concentration time data is typical for a clinical study: there are relatively few observations on each of a large number of patients and samples are not taken at the same time points (sparse and unbalanced data). Neither the NAD method nor the STS method can be used. A one compartment model with absorption compartment and first order elimination was fitted to the data by mixed effect modeling with NONMEM. Clearance and volume of distribution were described by

$$\mathrm{CL_i} = \theta_{\mathrm{CL}} \left(\frac{\mathrm{CL_{CR_i}}}{4\mathrm{L/h}} \right) \mathrm{e}^{\eta_{\mathrm{CL_i}}} \tag{4}$$

(equation 3 with $\theta_{\mathrm{CL_{CR}}} = 1$)

$$\mathrm{V_i} = \theta_{\mathrm{V}} \left(\frac{\mathrm{WT_i}}{70\,\mathrm{kg}} \right) (1 + (2 - \mathrm{SEX_i})\theta_{\mathrm{SEX}})\, \mathrm{e}^{\eta_{\mathrm{V_i}}} \ . \tag{5}$$

The model uses $\mathrm{CL_{CR}}$, WT (body weight [kg]) and SEX (1 = male and 2 = female) as covariates.

Alternatively, the volume model was simplified using LBM (lean body mass in kg) instead of WT and SEX. LBM is related to WT, HT (height in cm) and SEX in the following equation:

$$\mathrm{LBM}\begin{pmatrix}\text{male}\\ \text{female}\end{pmatrix} = \begin{pmatrix}1.1\\ 1.07\end{pmatrix}\mathrm{WT} - \begin{pmatrix}128\\ 148\end{pmatrix}\left(\frac{\mathrm{WT\,cm}}{\mathrm{HT\,kg}}\right)^2 \mathrm{kg}\ . \tag{6}$$

The model for V (volume of distribution) given as,

$$\mathrm{V_i} = \theta_{\mathrm{V}} \left(\frac{\mathrm{LBM_i}}{50\mathrm{kg}} \right) \mathrm{e}^{\eta_{\mathrm{V_i}}} \tag{7}$$

needs one parameter, θ_{SEX}, less than the model given in Equation 5. Now the PPK model uses in total only two covariates, i.e. CLCR and LBM.

RESULTS

PK Differences Between Male and Female Patients?

The volume given in Equation 5 as a full model (A) changes with $\theta_{\mathrm{SEX}} = 0$ to a reduced model (B). To perform the likelihood ratio test, both models were fitted with NONMEM and the OF of the full model (A) was 6.39 points lower than the OF obtained for the reduced model (B). This difference is highly significant, so the full model (A) is preferred when compared to a reduced model (B).

WT and SEX are combined in LBM. To simplify the model, we described the fixed effect on V only with LBM as a single measure of body size. Using Equation 7 in model (C), we repeated the NONMEM

Table 1 PK parameters from mixed effect modeling using equations 4 and 7. The absorption coefficient θ_{KA} was fixed to 1/h.

	Parameter	Mean	SEM
fixed effects θ	θ_{CL} [L/h]	5.3	0.3
	θ_{V} [L]	76	5
	θ_{KA} [1/h]	1	fix
random effects ω^2	ω_{CL}^2	0.12	0.02
	ω_{V}^2	0.11	0.04
residual error σ^2	σ^2	0.03	0.01

fit and compared the OF obtained for model (C) with the previous two fits. Model (A) was still 3.1 points better than model (C). We preferred model (C) because it uses only body size while model (A) uses body size and sex as demographic covariate in the V model. Table 1 resumes the values of the PPK parameter vector including θ (Vector of parameter, describing the fixed effect model), ω^2 and σ^2 calculated for model (C).

Concentration-time curves for three individuals with different kidney functions CL_{CR} are shown in Figure 1. The broken lines CL_{CR} represent the time dependent CL_{CR} as a measure of the kidney function. For the subject shown in the center panel, the CL_{CR} decreases at 3.5 days causing a steep increase in the drug concentration. Dots are observations CONC (observed concentration), full lines PRED (model predictions for the population with $\eta = 0$) correspond to the model predictions for a typical individual with a specific set of mean covariates CL_{CR}, WT and SEX (fixed effects). The broken lines IPRE (model predictions for the individual subject with random η_i) are the individual predictions for the subject taking the random effects on volume and clearance into account.

Once the model is in place, simulations can be performed in order to find or to verify the optimal dose regimen.

In Figure 2 mean C_{ss} (concentration at steady-state), given in Equation 8, as a function of CL_{CR} are shown.

$$C_{ss,i} = \frac{\text{dose rate}}{CL_i} \tag{8}$$

The circles correspondent to the observations. The lines are calculated using the fit parameters and Equation 2 for the 2.5%, 50% and 97.5% quantiles. All except two individuals are within these limits. As reveals from Figure 2, the selected dose regimen of 500 mg twice daily achieves even in more than 95% of male and female patients with normal kidney function C_{ss} concentrations above the MIC of 2mg/L.

Figure 3 shows the joint distribution of V and CL_{tot} (total clearance) for males and females as calculated by the model (C). Volume and clearance are distributed around mean values (center of the ellipse) and they are slightly correlated to each other. The 95% contour line of their joint probability of occurrence is shown as ellipses for male and female.

Simulations of the concentration time course under steady state conditions as predicted by the model for a 500 mg twice daily dose regimen are shown in Figure 4. The broad line corresponds to the typical male and

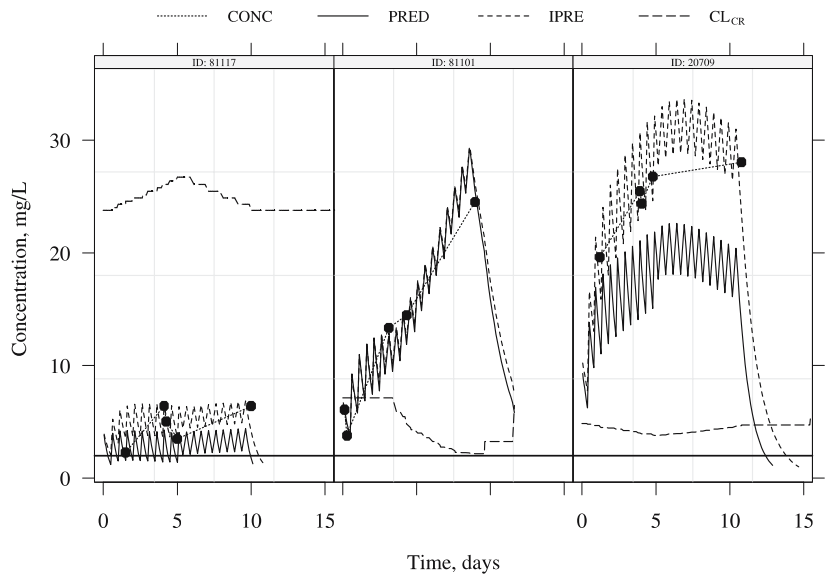

Fig. 1. Individual concentration time courses. CONC: creatinine clearance, PRED: model predictions for the population with $\eta = 0$, IPRE: model predictions for the individual subject with random η_i, CL_{CR}: creatinine clearance in L/h is calculated as left hand scale *10/4

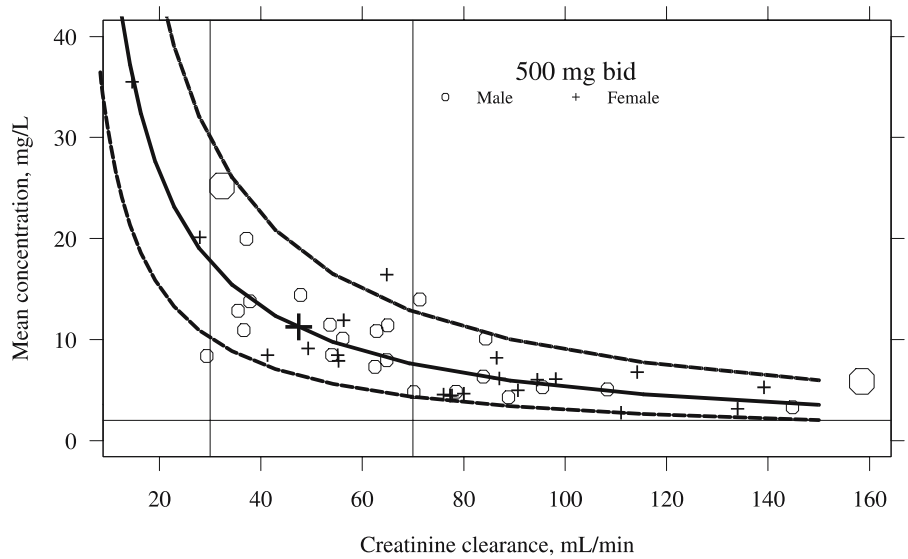

Fig. 2. Mean concentrations at steady state after twice daily 500 mg levofloxacin. The circles and crosses correspondent to the individual CL estimates in male and female patients, respectively. Three filled circles correspond to patients which PK is shown in Figure 1. The lines are calculated using the fit parameters given in Table 1 and Equation 2 for the 2.5%, 50% and 97.5% quantiles. All except two individuals are within these limits. The vertical solid lines mark patients with creatinine clearances of 30 ml/min and 70 ml/min, curves stop at 150 ml/min.

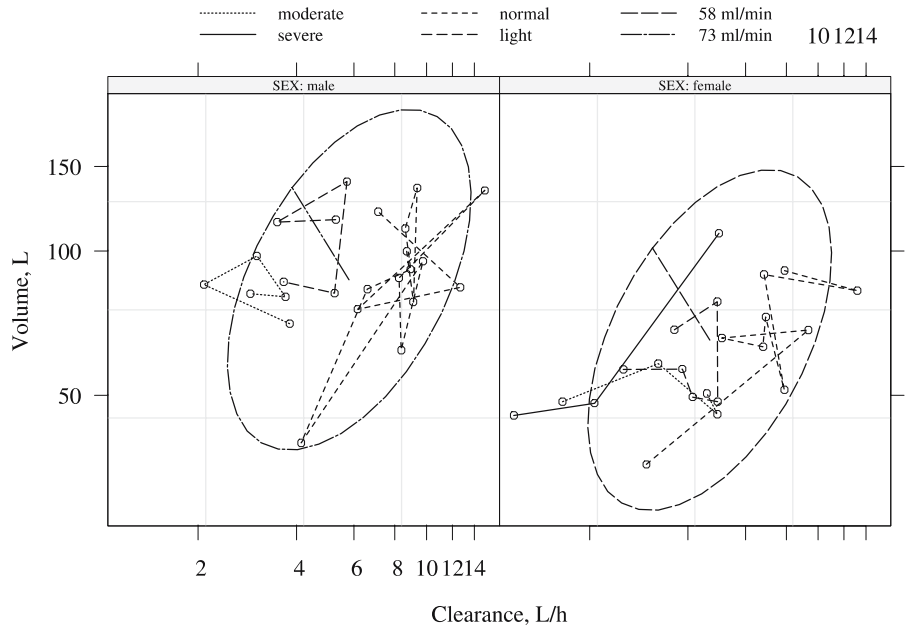

Fig. 3. Joint distribution of V and CL_{tot}: ellipse joint 95 % prediction interval in a subpopulation of male patients: CLCR = 72.53 ml/min & LBM = 57.63 kg and in a subpopulation of female patients: 58.32 ml/min & 43.12 kg. The individual V and CL_{tot} estimates calculated by NONMEM are grouped according to the degree of renal failure.

female patients. All other lines are calculated using only CL and V pairs of the 95% contour line of their joint probability of occurrence as shown in Figure 3 as ellipses. As reveals from Figure 4, concentration time courses remain within an interval describing the concentrations expected in 95% of male or female patients.

DISCUSSION

The PPK approach uses all the data observed at all sampling times and from all subjects enrolled in the satellite study in a single step to extract the information necessary to optimize a dose regimen.

For the example of levofloxacin given twice daily 500 mg, 95% of male and female patients achieved

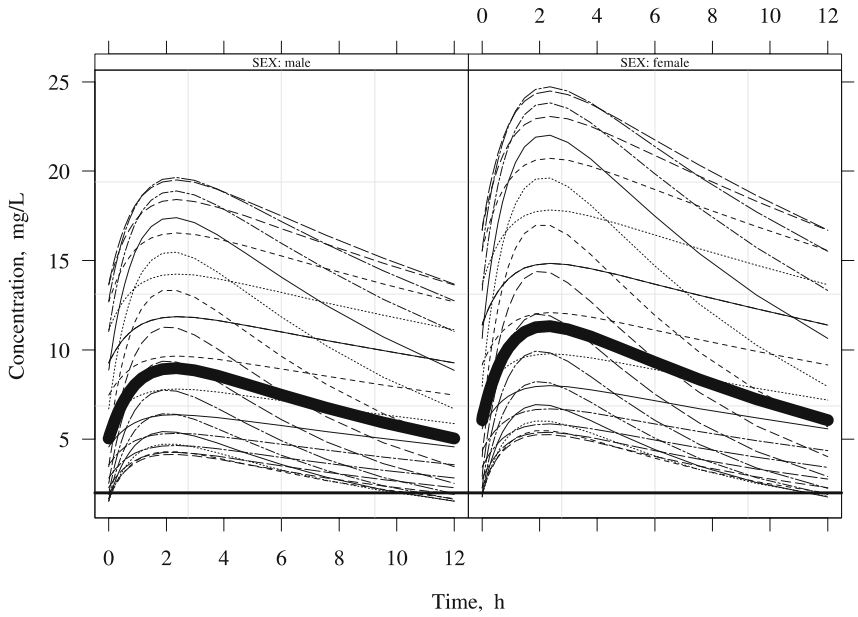

Fig. 4. Simulations of the concentration time course under steady state conditions as predicted by the model (C) for a 500 mg twice daily dose regimen. The broad line corresponds to the typical male (CLCR = 72.53 ml/min & LBM = 57.63 kg) and female (58.32 ml/min & 43.12 kg) patients. All other lines are calculated using only CL and V pairs of the 95% contour line of their joint probability of occurrence as shown in Figure 3 as ellipses. The horizontal solid line marks the MIC of 2 mg/L

the therapeutic goal and showed concentrations above 2 mg/L (MIC) for more than 10 hours of the 12 h dose interval. Due to their smaller volume of distribution, peak concentrations are higher and half-lives are shorter in female patients. The different extent of accumulation as an effect of differences in half-lives become evident when comparing the through levels. The highest concentrations reached are still below the safety limits. Therefore the same dose regimen for male and female patients was recommended.

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Section III
Safety Toxicology

Chapter III.A
Introduction

Dieter Mayer

In continuation of the text book "Drug Discovery and Evaluation, Pharmacological Assays" (Springer-Verlag Berlin Heidelberg New York) first edition by H. Gerhard Vogel and Wolfgang H. Vogel 1997, second edition 2002 by H. Gerhard Vogel, Coeditors: Wolfgang H. Vogel, Bernward A. Schölkens, Jürgen Sandow, Günter Müller, Wolfgang F. Vogel with contributions of authors mainly from Hoechst-Marion-Roussel it was decided to edit a new book entitled "Safety Assays in Drug Discovery and Development". This book covers all areas of modern toxicology (gene toxicity, toxicogenomics, toxicoproteomics) as well as the classical end-points like acute, sub-acute, chronic, carcinogenicity, reproductive toxicology, dermal toxicology, sensitization studies as well as pharmaco- and toxico-kinetic and metabolic methodologies suitable and/or necessary for the discovery and development of drugs under "state of the art" conditions in modern drug development.

Intent of the editors was to include chapters on safety pharmacology, which is part of the preclinical development package. Sometimes it is difficult to reasonably separate safety pharmacology from preclinical toxicology. However, from the editors' point of view it was considered necessary to devote a "stand alone" chapter to pre-clinical safety pharmacology. Safety pharmacology is more a pharmacology-based science than a pure toxicological discipline.

The editors and authors of the different chapters are scientist mainly active in the European pharmaceutical industry with a proven record of many worldwide approvals of new drugs, i.e. they have the practical experience to make a pharmaceutical product out of do a chemical compound.

Like in the former volumes dealing with pharmacological assays this book is intended to aid either the scientists or student. The reader can find methods to select candidates for drug development in early stages such as screening methods in dependence of the stage of development or methods up to advanced stages of development and which are considered necessary for international approval from the scientific and regulatory point of view.

Modern toxicology has, methodologically, experienced major achievements by the introduction of methods derived from molecular biology (toxicogenomics, proteomics) and mostly practiced by biologist. In contrast to that, the classical toxicological assays are relatively "stable" with regard to their methodology and have been modified relatively moderate with some exceptions or additions like immuno-toxicological endpoints.

Hundreds of national and international guidelines have been published, with the aim to "standardize" drug development on the basis of indication, duration and schedule of treatment of patients. However, it is the challenge and responsibility of the scientist to define finally the clinical profile of a drug candidate and to further profile it for a promising development up to approval.

It is the target of research and development to elaborate a strategy, which reaches the approval of an innovative therapeutic principle. It is the responsibility of investigators to contribute in early stages of development to the definition of a "target therapeutic profile" of a new drug.

Like pharmacologists, toxicologists have used methods from other disciplines. In contrast to former times, chemistry, biochemistry and molecular biology as neighboring disciplines play a dominant role in modern toxicology. The application of these sciences has contributed significantly to the elucidation of toxicological mechanisms and therefore to the deeper understanding of results. Further, these sciences have caused a dramatic change in the toxicology testing and for a scientific interpretation of results: from a pure empirical and descriptive science to a science which is capable to provide the necessary information with regard to extrapolation of results from animals to human patients. Results in pre-clinical toxicology studies as "stand alone" data without DMPK characteristics provide only little information with regard to characterize

a toxicological hazard to the patient. Especially in the development of modern drugs, which are frequently based on a highly sophisticated chemistry resulting in drug substances with a high molecular weight, require a profound DMPK analysis in order to improve the quality of animal data. What does "low toxicity" mean if a drug is not or poorly absorbed in animals but well absorbed in humans? What's the value of toxicology studies in pre-clinical studies when the drug is metabolized in animals and man in a different manner? The answer for both questions is: Close to Zero.

The mechanism of the pharmacological or biochemical activity is also a key element for the interpretation of animal studies in toxicology and requires a close co-operation between pharmacologists and toxicologists. Sub-acute or chronic effects in pre-clinical safety studies are often connected with the mechanistic activity of a drug. A typical example is the effect of H2-blockers in chronic and high dosed rodent carcinogenicity studies resulting in carcinoids in the stomach, which are related to the chronic increase of serum gastrin levels, which has no or little clinical relevance to humans. In the history of experimental toxicology many similar examples exist which further underline this idea.

In summary, the close co-operation in pharmacology, safety pharmacology, toxicology and DMPK, and in some cases also clinical pharmacology departments has been identified as an absolute key success factor in modern and efficiently working drug companies with intelligent development strategies.

The edition of this book covering preclinical drug safety (toxicology and safety pharmacology) and pre-clinical and clinical DMPK aspects is considered as a contribution to this principle.

Chapter III.B
International Guidelines for the Development of Pharmaceutical Compounds

Gerd Bode

III.B.1 General Considerations

Toxicology Studies support the detection of undesirable adverse effects of pharmaceutical or chemical compounds and identify hazards, they describe the type and degree of toxicity and asses the risks, they support management of the risks in humans, when participating in clinical trials or later as patients after market authorization, and analyze the mechanisms behind the alterations; they extrapolate preclinical hazards to humans and finally help to communicate these risks to populations concerned with exposure of that particular substance.

Toxicology should contribute to the safe use of pharmaceutical compounds in humans. It therefore plays a growing role in the development of pharmaceutical/chemical compounds and their acceptability by regulatory agencies or society.

For the safety of pharmaceutical compounds, a great number of in vitro and/or in vivo experiments are being conducted today. Test strategies for toxicological investigations have been refined and the extrapolation of preclinical results to humans improved. The prevention of toxic events in man became the main purpose of preclinical experimentation.

Considerable differences were observed in the creation of guidance and legal conditions. Accordingly, in different regions differently designed studies were requested. The repetition of similar studies in different regions did not support the safety but only increased the number of animals being used.

Accordingly, public resistance and concerns among researchers increased the desire to harmonize test procedures and recommendation worldwide. With the creation of the International Conferences of Harmonization (ICH) these initiatives were channeled into a global process, which is ongoing.

The ICH focuses on guidances for Quality, Preclinical Safety (= Toxicology) and Efficacy (clinical effective substances) of pharmaceutical compounds.

This section illustrates all ICH Guidelines important for the safe use of drugs worldwide.

III.B.2 Background to the ICH Conference

The International Conferences was jointly supported and organized by the Commission of the European Communities (CEC), the US Food and Drug Administration (FDA), the Japanese Ministry of Health and Welfare (MHW), together with the pharmaceutical industry, as represented by the International Federation of Pharmaceutical Manufacturers Associations (IFPMA), the European Federation of Pharmaceutical Industry Associations (EFPIA), the US Pharmaceutical Manufacturers Association (PMA) and the Japanese Pharmaceutical Manufacturers Association (PMA).

Many important initiatives have been undertaken between regulatory authorities and industry associations, particularly on a bilateral basis, to promote harmonization of regulatory requirements between the three regions Japan, USA and the European Community. ICH owes much to these initiatives of experts during international symposia.

The Main Objectives of the ICH

1. To provide a forum for a constructive dialogue between regulatory authorities and the pharmaceutical industry on the real and perceived differences in the technical requirements for product registration in the CEC, USA and Japan;

2. To identify areas where modifications in technical requirements or greater mutual acceptance of research and development procedures could lead to a more economical use of human, animal and material resources, without compromising safety; and

3. To make recommendations on practical ways to achieve greater harmonization in the interpretation and application of technical guidelines and requirements for registration.

III.B.3 Preparatory Activities for ICH Conferences

A Steering Committee was appointed with members from EU, FDA, MHW, EFPIA, JPMA, PMA and IFPMA, with observers from WHO, and from the regulatory authorities of Canada and Switzerland (for EFTA).

The Steering Committee set up three joint industry/regulatory Expert Working Groups to deal with the technical aspects of the three main subject areas – Quality, Safety and Efficacy – which were discussed in three parallel workshops during the conferences. Each of the Expert Working Groups has members, representing EU, FDA, MHW, EFPIA, PMA, and JPMA. With advice from these technical Working Groups, the Steering Committee was responsible for the selection and prioritization of the topics discussed at the Workshops at the ICH conferences.

The International Conference on Harmonisation differs from many other harmonization initiatives in that it has a recognized status and is backed by a commitment on the part of both industry and regulators, to facilitate greater harmonization of technical requirements in the three regions. Commitment to these objectives, set out in the Terms of Reference, was re-affirmed by the Steering Committee in a statement issued following the meeting held in Tokyo, October 1990.

III.B.4 Success of the ICHs

There is considerable success of the international discussions and constructive solutions documented in the guidelines. There are more than 20 guidelines focusing on issues concerning the quality of compounds. There are more than 15 guidelines dealing with problems of efficacy during clinical trials or after marketing authorisation. See Table 1.

III.B.5 Description of the ICH Guidelines on Preclinical Safety

III.B.5.1 Carcinogenicity

In the following a survey is given in regard to the guidelines on preclinical safety.

There are a number of endpoints which should and could not be tested in humans, these are mainly: genotoxicity, teratogenicity and cancerogenicity. Especially the cancerogenic risk can usually not be tested in humans, it is ethically forbidden and such a risk for patients is unacceptable.

The testing for carcinogenic potential today employs short-, mid- and longterm studies in rodents, which have a relatively high power of predictivity for the carcinogenic risk in humans.

Table 1 Summary of all guidelines which have been agreed upon in the area of qualitiy

ICH No.	Title	CPMP Doc. No.	Step
Q1A	Stability Testing of New Drug Substances and Products	CPMP/ICH/380/95	Step 5
Q1A (R)	Stability Testing of New Drug Substances and Products (Revision of CPMP/ICH/380/95)	CPMP/ICH/2736/99	Step 5
Q1B	Photostability testing of New Active Substances and Medicinal Products	CPMP/ICH/279/95	Step 5
Q1C	Stability Testing: Requirements for New Dosage Forms	CPMP/ICH/280/95	Step 5
Q1D	Bracketing and Matrixing Designs for Stability Testing of Drug Substances and Drug Products	CPMP/ICH/4104/00	Step 2
Q2A	Validation of Analytical Methods: Definitions and Terminology	CPMP/ICH/381/95	Step 5

Table 2 A summary of all guidelines for the area of Preclinical Safety

ICH No.	Title	CPMP Doc. No.	Step
Q2B	Validation of Analytical Procedures: Methodology	CPMP/ICH/281/95	Step 5
Q3A	Impurities in New Drug Substances	CPMP/ICH/142/95	Step 5
Q3A (R)	Impurities in New Drug Substances (Revision of CPMP/ICH/142/95)	CPMP/ICH/2737/99	Step 2
Q3B	Impurities in New Medicinal Products	CPMP/ICH/282/95	Step 5
Q3B (R)	Impurities in New Drug Products (Revision of CPMP/ICH/282/95)	CPMP/ICH/2738/99	Step 2
Q3C	Impurities: Residual Solvents	CPMP/ICH/283/95	Step 5
Q3C (M)	Impurities: Residual Solvents	CPMP/ICH/1940/00	Step 2
Q4	Pharmacopoeial Harmonisation		
Q5A	Quality of Biotechnological Products: Viral safety Evaluation of Biotechnology Products derived from Cell Lines of Human or Animal Origin	CPMP/ICH/295/95	Step 5
Q5B	Quality of Biotechnological Products: Analysis of the Expression Construct in Cell Lines used for Production of r-DNA derived Protein Products	CPMP/ICH/139/95	Step 5
Q5C	Quality of Biotechnological Products: Stability Testing of Biotechnological/ Biological Products	CPMP/ICH/138/95	Step 5
Q5D	Quality of Biotechnicological Products: Derivation and Characterisation of Cell Substrates used for Production of Biotechnological/ Biological Products	CPMP/ICH/294/95	Step 5
Q6A	Specifications: Test procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances	CPMP/ICH/367/95	Step 5
Q6B	Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products	CPMP/ICH/365/96	Step 5
Q7A	Good Manufacturing Practices for Active Pharmaceutical Ingredients	CPMP/ICH/4011/00	Step 5

III.B.5.1.1 General Regulatory Background

Treatment with compounds associated with carcinogenic potential is unacceptable for banal indications; for severe indications like life-threatening cancer diseases the treatment with carcinogenic compounds often does not increase the overall risk of the underlying disease.

Carcinogenicity studies are the longest (life long or of 2 year duration) and most expensive (approximately 1.5 to 4 million dollars or euros) preclinical studies. They should therefore be well designed and conducted in such a way that they clearly indicate any risk involved. These conditions explain why a great number of international and regional guidelines exist and give best advice for the researchers.

III.B.5.1.2 Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals (S1A)

This guideline was adopted in 1997 and implemented in all regions.

The objectives of carcinogenicity studies are:

- To identify a tumorigenic potential in animals
- To assess the relevance of these identified risks for humans. Any cause for concern derived from laboratory investigations, animal toxicology studies, and data in humans, may lead to a need for carcinogenicity studies.

Carcinogenicity studies should be performed for any pharmaceutical whose expected clinical use is continuous for at least 6 months. Certain classes

Table 3 A summary of all guidelines which have been issued for the clinical Efficacy

ICH No.	Title	CPMP Doc. No.	Step
S1A	The Need for Carcinogenicity Studies of Pharmaceuticals	CPMP/ICH/140/95	Step 5
S1B	Carcinogenicity: Testing for Carcinogenicity of Pharmaceuticals	CPMP/ICH/299/95	Step 5
S1C	Carcinogenicity: Dose selection for carcinogenicity studies of pharmaceuticals	CPMP/ICH/383/95	Step 5
S1C (R)	Dose Selection for Carcinogenicity Studies of Pharmaceuticals: Addition of a Limited Dose and related Notes	CPMP/ICH/366/96	Step 5
S2A	Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals	CPMP/ICH/141/95	Step 5
S2B	Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals	CPMP/ICH/174/95	Step 5
S3A	Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies	CPMP/ICH/384/95	Step 5
S3B	Pharmacokinetics: Guidance for Repeated Dose Tissue Distribution Studies	CPMP/ICH/385/95	Step 5
S4A	Duration of Chronic Toxicity Testing in Animals (Rodent and non Rodent Toxicity Testing)	CPMP/ICH/300/95	Step 5
S5A	Reproductive Toxicology: Detection of Toxicity to Reproduction for Medicinal Products	CPMP/ICH/386/95	Step 5
S5B (M)	Reproductive Toxicology: Toxicity on Male Fertility (Modification)	CPMP/ICH/136/95	Step 5
S6	Preclinical Safety Evaluation of Biotechnology-Derived Products	CPMP/ICH/302/95	Step 5
S7A	Safety Pharmacology Studies for Human Pharmaceuticals	CPMP/ICH/539/00	Step 5
S7B	Safety Pharmacology Studies Assessing the Potential For Delayed Ventricular Repolarization (QT Interval prolongation) by Human Pharmaceuticals	CPMP/ICH/433/02	Step 5
S8	Immunotoxicity		Step 5

Table 4 A summary of all guidelines which have been agreed upon on multidisciplinary subjects

ICH No.	Title	CPMP Doc. No.	Step
E1	Population Exposure: The extent of Population Exposure to assess Clinical Safety	CPMP/ICH/375/95	Step 5
E2A	Good Clinical Safety Data Management: Definitions and Standards for Expedited Reporting	CPMP/ICH/377/95	Step 5
E2B E2B(M)	Clinical Safety Data Management: Data Elements for Transmission of Individual Case Safety Reports	CPMP/ICH/287/95	Step 5
E2C	Clinical Safety Data Management: Periodic Safety Update Reports for Marketed Drugs	CPMP/ICH/288/95	Step 5
E3	Structure and Content of Clinical Study Reports	CPMP/ICH/137/95	Step 5
E4	Dose Response Information to support Drug Registration	CPMP/ICH/378/95	Step 5
E5	Ethnic Factors in the Acceptability of Foreign Clinical Data	CPMP/ICH/289/95	Step 5
E6	Good Clinical Practice	CPMP/ICH/135/95	Step 5
E7	Studies in support of Special Populations: Geriatrics	CPMP/ICH/379/95	Step 5
E8	General Considerations for Clinical Trials	CPMP/ICH/291/95	Step 5
E9	Statistical Principles for Clinical Trials	CPMP/ICH/363/96	Step 5
E10	Choice of Control Group for Clinical Trials	CPMP/ICH/364/96	Step 5
E11	Clinical Investigation of Medicinal Products in the Paediatric Population	CPMP/ICH/2711/99	Step 5
E12A	Principles for Clinical Evaluation of New Antihypertensive Drugs	CPMP/ICH/541/00	Principle document
E14	Clinical QT		Step 2

Table 5 A summary of the guidelines focussing in Carcinogenicity

ICH No.	Title	CPMP Doc. No.	Step
M1	Medical Terminology: Medical Dictionary for Regulatory Activities Terminology (MedDRA)		
M2	Recommendations on Electronic Transmission of Individual Case Safety Reports Message Specification	CPMP/ICH/285/95	Step 5
M3 (M)	Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals (Modification)	CPMP/ICH/286/95	Step 5
M4	Common Technical Document for the Registration of Pharmaceuticals for Human Use	CPMP/ICH/2887/99	Step 4

of compounds may not be used continuously over a minimum of 6 months but may be expected to be used repeatedly in an intermittent manner. For pharmaceuticals used frequently in an intermittent manner during the treatment of chronic or recurrent conditions, carcinogenicity studies are generally needed. Examples of such conditions include allergic rhinitis, depression, and anxiety.

Pharmaceuticals administered infrequently or for short duration of exposure (e.g., anesthetics and radiolabel imaging agents) do not need carcinogenicity studies unless there is cause for concern.

Carcinogenicity studies may be recommended if there is cause for concern e.g. due to

- Previous demonstration of carcinogenic potential in the product class that is considered relevant to humans.
- Structure-activity relationship suggesting carcinogenic risk.
- Evidence of preneoplastic lesions in repeated dose toxicity studies; and
- long-term tissue retention of parent compound or metabolite(s) resulting in local tissue reactions or other pathophysiological responses.
- Unequivocally genotoxic compounds need not to be subjected to longterm carcinogenicity studies. However, if such a drug is intended to be administered chronically to humans a chronic toxicity study (up to one year) may be necessary to detect early tumorigenic effects. In practice, this option has not been used since 1997. Main concern of industry is the lack of historical comparison data. The tumor evaluation was always based on 2 year data.
- In instances, where the life-expectancy in the indicated population is short (i.e., less than 2–3 years) no long-term carcinogenicity studies may be required. For example, oncolytic agents intended for treatment of advanced systemic disease do not generally need carcinogenicity studies.
- In cases, where the therapeutic agent for cancer is generally successful and life is significantly prolonged, there may be requirements to provide knowledge about the tumorigenic risk.
- When such pharmaceuticals are intended for adjuvant therapy in tumor free patients or for prolonged use in non- cancer indications, carcinogenicity studies are usually needed.
- Pharmaceuticals showing poor systemic exposure from topical routes in humans may not need studies by the oral route to assess the carcinogenic potential to internal organs.
- Carcinogenicity studies are not generally needed for endogenous substances, when given essentially as replacement therapy (i.e., physiological levels), particularly, where there is previous clinical experience with similar products (for example animal insulins, pituitary hormones).

III.B.5.1.3 Testing for Carcinogenicity of Pharmaceuticals (S1B)

S1B was adopted and implemented in 1997.

Historically, the regulatory requirements for the assessment of the carcinogenic potential was the conduct of long-term carcinogenicity studies in two rodent species, usually the rat and the mouse. It was the mission of ICH to examine whether this practice could be reduced without compromising human safety. The discussion in the Expert Working Group soon revealed that the rule of testing in two species had to continue, the American consumer societies demanded continuation with the same standard of safety, based on 2 species testing.

As the new experimental approach to testing for carcinogenic potential, a basic scheme was set up to comprise one long-term rodent carcinogenicity study, plus one other study of the type that supplements the long term carcinogenicity study and provides additional information that is not readily available from the long term assay:

S1B: Basic Principle for Testing the Carcinogenic Potential

- 1 long-term rodent carcinogenicity study

plus

- 1 short or medium-term study that
  - supplements the long-term carcinogenicity study and
  - provides additional information not readily available from the long-term assay.

The species selected should be appropriate, based on considerations on pharmacology, repeated-dose toxicology data, metabolism (see also Guidelines S1C and S3A), toxicokinetics (see also Guidelines S1C, S3A, and S3B), and route of administration (e.g., less common routes such as dermal and inhalation). In the absence of clear evidence favoring one species, it is recommended that the rat be selected for the long term study.

Additional tests may either be short or medium-term in vivo rodent test systems, usually using the mouse. These models of carcinogenesis may use transgenic or neonatal rodents. The guideline also includes models

of initiation promotion in rodents; these models are today considered to be useful models for hepatic carcinogenesis or adequate mechanistic studies, but not as assays appropriate as general screen for drug-induced carcinogenesis.

A long-term carcinogenicity study in a second rodent species (e.g., mice) is still considered acceptable.

The guideline describes in the "Notes" important information about the new models. Note 1 informs about the SHE assay; Note 2 on conditions to limit testing with 1 species only: if the findings of a short or long-term carcinogenicity study and of genotoxicity tests and other data indicate that a pharmaceutical clearly poses a carcinogenic hazard to humans, a second carcinogenicity study would not usually be useful; Note 3 provides details about the short- or mid-term models. Table 6 is a representative list of some approaches that may meet these criteria.

Table 6 New Approaches for Testing the Carcinogenic Potential

Approach	Including
Several transgenic mouse assays	σp53+/− deficient model σ Tg.AC model σ TgHras2 model σ XPA deficient model, etc.
The neonatal rodent tumorigenicity model	

Evidence of tumorigenic effects of the drug in rodent models should be evaluated in light of the tumor incidence and latency, the pharmacokinetics of the drug in the rodent models as compared to humans, and data from any ancillary or mechanistic studies that are informative with respect to the relevance of the observed effects to humans.

The results from any tests cited above should be considered as part of the overall "weight of evidence" taking into account the scientific status of the test systems.

These new models have been accepted by Regulatory Agencies.

Table 7 Transgenic mouse models

Model	Including
Activated oncogenes	TgHras2 model (Japan) Tg.AC skin model (USA) also gavage
Inactivated tumor suppressor gene	p53 knock out (=p53+/−) model (USA)
Inactivated DNA repair gene	XPA−/− (NL)

The FDA considerations for assay selection are:

- P53+/-: if clearly or equivocally genotoxic
- TgAC: for dermally applied products
- Neonatal: if clearly or equivocally genotoxic
- TgRasH2: for genotoxic or nongenotoxic products.

The EU evaluation as discussed in the Safety Working Party EMEA reaches the following conclusions:

- p53 and Tg.RasH2 are equally sensitive to genotoxic compounds (some false positives and false negatives)
- TgRasH2 is more sensitive to peroxisome proliferators
- p53 and TgRasH2 are acceptable in a regulatory context.

III.B.5.1.4 Dose Selection for Carcinogenicity Studies of Pharmaceuticals (S1C)

This guideline was adopted and implemented in the different regions in 1997.

Traditionally, carcinogenicity studies for chemical agents have relied upon the maximally tolerated dose (MTD) as the standard method for high dose selection.

The MTD is generally chosen based on data derived from toxicity studies of 3 months' duration. Testing options for dose-range-finding studies are as follows:

- Usually:
  - 3 months for long term studies
  - 1 month for neonatal or transgenic mice
- Range of different dose levels, often 5
- Focus on toxicity endpoints, determination of MTD
- Profiling of AUC
  - e.g. 1 and 4 months for rats,
  - e.g. 1 and 4 weeks for alternatives

(transgenic = wild type).

Table 8 illustrates the general design of long term tumorigenicity assays.

Ideally, the doses selected for rodent bioassays for non-genotoxic pharmaceuticals should provide an exposure to the agent, and adequate margin of safety, no significant chronic physiological dysfunction and compatible with good survival.

The guideline calls for a flexible approach to dose selection. The guideline proposes 5 different approaches:

Table 8 General Design of longterm Cancerogenicity Studies

Aspect	Parameters
Species	rats, mice, rarely hamster
Duration	24 month rats, 24 months mice
Route	as in clinical conditions, in feed, water, by gavage, inhalation
Dose levels	mostly 3, mostly with a factor (e.g. 1:3:9)
Animal numbers	50/group/sex
Results available	prior to NDA
Cost	1.0–1.5 Million Euros

1) toxicity based endpoints (MTD = Maximum Tolerated Dose or Minimum Toxic Dose)

2) pharmacokinetic endpoints (25 times the human AUC)

3) saturation of absorption

4) pharmacodynamic endpoints

5) maximum feasible dose.

Ad 1) The ICH Expert Working Group on Safety has agreed to continue use of the MTD as an acceptable toxicity-based endpoint for high dose selection for carcinogenicity studies.

The MTD is defined as the top dose or maximum tolerated dose that produces a minimum toxic effect over the course of the carcinogenicity study. Factors to consider are alterations in physiological function, which would alter the animal's normal lifespan or interfere with interpretation of the study. Such factors include: no more than 10 % decrease in body weight gain relative to controls; target organ toxicity or significant alterations in clinical pathological parameters.

Ad 2) A systemic exposure representing a 25 times multiple of the human AUC (at the maximum recommended daily dose) may be an appropriate endpoint for dose selection for carcinogenicity studies for non-genotoxic pharmaceuticals, as a pragmatic solution.

Ad 3) High dose selection based on saturation of absorption measured by systemic availability of drug-related substances is acceptable. The mid and low doses selected for the carcinogenicity study should take into account saturation of metabolic and elimination pathways.

Ad 4) Pharmacodynamic endpoints for high dose selection will be highly compound-specific. The high dose selected should produce a pharmacodynamic response in dosed animals of such magnitude as would preclude further dose escalation. However, the dose should not produce disturbances of physiology or homeostasis. Examples include hypotension, inhibition of blood clotting or insulin-like effects.

Ad 5) The maximum feasible dose by dietary administration was considered 5 % of diet. By many scientists this amount of drug is considered to be too high. Therefore, a new and more reasonable solution was formulated in the Guideline ICH/S1C(R), which follows.

III.B.5.1.5
Addendum to Dose Selection for Carcinogenicity Studies of Pharmaceuticals

Addition of a limit dose and related notes of pharmaceuticals. This addendum S1C (R) was adopted in 1998.

In determining the high dose for carcinogenicity studies, it may not be necessary to exceed a dose of 1500 mg/kg/day. This limit dose applies only in cases where there is no evidence of genotoxicity and where the maximum recommended human dose does not exceed 500 mg/day.

III.B.5.1.6
Genotoxicity Guidelines (ICH/S2A and S2B)

A permanent alteration of genes or chromosomes can cause heritable effects leading to malformations and dysfunctions or inducing tumors.

Testing of new drugs for their genotoxic potential is therefore an important contribution to the safety of humans. The appropriate approaches for assessing the genotoxic potential of pharmaceutical compounds are recommended in two ICH guidelines: ICH/S2A "Guidance on specific aspects of regulatory genotoxicity tests for pharmaceuticals (CPMP/ICH/141/95)" and ICH/S2B "Genotoxicity: A standard battery for genotoxicity testing of pharmaceuticals (CPMP/ICH/174/95)".

Valid information on gene mutations, structure chromosome aberrations (clastogenicity) and numerical chromosome aberrations (aneugenicity) is required. No single test is capable of detecting all relevant genotoxic agents, therefore, a battery of tests is considered appropriate.

ICH/S2B recommends a standard battery which is considered to be sufficient for most pharmaceuticals consisting of three assays:

1. a test for gene mutations in bacteria (Ames), combined with
2. an in vitro test with cytogenetic evaluation of chromosomal damage with mammalian cells *or* an in vitro mouse lymphoma *tk* assay and
3. an in vivo test for chromosomal damage using rodent hematopoietic cells.

Table 9 Genotoxicity Guidelines (ICH/S2A and S2B)

ICH No.	Title	CPMP Doc. No.	**Step**
S2A	Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals	CPMP/ICH/141/95	Step 5
S2B	Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals	CPMP/ICH/174/95	Step 5

Table 10 Toxicokinetics/Pharmacokinetics (ICH/S3A and S3B)

ICH No.	Title	CPMP Doc. No.	**Step**
S3A	Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies	CPMP/ICH/384/95	Step 5
S3B	PharmacoKinetics: Guidance for Repeated Dose Tissue Distribution Studies	CPMP/ICH/385/95	Step 5
S4A	Duration of Chronic Toxicity Testing in Animals (Rodent and non Rodent Toxicity Testing)	CPMP/ICH/300/95	Step 5

The in vitro tests are carried out with and without extrinsic metabolic activation, usually provided by a rat liver S9-mix.

Modifications of the standard battery may be necessary for some classes, e.g., antibiotics which are toxic to bacteria or e.g., for compounds like topoisomerase inhibitors which interfere with the mammalian cell replication system. A selection of additional assays is being proposed, further modifications may be acceptable via discussion in the ICH Maintenance Process. Alternative strategies may consider assays like the in vivo Comet assay (single cell gel electrophoresis measuring DNA strand breaks) or gene mutation tests with transgenic animals or in vivo DNA adduct studies.

The ICH guidances complement the details of study designs outlined in the OECD guidelines. Additionally, they inform about the top concentrations for in vitro tests and the expected levels of cytotoxicity, and about repeat or confirmatory testing and the proof of target tissue exposure to the test article in the in vivo studies.

Support for the interpretation of positive test results and considerations on conditions leading to false positive data can be found.

III.B.5.1.7 Toxicokinetics/Pharmacokinetics (ICH/S3A and S3B)

Table 10 cites the toxicokinetic and pharmacokinetic guidelines.

ICH/S3A

The objectives of Toxicokinetics are primarily to describe the systemic exposure achieved in animals and its relationship to dose level and the time course of the toxicity study. And further, to relate exposure levels to toxicological findings, to assess the relevance of these findings to clinical safety and to support the design as the choice of species and treatment regimen in non-clinical studies.

If animals or humans are exposed to pharmaceutical compounds, they will either elicit a pharmacodynamic effect, e.g., show a suppression of blood pressure, or reveal in analytical blood samples exposure levels of the compound. This kinetic information of the parent compound and its metabolites is an important contribution for the extrapolation of safety data from animal studies to humans. Species differ considerably in regard to their kinetic conditions as C_{max}, T_{max}, Area Under the Curve (AUC), $t_{1/2}$ and ADME (Absorption, Distribution, Metabolism and Excretion).

Therefore, it is important to know what a drug does with the body (what pharmacology and toxicology is induced?); it is also crucial to know what the body does with the drug.

The following toxicokinetic studies are usually distinguished: concomitant toxicokinetics, which are normally integrated in the toxicity studies and other supportive studies which mimic the conditions of the toxicity study.

The focus before IND is on T_{max}, C_{max} and AUCs, while the complexity of pharmacokinetic characterization (like oral bioavailability, plasma half life, volume of distribution, mean residence time, absorption, solubility and concentration) is built up during clinical trials and on the basis of comparable human data.

Kinetic data should be considered in repeat dose toxicity studies, in genotoxicity studies when there are negative results and exposure can be characterized in the indicator tissue, in cancerogenicity studies where

monitoring is appropriate but not beyond 6 months duration.

The compound can be bound to plasma proteins, cells or tissues. Therefore, a distinction between "unbound drug" or "free fraction" is relevant. Distribution studies help to design preclinical studies. Demonstration of accumulation can, for example, explain toxicity at the site of increased compound accumulation.

ICH/S3B

Single dose studies provide usually sufficient information about tissue distribution but there may be cases where assessments after repeated dosing may provide better information. Such studies are necessary when

1) single dose distribution studies suggest that the half-life of the test compound and/or metabolites in organs or tissues significantly exceeds the half-life of the elimination phase in plasma or

2) steady-state levels of a compound/metabolite in the circulation, determined in repeated dose pharmacokinetic or toxicokinetic studies, are markedly higher than those predicted from single dose kinetic studies or

3) when histopathological changes were observed that were not predicted from short-term toxicity studies.

This information is provided in the ICH-Guideline S3B: "Pharmacokinetics: Guidance for repeated dose distribution studies (CPMP/ICH/395/95)". Other relevant kinetic questions are the investigations of the potential of compounds to penetrate the barriers of placenta, blood-brain or excretion into milk.

Knowledge about metabolites and their activity is further an important criteria for the assessment of species-specific effects and differences, e.g. the search for the most human-like test model as the best predictor for human reactions, focuses on such differences. Metabolism can lead to pharmacologically active metabolites; such knowledge is desirable early in development. In vitro metabolism studies normally precede in vivo preclinical safety assessments.

For safety reasons, it is important to identify, and perhaps eliminate, drugs from further development if they are subject to polymorphic metabolism or extensive metabolism by key human enzymes. Knowledge about the cytochrome P450 (CYP450) superfamily of drug metabolising enzymes is of particular interest.

ICH/S4A

This is one of the guidelines where harmonisation continued to be difficult. There was clear agreement that rodent studies were only needed for a maximum duration of six months. When a 6 month study in rodents is needed, then continuous application of pharmaceutical compounds will take place. Under such conditions, a long-term carcinogenicity study will usually be performed. Long-term exposure and life-long observation is therefore possible. For non-rodents, life-long exposure is normally not required. The EU proposed recommending a common maximum duration of 6 month studies for rodents and no-rodents, but there were 16 cases where unexpected findings appeared after the treatment time of 6 months. In face of this, the Guideline S4A recommends the following: For non-rodents, 12 month studies are usually not necessary, in the EU 6 month studies are acceptable based on the Directive 75/318/EEC. Accordingly, as a compromise, the Expert Working Group agreed that 9 month studies are recommended in general. In the US Federal Register this guideline was published with an FDA Note on ICH/S4A as follows: 9 month studies are acceptable for most development plans; shorter ones like 6 month studies, may be acceptable for some drugs, while longer durations, e.g. 12 months, may be more appropriate for others. This advice is characterized by the FDA as their current line of thinking. In practice, FDA agreed with the sponsors in 50 % for 9 month studies and in 32 % for 6 month studies, while 12 month studies were only requested for compounds with novel mechanisms, or when only sparse clinical data were available, as in indications like HIV. Aids patients should be provided early on with the new treatment and the lack of complex clinical data is compensated by longer non-rodent studies. Sponsors are advised by the FDA to get in contact with the Agency when the maximum duration needs to be determined for non-rodents.

III.B.5.1.8
Reproductive Studies (ICH/S5 A+B) and Preclinical Evaluation of Biotechnology-derived Products (ICH/S6)

ICH/S5A+B

The special toxicology discipline "Reproductive and Developmental Toxicity" focuses on undesirable adverse effects on male and female fertility, birth defects (developmental toxicity, malformations, teratogenicity) and non-physiological changes that appear shortly before, during and after birth, and during the weaning period. The relevant ICH Guidelines describing the requirements for such studies are the ICH Guideline S5A and S5B.

In humans, malformations and changes in development are relatively rare (around 6 %) and often caused

Table 11 Reproductive Studies (ICH/S5 A+B) and Preclinical Evaluation of biotechnology-derived Products (ICH/S6)

ICH No.	Title	CPMP Doc. No.	Step
S5A	Reproductive Toxicology: Detection of Toxicity to Reproduction for Medicinal Products	CPMP/ICH/386/95	Step 5
S5B (M)	Reproductive Toxicology: Toxicity on Male Fertility (Modification)	CPMP/ICH/136/95	Step 5
S6	Preclinical Safety Evaluation of Biotechnology-Derived Products	CPMP/ICH/302/95	Step 5

by accidental genetic errors; some are caused by external factors, e.g. chemical drugs. If a compound is labeled as a developmental toxicant, then the occurrence of structural or functional abnormalities in offspring is significantly increased at a dose level which does not induce severe maternal toxicity. If malformations are developed, then exposure levels of the individual foetuses are important, but also at which time of the development exposure was high. The inclusion of kinetic data helps to support the evaluation.

In general, there is a distinction between the following time periods:

Segment I: the early embryonic development (fertilized egg or zygote, prior to implantation and prior to formation of the three primary germ layers),

Segment II: the embryo-foetal development (embryo during major organ development i.e. organogenesis, the foetus in the post-embryonic period) and

Segment III: the prenatal and postnatal development (neonate or postnatal offspring).

These segments can be tested separately or in a combined manner. All stages of development from conception to maturity and the detection of acute and delayed effects of exposure through one complete life cycle should be examined .The standard species are rodents, rats as the preferred rodent species for all study types and, the rabbit as the second non-rodent species for the embryo-toxicity studies. In some rare cases mice or monkeys are used too, if special conditions – usually kinetic data – justify such species.

The route of administration should be similar to the intended human usage.

ICH/S6 – Preclinical Safety Evaluation of Biotechnology-derived Products

S6 outlines the products for which this guidance is applicable, such as recombinant DNA proteins, vaccines, peptides, plasma derived products, endogenous proteins extracted from human tissues or oligonucleotide drugs etc., while heparin, vitamins, and cellular blood components, for example, are not covered.

In general, for biotech products high flexibility for the developmental scheme is recommended and should be reconsidered on a case-by-case basis.

Usually, as in other toxicity studies, 2 species should be used, but when the biological activity is well understood or when in short term toxicity studies the effects were similar in both species, then longer term studies could be run only with one species.

When no relevant species can be identified, one should consider the use of homologous proteins or transgenic animals.

With regard to Safety Pharmacology, testing of the vital function such as cardiovascular, respiratory and CNS functions is recommended, but S 6 also mentions renal function, which from today's perspective would not be necessary before first administration of a compound in humans if there is no specific concern.

Some information on absorption, disposition and clearance in the animals models is desirable before clinical trials and systemic exposure of the compound should be monitored as well as the appearance of antibodies and their ability to neutralize the intended effect.

The immune response could also alter the pharmacokinetic or pharmacodynamic effects. Anaphylactic responses tested in the guinea pig, at any rate, are not predictive for humans and therefore not necessary. The same holds true for the standard testing batteries for immunotoxicity, these are not recommended.

Studies with a duration of between 2 weeks and 3 months are often sufficient and it is only with chronic use in humans that 6 month studies should be considered.

Flexibility may allow further reduction of the traditional testing program for the reproductive endpoints. Such studies may not be necessary if a new one, related to well-known compounds, shows similar effects.

The same holds true for genotoxicity. The standard studies are not appropriate, but testing on a case-by-case basis of impurities or promoter studies might be helpful.

Long-term carcinogenicity studies are usually not appropriate, but when there is cause for concern,

Table 12 Safety Pharmacology (ICH/S/A+B) and Immunotoxicology Studies (ICH/S8)

ICH No.	Title	CPMP Doc. No.	Step
S7A	Safety Pharmacology Studies for Human Pharmaceuticals	CPMP/ICH/539/00	Step 5
S7B	Non-Clinical Studies for Assessing Risk of Repolarisation – Associated Ventricular Tachyarrhythmia for Human Pharmaceuticals	CPMP/ICH/423/02	Step 5
S8	Immunotoxicology Studies		Step 5

studies with a single rodent species are sufficient. Cause for concern may have arisen in general toxicity studies, or else a stimulation of the growth of normal as well as malignant cells can be assumed.

III.B.5.1.9 Safety Pharmacology (ICH/S/A+B) and Immunotoxicology Studies (ICH/S8)

These 3 guidelines are the most recent ones. S7A was implemented in 2001 and the S7B and S8 reached step 4 in June 2005 and will be implemented in 2006.

S7A informs in general about the requirements necessary for testing the vital functions usually in single dose studies in Safety Pharmacology.

S7A differentiates between 3 types of studies: core battery, follow-up and supplemental studies. The core battery of tests/S7A consistes of an investigation of the effects of a test substance on vital functions: central nervous system; cardiovascular system; respiratory system; other systems as appropriate. The exclusion of a system or function should be justified. Safety Pharmacology studies carried out as necessary as:

- Follow-up studies for core battery (they provide a greater depth of understanding than, or additional knowledge to, that provided by the core battery [e.g. mechanistic studies])
- Supplemental studies, evaluate effects of the test substance on systems not addressed by the core battery when there is cause for concern not addressed elsewhere (e.g. in toxicology).

S7A expresses very clearly when such studies should be available and what conditions should be considered in regard to good laboratory procedures (see Table 13).

Special focus is given to the cardiovascular system. For the core battery of the cardiovascular system according to S7A blood pressure, heart rate and electrocardiogram should be assessed, but also in vivo, in vitro and/or ex vivo evaluations, including methods for repolarization and conductance abnormalities should be considered.

This text was finalized at a time when the details of S7B were not yet outlined. During recent years there has been an increase of regulatory concern. The awareness that non-cardioactive drugs, used for sometimes non-life threatening diseases, can cause QT prolongation, and serious dysrhythmias such as TdP was intensified.

A greater number of compounds became known to be associated with QT prolongation and the potential to cause Torsades de Pointes.

Accordingly, the ICH Expert Working Group for S7B was created to work on this specific concern.

ICH S7B – Non-Clinical Studies for Assessing Risk of Repolarisation – Associated Ventricular Tachyarrhythmia for Human Pharmaceuticals

The background of S7B is summarized as follows:

- The QT interval (time from the beginning of the QRS complex to the end of the T wave) of the electrocardiogram (ECG) is a measure of the duration of ventricular depolarization and repolarization.
- QT interval prolongation can be congenital or acquired (e.g., pharmaceutical-induced).
- When the QT interval is prolonged, there is an increased risk of ventricular tachyarrhythmia, including Torsade de Pointes (TdP), particularly when combined with other risk factors (e.g., hypokalemia, structural heart disease, bradycardia), see Figure 1.

Fig. 1.

To cope with this issue, a relatively flexible approach is given with the ICH/S7B non-clincal testing strategy as seen in Figure 2.

The basis for Integrated Risk Assessment are in vitro and in vivo assays, supported by any knowledge about the chemical/pharmaceutical class. This first risk assessment may later be modified when results from follow-up studies or relevant non-clinical or

Table 13

Guideline	Timing
ICH/S7A	Before first administration to humans Core battery tests and follow-up/supplemenal studies as appropriate During clinical development. Additional studies as required to clarify observed or suspected undesirable effects in animals or humans Before approval. Effects on all organ systems, if not covered elsewhere (e.g. toxicology)
GLP/S7A	Core battery tests should be conducted according to GLP Follow-up and supplemental studies should be conducted according to GLP as far as possible deviation should be justified and impact discussed Primary and secondary pharmacodynamic studies need not be conducted according toGLP

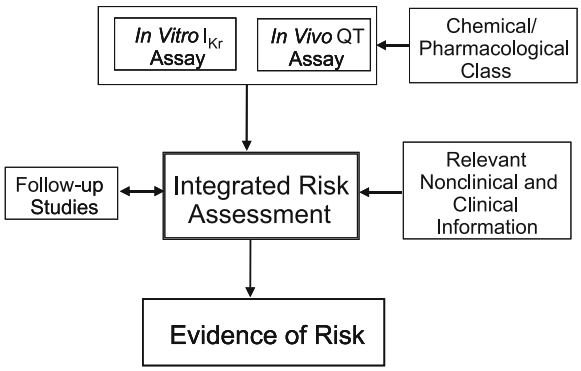

Fig. 2. Nonclinical Testing Strategy

clinical information becomes available. The Evidence of Risk summarizes the preclinical evaluation of the pro-arrhythmic potential for clinicians.

Parallel to the development of S7B a clinical guideline (ICH/E14) was drafted and reached step 4, also in 2005. During the discussion between these 2 expert groups, the question was raised again and again, if toxicologists could exclude any risk for QT prolongation for humans in their testing strategies. Of course they cannot, as clinicians cannot exclude any such risk for future patient generations based only on their clinical trial results. In addition, the Food and Drug Administration (FDA) is comparing preclinical data with clinical results and seems to have identified a few cases where QT prolongation was observed under clinical conditions while the preclinical tests were negative. This discrepancy is the basis for the diplomatic text in regard to the need for availability of S7B QT studies.

Timing of S7B Non-clinical Studies and Integrated Risk Assessment in Relation to Clinical Development/Step 4, June 2005, Brussels:

- Conduct of S7B non-clinical studies assessing the risk for delayed ventricular repolarization and QT interval prolongation prior to first administration in humans should be considered.
- These results, as part of an integrated risk assessment, can support the planning and interpretation of subsequent clinical studies.

The term "should be considered" allows flexibility either to do the studies before first time in humans or at a later stage of development. In practice, these studies are most often available before IND, because one wants to cope with this issue in time and wants to provide best safety to volunteers and patients.

But in conclusion is has to be stated that:

S7B proposes a series of non-clinical tests which it is believed can predict the likelihood that a compound will prolong cardiac repolarisation *in vivo*, in animals and in humans.

These data currently seem to have little impact on the clinical development proposals contained in the draft E14 guideline.

Immuntoxicology Studies (ICH/S8)

The guideline is restricted to unintended immunosuppression and immunoenhancement, excluding allergenicity or drug-specific autoimmunity. The guideline applies to new pharmaceuticals intended for use in humans, as well as to marketed drug products proposed for different indications or other variations on the current product label. The guideline does not apply to biotechnology-derived pharmaceutical products covered by ICH S6 and other biologicals.

Immunosuppression or enhancement can be associated with two distinct groups: (1) Drugs intended to modulate immune function for therapeutic purposes (e.g. to prevent organ transplant rejection) where adverse immunosuppression can be considered exaggerated pharmacodynamics. (2) Drugs not intended to affect immune function but cause immunotoxicity due, for instance, to necrosis or apoptosis of immune cells or interaction with cellular receptors shared by

both target tissues and non-target immune system cells.

Methods include standard toxicity studies (STS) and additional immunotoxicity studies conducted as appropriate. Whether additional immunotoxicity studies are appropriate should be determined by a weight of evidence review of cause(s) for concern.

Findings from standard toxicity studies (STS) pharmacological properties of the drug intended patient population structural similarities to known immunomodulators disposition of the drug clinical information.

Findings from standard toxicity studies (STS) pharmacological properties of the drug intended patient population structural similarities to known immunomodulators disposition of the drug clinical information.

The immunotoxicity evaluation follows a straightforward strategy which is detailed in Figures 3 and 4 below.

Hematological changes such as leukocytosis, granulocytopenia, or lymphopenia.

Alterations in immune system organ weights and/or histology (e.g. changes in thymus, spleen, lymph nodes, and/or bone marrow); changes in serum globulins that occur without a plausible explanation, such as effects on the liver, can be an indication that there are changes in serum immunoglobulins. Increased incidence of infections. Increased occurrence of tumors may be viewed as a sign of immunosuppression in the absence of other plausible causes such as genotoxicity, hormonal effects, or liver enzyme induction.

The assessment of immunotoxicity should include the following:

- statistical and biological significance of the changes
- severity of the effects dose/exposure relationship
- safety factor above the expected clinical dose
- treatment duration
- number of species and endpoints affected
- changes that may occur secondarily to other factors (e.g. stress)
- possible cellular targets and/or mechanism of action
- doses which produce these changes in relation to doses which produce other toxicities and reversibility of effect(s).

Additional immunotoxicity testing should be considered:

- If the pharmacological properties of a test compound indicate it has the potential to affect immune function (e.g. anti-inflammatory drugs). If the majority of the patient population for whom the drug is intended is immunocompromised by a disease state or concurrent therapy.
- If a compound is structurally similar to compounds with known immunosuppressive properties.
- If the compound and/or its metabolites are retained at high concentrations in cells of the immune system.
- If clinical findings suggestive of immunotoxicity in patients exposed to the drug occur.

If the weight-of-evidence review indicates that additional immunotoxicity studies are needed, there are a number of assays which can be used. It is recommended that an immune function study be conducted, such as a T-cell dependent antibody response (TDAR). If specific cell types are affected in STS not involving cells participating in a TDAR, assays that measure

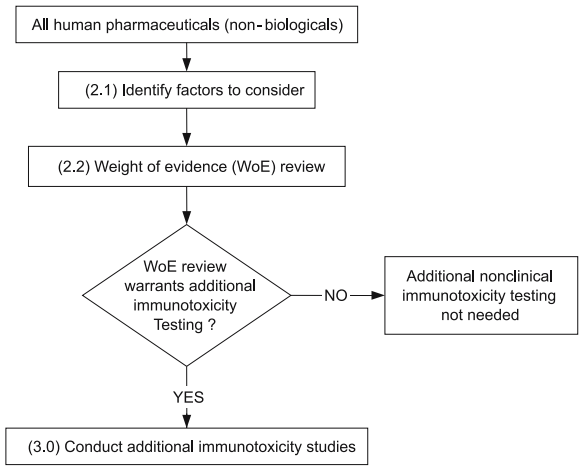

Fig. 3. ICH S8: Flow Diagram for Recommended Immunotoxicity Evaluation

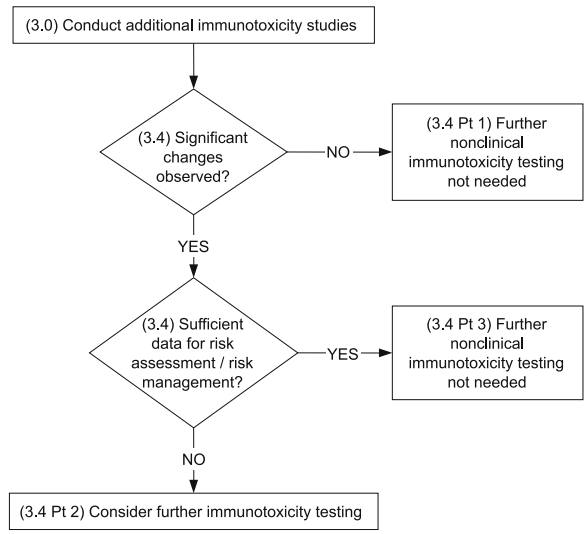

Fig. 4. ICH S8

Table 14 Standard toxicity studies

Area	Subject
Hematology	Total leukocyte counts and absolute differential leukocyte counts
Clinical Chemistry	Globulin levels and A/G ratios
Gross pathology	Lymphoid organs/tissues
Organ weights	thymus, spleen, (optional: lymph nodes)
Histology	thymus, spleen, draining lymph node and at least one additional lymph node, bonemarrow, Peyer's patch*, NALT**, BALT**

* oral administration only; ** inhalation administration only

function of that specific cell type might be conducted. Immunophenotyping of leukocyte populations, a non-functional assay, may be conducted to identify the specific cell populations affected and may provide useful clinical biomarkers.

Generally accepted: 28 consecutive daily doses in rodents. Adaptations of immunotoxicity assays have been described using non-rodent species. The species, strain, dose, duration, and route of administration used in immune function assays should be consistent, where possible, with the non-clinical toxicology study in which an adverse immune effect was observed.

Usually both sexes should be used in these studies, excluding non-human primates. The high dose should be above the no observed adverse effect level (NOAEL) but below a level inducing changes secondary to stress. Multiple dose levels are recommended in order to determine dose-response relationships and the dose at which no immunotoxicity is observed.

Additional studies may show that no risk of immunotoxicity can be detected and no further testing is needed.

Additional studies may demonstrate a risk of immunotoxicity, but there is not sufficient data to make a reasonable risk-benefit decision. In this case further testing may be of benefit to provide sufficient information for the risk-benefit decision. If the overall risk-benefit analysis suggests that the risk of immunotoxicity is considered acceptable and/or can be addressed in a risk management plan (see ICH E2E), then no further testing in animals might be called for.

If the weight-of-evidence review indicates the need for additional immunotoxicity studies, these should be completed before exposure of a large population of patients, usually Phase III. If the target patient population is immunocompromised, immunotoxicity testing can be initiated at an earlier time point in the development of the drug.

The different methods which are recommended for the evaluation of immuntoxic effects are summarized in Table 14.

Methods to Evaluate Immunotoxicity: Additional Immunotoxicity Studies

- T-cell Dependent Antibody Response (TDAR)
- Immunophenotyping (lymphocyte subsets)
- Natural Killer Cell Activity Assays
- Host Resistance Studies
- Macrophage/Neutrophil Function
- Assays to Measure Cell-Mediated Immunity.

This new ICH guideline replaces all guidances from EU, USA and Japan. It represents a very pragmatic approach and uses studies, e.g standard toxicity studies, which are conducted anyhow (see Table 15). There is great confidence in the prediction of these assays for any potential of new compounds to induce immune suppression or immune stimulation. This guideline helps to reduce the number of animals and requires additional studies only in special cases for concern.

III.B.5.1.10 ICH Multidisciplinary Guidelines M3 (Timing) and M4 (Common Technical Document)

ICH/M3 provides information about which studies – and of what duration – are needed before the different clinical phases of development can be started. In addition to this, timing of preclinical studies in dependence of clinical development plans, there are recommendations under which conditions different population can be included into clinical trials, populations such as men, women of childbearing potential or pregnant women and finally pediatric populations.

The objectives of M 3 are: To reduce differences between regions; to facilitate timely conduct of clinical trials; to reduce unnecessary use of animals and other resources; to promote early availability of new drugs.

The background of this guidance was that Regulatory recommendations differed among regions of Eu-

Table 15

ICH No.	Title	CPMP Doc. No.	Step
M3	Non-clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals		Step 5
M4	Common Technical Document		Step 5

rope, USA and Japan. Would it be possible to develop a mutually acceptable guidance?

The guideline provides general guidance for drug development. A guideline is not a legal requirement. Approaches should be scientifically and ethically appropriate.

The different endpoints are addressed in details in this note. Table 16 summarizes all study requirement before the first starting dose in humans.

The core battery of Safety Pharmacology is recommended to be conducted prior to first administration in humans. Any follow-up or supplemental studies as appropriate. During clinical development, a clarification of observed or suspected adverse effects in animals or during clinical trials may be needed. Before NDA, an assessment of effects on all systems should be provided.

For kinetics, an information on exposure data (AUC) in animals prior human clinical trials is needed; while ADME data are needed at completion of Phase I (Human Pharmacology) studies.

The duration for Repeat Dose Toxicity Studies is related to the duration of clinical trials and their therapeutic indication. In principle, the duration of animal studies are equal to or exceed the duration of the human clinical trials.

In general, there is a relationship of 1:1 ratio for studies in two mammalian species (one non-rodent). The details can be found in Table 16.

This table was updated by thorough additional Japanese studies in 2000 during the 5. ICH Conference in San Diego, USA. The Japanese scientists compared the utility of routine 4 week toxicity studies with 2 week studies and concluded that in regard to the prediction of toxicities to the male reproductive organs, 2 weeks studies were as valid as 4 week studies, therefore the former regional Japanese requirement to ask for a minimum duration of 4 week studies before starting trials in men was dropped for a global consensus that the minimum duration of non-clinical studies is 2 weeks in rodents and 2 weeks in non-rodents.

On the other hand, regional differences continue in regard to Single dose animal studies supporting single dose studies in humans:

In the US, single dose toxicity studies with extended examinations can support single-dose human trials. This concept encouraged the EU to offer comparable options with the Microdosing concept. This principle may be especially valuable for gaining early data for Go/No go decisions, when several candidates are being developed in parallel.

Results from 2 in vitro Genotoxicity studies are recommended to be available prior to first administration to humans, while the standard battery should be completed prior to initiation of Phase II studies.

Carcinogenicity studies do not need to be completed in advance of the conduct of clinical trials unless there is cause for concern (ICH: S1A).

For pharmaceuticals to treat certain serious diseases, carcinogenicity testing, if needed, may be concluded post-approval.

The inclusion of different patient populations reveals regional differences, especially for women with child bearing potential. There is a high level of concern for unintentional exposure of an embryo/fetus.

The currently regional differences in the timing of reproduction toxicity studies to support the inclusion of women with childbearing potential are:

1. **Japan:** assessment of female fertility and embryo-fetal development should be completed prior to the inclusion of women of childbearing potential using birth control in any type of clinical trial
2. **EU:** assessment of embryo-fetal development should be completed prior to Phase I trials in women and female fertility studies prior to Phase III trials
3. **US:** women of childbearing potential may be included in early, carefully monitored studies without reproduction toxicity studies provided appropriate precautions are taken to minimise risk
4. **US:** assessment of female fertility and embryo-fetal development should be completed before Phase III trials

The inclusion of children into clinical trials has gained tremendous interest. The following data are requested:

Table 16 Duration of Repeated Dose Toxicity Studies to Support Phase I and II Trials in EU and Phase I, II and III Trials in the US and Japan

Duration of Clinical Trials Single Dose	Minimum Duration of Toxicity Rodents	Non-rodents
Up to 2 Weeks	2 Weeks	2 Weeks
Up to 1 Month	2 Weeks	2 Weeks
Up to 3 Months	1 Month	1 Month
Up to 6 Months	3 Months	3 Months
> 6 Months Chronic	6 Months	6 Months
	6 Months	6–12 Months

- Safety data from previous adult human exposure:
  - most relevant information
  - necessary before pediatric clinical trials
- Prior trials:
  - appropriate repeated dose toxicity studies
  - all reproduction toxicity studies
  - standard battery of genotoxicity tests
- Juvenile animal studies should be considered
- Carcinogenicity testing:
  - prior to long-term exposure of children
  - cause for concern

III.B.5.1.11 Common Technical Document (ICH/M4)

M4 is another very important multidisciplinary guideline, which combines information for the 3 disciplines within the ICH Process: for Quality, Safety and Efficacy. The following section focuses predominantly on the preclinical safety issues. The total document is divided into 5 modules: Module 1 contains regional specific aspects, it provides for the European Union e.g. the European Community specific data. This module therefore is not harmonized but region-specific. Module 2 provides the Summaries for Quality, for Safety and Efficacy. The quality part uses as a headline "Quality Overall Summary", for safety and efficacy the terms "Non-Clinical or Clinical Overview". The different names signal that the quality part is a clear summary, while the non-clinical and clinical part should be critical evaluations. Module 3 provides chemical, pharmaceutical and biological information. Module 4 contains the non-clinical reports and Module 5 provides clinical study reports.

The Objectives of M4 are to assist authors in the preparation of nonclinical pharmacology, pharmacokinetics, and toxicology written summaries in an acceptable format. The CTD is not intended to indicate what studies are required, but provides an appropriate format for the nonclinical data. The Common Technical Document is nothing other than a placeholder for the different parts of a documentation for the market authorization process.

No guideline can cover all eventualities; common sense and a clear focus on needs of regulatory authority assessor are best guides to constructing an acceptable document. Therefore, modify the format if needed with the aim to provide best possible presentation and facilitate the understanding for the evaluation of the results.

The CTD-S is organized as follows:

F. Non-clinical summary

1. Pharmacology
a. Written summary
b. Tabulated summary

2. Pharmacokinetics
a. Written summary
b. Tabulated summary

3. Toxicology
a. Written summary
b. Tabulated summary

This organization is kept up in all parts of the dossier, it is repeated for the overview, the summaries, and the reports.

The detailed organization for Pharmacology, Kinetics and Toxicology are as follows:

1. Pharmacology Written Summary
   - Brief Summary
   - Primary Pharmacodynamics
   - Secondary Pharmacodynamics
   - Safety Pharmacology
   - Pharmacodynamic Drug Interactions
   - Discussion and Conclusions
   - Tables and Figures (either here, or included in text)

2. Pharmacokinetics Written Summary

- Brief Summary
- Methods of Analysis
- Absorption
- Distribution
- Metabolism
- Excretion
- Pharmacokinetic Drug Interactions (Non human)
- Other Pharmacokinetic Studies
- Discussion and Conclusions

3. Toxicology Written Summary

- Brief Summary
- Single-Dose Toxicity
- Repeat-Dose Toxicity
- Genotoxicity
- Carcinogenicity
- Reproduction Toxicity Local Tolerance
- Other Toxicity Studies
- Discussion and Conclusions

Examples of detailed advice for sections on discussion and conclusion of Pharmacokinetics: Information should be integrated across studies and across species, exposure in the test animals should be related to exposure in humans given the maximum intended doses.

Similar examples for Toxicology: in vitro studies should precede in vivo studies. Where multiple studies of the same type need to be summarized within the Pharmacokinetics and Toxicology sections, studies should be ordered by species, by route, and then by duration (shortest duration first).

The species should be ordered as follows: 1. mouse; 2. rat; 3. hamster; 4. other rodent; 5. rabbit; 6. dog; 7. non-human primate; 8. other non-rodent mammal; 9. non-mammals (see also Table 17.)

It is also recommended to limit the information in the summaries and overview. The Overview should contain the essential and critical results on approximately 30 pages. The length of the Nonclinical Written Summaries should in general not exceed 100–150 pages.

The brief Summaries for Pharmacology should be written on 2–3 pages, for Pharmacokinetics the same length and for Toxicology approximately 6 pages.

Module 2 contains, in addition, 34 templates and 31 examples.

The examples have been taken from real dossiers and give good orientation on how to fill out the templates.

For illustration of the templates see Table 18 for Pharmacology, Table 19 as summary for Toxicology and Table 20 with details for a Repeat Dose Toxicity Study.

Finally, the following list shows examples of recommendations for Module 4:

The appropriate location for individual-animal data is in the study report or as an appendix to the study report.

4.1 Table of Contents

A Table of Contents should be provided that lists all of the nonclinical study reports and gives the location of each study report in the Common Technical Document.

Study Reports

The study reports should be presented in the following order:

4.2 Pharmacology

4.2.1 Primary Pharmacodynamics

4.2.2 Secondary Pharmacodynamics

4.2.3 Safety Pharmacology

4.2.4 Pharmacodynamic Drug Interactions

4.3 Pharmacokinetics

4.3.1 Analytical Methods and Validation Reports (if separate reports are available)

4.3.2 Absorption

4.3.3 Distribution

4.3.4 Metabolism

4.3.5 Excretion

4.3.6 Pharmacokinetic Drug interactions (nonclinical)

4.3.7 Other Pharmacokinetic Studies

4.4 Toxicology

4.4.1 Single-Dose Toxicity (in order by species, by route)

4.4.2 Repeat-Dose Toxicity (in order by species, by route, by duration; including supportive toxicokinetics evaluations)

4.4.3 Genotoxicity

4.4.3.1 In vitro

4.4.3.2 In vivo (including supportive toxicokinetics evaluations)

4.4.4 Carcinogenicity (including supportive toxicokinetics evaluations)

4.4.4.1 Long-term studies (in order by species; including range-finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics)

4.4.4.2 Short- or medium-term studies (including range-finding studies that cannot appropriately be included under repeat-dose toxicity or pharmacokinetics)

4.4.4.3 Other studies

Table 17 Recommendations on how to summarize studies and tables

Studies	Recommendations
Repeat-Dose Toxicity (including supportive toxicokinetics evaluation)/CTD-S	summarise in order by species, by route, and by duration give brief details of the methodology highlight important findings (e.g., nature and severity of target organ toxicity, dose (exposure)/response relationships, no observed adverse effect levels, etc.) non-pivotal studies can be summarized in less detail (pivotal studies are the definitive GLP studies specified by ICH Guideline M3).
Carcinogenicity (including supportive toxicokinetics evaluations)/CTD-S	a brief rationale should explain why the studies were chosen the basis for high-dose selection. Individual studies should be summarised inthe following order: • Long-term studies (in order by species; including range-finding studiesthat cannot appropriately be included under repeat-dose toxicity orpharmacokinetics) • Short- or medium-term studies (including range-finding studies that cannotappropriately be included under repeat-dose toxicity or pharmacokinetics) • Other studies
Other Toxicity Studies (if available)/CTD-S	If other studies have been performed, they should be summarized. When appropriate, the rationale for conducting the studies should be provided. • Antigenicity • Immunotoxicity • Mechanistic studies (if not reported elsewhere) • Dependence • Studies on metabolites • Studies on impurities • Other studies

Table 18

1 Pharmacology			**Overview**	**Test Article**:
Type of Study	**Test System**	**Method of Administration**	**Study Number**	**Location Vol. Page**
1.1 Primary Pharmacodynamics				
1.2 Secondary Pharmacodynamics				
Safety Pharmacology				

4.4.5 Reproductive and Developmental Toxicity (including range-finding studies and supportive toxicokinetics evaluations). (If modified study designs are used, the following subheadings should be modified accordingly.)

4.4.5.1 Fertility and early embryonic development

4.4.5.2 Embryo-fetal development

4.4.5.3 Prenatal and postnatal development, including maternal function

4.4.5.4 Studies in which the offspring (juvenile animals) are dosed and/or further evaluated.

4.4.6 Local Tolerance

4.4.7 Other Toxicity Studies (if available)

4.4.7.1 Antigenicity

4.4.7.2 Immunotoxicity

4.4.7.3 Mechanistic studies (if not included elsewhere)

4.4.7.4 Dependence

4.4.7.5 Metabolites

4.4.7.6 Impurities

4.4.7.7 Other

4.5 Key Literature References

This CTD has been tested in practice for several years. Although not perfect for every case, it has proved its usefulness. Industry knows where to place specific information and data, and regulators know where to find them. This has facilitated the review process tremendously, only one dossier is necessary for international registration, a lot of resources can be diverted to more important issues.

But the CTD is a living document, its weakness is apparent. In order to improve its quality and practicability, suggestions and proposals for improvements are invited from the public. These modifications are

Table 19

3A Toxicology Type of Study	Species and Strain	Method of Administration	Overview Duration of Dosing	Doses mg/kg	GLP Compliance	Test Article: Testing Facility	Study Number	Location Vol.
nsingle-Dose Toxicity								
3.2 repeat-Dose Toxicity								
Genotoxicity								
3.4 Carcinogenicity								
3.5 Reproductive & Developmental Toxicity								
3.6 Local Tolerance								
3.7 Other Toxicity Studies								

Table 20

3.2 Repeat-Dose Toxicity	Report Title:							Test Article
Species/Strain:	Duration of Dosing							Study No.
Initial Age:	Duration of Postdose							Location in CTD: Vol. page
Date of First Dose:	Method of Administration Vehicle/Formulation							
Special Features:								
No Observed Adverse-Effect Level:								
Daily Dose (mg/kg)	0 Control							
Number of Animals	M	M:	F:	M:	F:	M:	F:	M:
Toxicokinetics: AUC ( )								
Noteworthy Findings								
Died or Sacrificed Moribund								
Body Weight (%[a])								
Food Consumption (%[a])								
Water Consumption ( )								
Clinical Observations								
Ophthalmoscopy								
Electrocardiography								

being dealt with within the process of "Questions and Answers", the ICH experts publish their conclusions regularly.

III.B.5.1.12 Outlook and Future of ICH

ICH can look back to 15 years of achievements. As the most successful international harmonization initiative in the world, its achievements include: an understanding of innovation; a common regulatory platform based on more than 40 guidelines; improvement of scientific guidelines; facilitating communication between the regulators, and between industry and the regulatory authorities; effective use of research and development (R&D) resources; greater mutual acceptance of R&D data realized.

In addition, the drafting process of guidelines has identified gaps in science, new studies analysing problems have been conducted to validate the assays being used to confirm safe use of drug in humans, like validity of 2 weeks studies to assess male fertility or the validity of transgenic mouse models for assessing the carcinogenic potential within the ILSI/HESI evaluation process.

CHMP Guidance Documents in Preparation/Released for Consultation:

1. Guideline on Environmental Risk Assessments for Pharmaceuticals
2. Guideline on the Limits for Genotoxic Impurities

3. Guideline on the Need for Pre-clinical Testing of Human pharmaceuticals in Juvenile Animals
4. Guideline on Drug-induced Hepatotoxicity
5. Guideline on the Non-Clinical Development of Fixed Combinations of Medicinal Products
6. Guideline on Risk Assessment of Medicinal Products on Human Reproductive and Development Toxicities: from Data to Labelling1. Guideline on Environmental Risk Assessments
7. Guideline on the Investigation of Dependance Potential of Medicinal Products
8. Guideline on the Assessment of Carcinogenic and Mutagenic Potential of Anti-HIV Medicinal Products
9. Guideline on the Non-Clinical Testing for Inadvertent Germ line Transmission of Gene Transfer Vectors
10. Guideline on Adjuvants in Vaccines
11. Points to Consider Document on Xenogenic Cell Therapy
12. Guideline on the Comparability of Biotechnology Products, Preclinical and Clinical Issues
13. Guideline on the Evalutaion of Medicinal Products intended for Treatment of Chronic Hepatitis B

REFERENCES

From http://www.emea.eu.int/htms/human/ich/safety/ichfin.htm:
Topic S1A Note for Guidance on the need for Carcinogenicity Studies of Pharmaceuticals
Topic S1B Note for Guidance on Carcinogenicity: Testing for Carcinogenicity of Pharmaceuticals
Topic S1C Note for Guidance on Dose Selection for Carcinogenicity Studies of Pharmaceutical
Topic S1C(R) Note for Guidance on Dose Selection for Carcinogenicity Studies of Pharmaceuticals: Addition of a Limited Dose and related Notes
Topic S2A Note for Guidance on Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals
Topic S2B Note for Guidance on Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals
Topic S3A Note for Guidance on Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies
Topic S3B Note for Guidance on PharmacoKinetics: Guidance for Repeated Dose Tissue Distribution Studies
Topic S4A Note for guidance on Duration of Chronic Toxicity Testing in Animals (Rodent and non Rodent Toxicity Testing)
Topic S5A Note for Guidance on Reproductive Toxicology: Detection of Toxicity to Reproduction for Medicinal Products
Topic S5B Note for Guidance on Reproductive Toxicology: Toxicity on Male Fertility
Topic S6 Note for Preclinical Safety Evaluation of Biotechnology-Derived Products
Topic S7A Note for Guidance on Safety Pharmacology Studies for Human Pharmaceuticals
Topic S7B Note for Guidance on Non-Clinical Evaluation of the potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals
Topic S8 Immunotoxicity Studies for Human Pharmaceutical

Chapter III.C
General Toxicity

Dieter Mayer

III.C.0.1
Acute Toxicity

PURPOSE AND RATIONALE

Acute toxicity testing determines the toxicity of a chemical/drug substance after single administration using different routes of administration (oral, dermal, sub-cutaneous, intra-venous, intra-peritoneal, via inhalation = pulmonary). The main purpose of acute toxicity studies is to evaluate the degree of toxicity in a quantitative and qualitative manner with the purpose of comparing it with other drug substances (e.g. other drug candidates for the same indication). Further, acute toxicity testing provides information about the acute toxic effects of a chemical in a qualitative manner i.e. it generates information about acute mechanisms of toxicity. Another purpose is to develop first ideas about dose levels to be tested in studies with multiple administrations (sub-chronic, chronic studies). The time between administration, onset, and disappearance of toxic signs can provide valuable information about the toxic-kinetic behavior of the compound (Dekant and Vamvakas 1994).

Testing of combination of drug substances gives information about additive, over-additive or under-additive toxic effects of the combination partners.

The method of determination has changed in the last three decades mainly for animal welfare reasons. Producing mortality in animals in order to determine an LD50 (dosis letalis media) is no longer the main purpose of acute toxicity testing. Today, acute toxicity focuses on levels of acute tolerance, nature of acute toxic symptoms in the sub-lethal range, and dose levels which cause mortality in few animals, i.e. quality has replaced quantity. This principle can be followed by using 3–5 animals per sex and dosage group.

PROCEDURE

Drug Substance

For toxicity testing, the quality of the drug substance should be similar to that intended to be used in clinical trials, and, after approval, to that for marketing purposes, i.e. the pattern of impurities should be similar. This is the experience of three decades of work in drug development. The quality of a drug product for toxicity testing should not be too sophisticated because changes of the route of synthesis may occur during development and scaling up of synthesis. If the content of impurities changes later in the bigger scale production, the question is raised whether the toxicity data with no or fewer impurities are valid for the drug product emerging from the large scale production process with a higher quantity of impurities. A similar philosophy should be applied also for degradation products, i.e. if degradation occurs, the toxicity of degradation products should be covered as well. Also physical properties, such as particle size of insoluble drug substances, which are administered in the form of a suspension, should be similar because differences of particle size can significantly alter the degree of intestinal absorption.

In case of drug combinations each drug substance has to be tested separately in addition to the determination of the combination. Additive effects (over, under-additive) can be investigated by comparing the single constituents with the combination.

Animals

For pharmaceuticals at least two mammalian animal species are used. The studies should be conducted with established animal strains. Normally, rats (e.g. Sprague-Dawley, Wistar or Fisher) and mice (e.g. NMRI or CD1) or hamsters of both sexes are used.

In addition, the animals used should be characterized with regard to age, sex, body weight, breeding facility (origin of the animals and acclimatization (at least 14 days) to the environment in the animal facility.

Other factors like feeding conditions (fed or fasted) should be standardized as well. Usually, animals are fasted over night before they are treated. Feeding conditions as well as environmental factors (temperature in the animal room, humidity in the ambient air) can significantly alter the results of the toxicity testing.

Allocation of Animals to Treatment Groups

All test animals, from acute to chronic and carcinogenicity studies, should randomly be attributed to the test group. Randomization avoids influences of the selection process of the animals during the allocation process. Random tables exist in computer programs designed for toxicity studies. No in vivo study should be conducted without attributing animals to groups by means other than random events. Otherwise the study is severely compromised.

Mode of Administration

In rodents, two routes of administration have to be chosen, one should be similar to the intended route in human patients and the second should guarantee the full uptake (100 % absorption) of the drug substance, e.g. in case the intended clinical route of administration is the oral route, the first route in animals is the oral route and the second the i.v. administration. If the intended clinical route is parenteral (e.g. intravenous) the determination of acute toxicity must be performed by this route only (Stötzer 1989).

Conditions of Administration

The pH value of solutions should be considered. Deviations from the physiological pH (= 7,4) should be avoided. In general, solutions with an acid pH are better tolerated than solutions with an alkaline pH. Deviations from the "physiological" pH should be avoided for the parenteral route of administration because local reactions at the site of injection can significantly influence the results. In addition, animal wellfare aspects play an important role with regard to severe local reactions (irritation, corrosion) induced by administration. In case of i.v. administrations, osmolarity of the solutions should not deviate significantly from the physiological range. Otherwise hemolysis occurs. The volume of a preparation of a drug substance should not exceed 10 ml/kg body weight in case of oral administration and for parenteral 2–5 ml/kg body weight.

Dose Levels

The dose levels should be chosen in a manner that significant toxicity can be induced. In rodents, a quantitative evaluation of the approximative lethal dose should be performed.

Observations

All animals used for the study, those surviving for a period of about 14 days after treatment and those being lethally intoxicated have to be necropsied. In addition, body weight development, food and water consumption should be noted at weekly intervals, urine and faeces analysis, cardiovascular functions, hematological parameters respiration and spontaneous behavior such as motor and sensory functions. All tissues showing macroscopically visible changes should be analyzed histologically (Dekant and Vamvakas 1994).

Reporting and Evaluation

All results have to be described in detail with regard to body weight development, feed consumption, symptoms of intoxication and morphological investigation. In case the results allow for a statistical evaluation adequate methods exist (FDA 1996).

MODIFICATION OF THE METHOD

The Traditional Method

Until the end of the 70s it was obligatory to determine the "dosis letalis media", the LD50, or LC50 (for aerosols), the dose level which intoxicates 50 % of the animals lethally. The exact determination of the LD50 is still needed for chemicals, like pesticides and others like pharmaceuticals, for the approval in certain countries (e.g. Japan). The determination of the LD50 (LC50) required more animals than the new, approximative method. Five or ten animals per dose level and

sex were treated with one dose level. At least three to four doses had to be tested in order to establish a dose response relationship. With the "Probit Analysis", an approximative statistical method, the LD50 value with the 95 % confidence interval could be calculated, i.e. the likely hood was 95 % that the real LD50 is lies between the upper and the lower limit of the confidence interval. However, toxicologists found that this accuracy is an artificial one, because other variables of the methodology, like strain of animals used, feeding status, housing conditions, volume of administration, influenced the LD50 value more than the number of animals used. In 60 laboratories around the world, defined chemicals were tested using a more or less standardized protocol. The LD50 values determined varied by a factor of 14, demonstrating that accuracy of the traditional method is highly artificial and does not contribute significantly to the safety of drugs. For the development of new drugs the determination of "no observable toxic effect levels" (NOTEL) and the symptoms of toxicity after multiple administration are more important than the establishment of an LD50 in a rodent experiment.

III.C.0.2 Subacute and Chronic Toxicity Studies

PURPOSE AND RATIONALE

The development of a pharmaceutical is a step by step process with evaluation of both animal and human safety information.

The purpose of the non-clinical sub-acute and chronic testing is the identification of target organs of toxicity, response to the administered dose levels, relationship to "internal exposure" and potential reversibility. "Internal exposure" means blood or tissue levels achieved in toxicological animal experiments after administration of different dose level of the drug. The comparison of "internal" exposure in animal test species and humans results in "safety factors" which are more reliable for the toxicological risk estimation in clinical trials and in the marketing phase of drugs. This information is important for the estimation of an initial starting dose for the human trials and the identification of target organs and target cells (e.g. blood cells) for clinical monitoring of potential adverse effects (Rennwick 1991).

The purpose of sub-acute to chronic toxicology studies is to provide a reliable set of information on the dose levels to be administered in the different phases of clinical development. Further, these studies are a prerequisite for international approval.

The Importance of Metabolism for the Selection of an Adequate Animal Model

Toxicity is considerably influenced by the metabolic transformation of drug substances. If the animal experiment does not simulate the metabolism in humans, important toxicity can be missed, or visa versa, toxicity in animals occurs but not in humans if animals generate a toxic metabolite not occurring in humans. Therefore, it is necessary to perform metabolism studies in animals prior to human phase I studies. The results of these animal studies can be compared with first studies in human hepatocytes in culture or with the S9 fraction of human liver. The availability and use of human hepatocytes or/and human hepatic metabolically active S9 fraction and its use to investigate comparative hepatic metabolism caused a "quantum leap" in the safe use of drugs in the development phase. The comparison of human in vitro metabolism with the metabolism in different animal species and the selection of animal species according to human metabolism has become a extremely important procedure and has increased the validity of animal results in toxicological studies in general (e.g. also in pharmacology studies (see also the section on preclinical metabolism studies with human hepatic tissue).

PROCEDURE

Drug Substance

As described in the "Acute Toxicity" section the drug substance should be qualified at least to the same degree as required for the acute toxicity testing. In this phase, the chemical and analytical development should be at an advanced stage and a decision on a more or less definitive salt should have been performed. In this phase of development the drug substance is prepared for being administered first time in man. A lot of other activities are going on in addition to drug safety. Therefore, the salt selection is an important decision. In case of changes of the salt form, toxicity studies frequently have to be repeated, especially when the change of the salt influences the bioavailability. Regarding purity of the drug substance, the same holds true as for the acute studies. The availability of an analytical certificate according to GLP guidelines is of greatest importance.

Animals

For testing pharmaceuticals, two animal species are required, one rodent and one non-rodent species, usually the rat and the dog. The decision on the selection of the animal species is based on the kinetic and metabolic characteristics of the drug substance.

Regarding metabolism, a comparison of the metabolic fate is conducted based on results in animal and human hepatocytes or S9 fraction derived from human and test animal species. If a major metabolite occurs in man, but not in the animal species selected for toxicity testing, other species have to be selected according to metabolic characteristics observed in man. Alternative animal species are the mouse, hamster, or the monkey (Rhesus, Cynomolgus, Baboon) the pig (Göttinger Minipig) (non-rodent) etc. The selection of the species can be difficult. In case a high first pass effect is seen in animal but not in man, e.g. exposure to the original drug substance is low or not measurable in animals but in man, the responsible toxicologist must address the issue. One possible solution in this situation is testing separately the metabolite which occurs only in man.

Strains of animals used most frequently and available on the market are:

- Swiss mice
- NMRI mice
- Wistar rats
- Sprague Dawley rats
- Beagle dogs
- Guinea pigs
- Himalayan rabbits
- New Zealand white rabbits
- Cynomolgus monkeys
- Rhesus monkeys
- Baboons
- Mini pigs.

At the start of the toxicological tests the rodents should have an age from 6–8 weeks, the dog about 8–12 months. With regard to other non-rodent species, the animals should be young but sexually mature.

The animals should be purchased from a certified breeder which guarantees a standardized good quality (proof of absence of species specific diseases) otherwise problems with the acceptance of the study-results can occur.

For rodent studies the number of animals used in a study depends on the species and duration of administration. For 14-day studies at least 10 animals per sex and dose level, including the same number of animals for untreated controls, should be used. Of great scientific interest can be the question whether drug induced symptoms disappear after the end of the treatment period. Therefore, additional animals, at least 5 per dose group, should be used to investigate recovery from the symptoms. In longer term studies (e.g. 3 and 6 months) animal numbers in rodent studies should be 20 to 25 animals per dose group and sex.

In case of non-rodent studies the minimum requirement is 3 animals per dose level and sex (one month studies). In longer term studies animal numbers should be increased up to 6 animals per dose and sex (e.g. in chronic studies).

The rationale for the need of recovery animals was frequently discussed. For drug substances which require chronic (live time) treatment (e.g. oral anti-diabetics, drugs for treatment of hypertension, anti-Parkinson drugs, etc.) the question of recovery is less important than in the case of anti-infectives with, in most cases, short treatment periods where mild symptoms of intolerance, e.g. diarrhoea, are observed. However, inclusion of recovery animals is recommended in general because at the stage of development where first clinical studies are conducted, the whole set of indications is not finally known and line extensions can happen. One example is the use of quinolones and other anti-infectives for the treatment of cystic fibrosis. Another example is drugs used for chemotherapy of malignant diseases where recovery has to be studies anyway.

The longer term toxicology studies have to be conducted under special housing condition. For rodents, the room temperature should be in the range of 22 °C and a humidity of about 55 %. In non-rodents (e.g. dog) the study must not be conducted in strictly controlled animal rooms, however, the room temperature should not be lower than 18 °C. In Europe, especially in Germany, a dog must have access to open runs at least for 6 hours per day.

Rodents can be kept in groups of 5 animals per sex. However, in longer term studies with treatment periods of longer than 6 month, rodents should be kept single in order to avoid loss of animals by cannibalism. For non-rodent species, their natural behavior guides the conditions of housing (e.g. dogs are kept in groups). There are often situations where national veterinary animal welfare considerations are in conflict with scientific needs of a toxicology study. In general, the acceptance of such conditions by the animal welfare administration can be achieved if the scientific need for special maintenance can be provided.

Conditions and Mode of Administration

In general, the mode of administration should be the same as intended for human therapy. The situation for the oral (gavage), pulmonary and dermal route of administration does not cause problems because daily treatments can be performed without methodological difficulties. In case of parenteral administration, such as intravenous, intra-peritoneal, subcutaneous and

intramuscular, local tolerance plays an important role. Although veins in man and large animals (dog, pig, monkey, injection is preferably done in the cephalic ante-brachii or femural vein) show relatively good tolerance regarding osmotic and pH conditions of the i.v. formulation, in the rodents local tolerance in a small blood vessel, the tail vein of rodents, can cause problems. Inflammation and finally necrosis of the tail vein compared to the diameter of the injection needle (recommended for i.v. administration) prohibit repeated i.v. treatments in many cases. In such cases, the mode of administration can be changed from intravenous to intra-peritoneal or subcutaneous treatments. Kinetic considerations should dominate the decision on the alternative mode of administration. Intravenous treatment causes high blood levels immediately after administration, and in most cases, also intra-peritoneal administrations, but not sub-cutaneous administration. In case intravenous injection has to be replaced due to limiting venous tolerance, the alternative is in many cases the intraperitoneal route. However, chronic adhesive peritonitis can occur, especially if the technician conducting the daily injections is not experienced. Insolubility of the drug substance in "physiological" vehicles (e.g. water) prohibits intravenous administration. In such cases, the intra-peritoneal or the sub-cutaneous route has to be chosen.

The use of gelatine capsules filled with the bulk drug substance can be applied for large animals, especially dogs. Another method is the intra-oesophageal administration of the dissolved drug or the drug as a suspension by means of a rubber hose.

For dermal toxicity testing the drug substance is applied by innunction of a certain volume onto a depilated (mechanically or by depilatories) skin areas. However, these methods can alter the permeability of the skin. In order to avoid licking off from the treated skin areas, "Elizabethan" collars can be fixed around the neck of the animals.

For subcutaneous injections a large skin surface area is available for daily applications. It is recommended to change the site of injections continuously because repeated injection to the same site can cause fibrotic reactions and, in case of treatments for a period of 1 year and longer, induce subcutaneous tumors especially in small rodents. The risk of inducing local skin tumors is high if the pH value of the administration solution is below 5.

Implantation of bio-polymers loaded with the drug substance is often used, especially if this mode of treatment is also planned for clinical use. However, this technique requires special stability studies and information of the release of the dug substance from the device. This technique has been shown to be very useful for testing highly active peptides such as hormones (e.g. gonadotropins and their antagonists and other "biological" proteins).

Continuous infusion may also be a mode of administration if the same mode administration is planned for clinical use or if the solubility is too low to achieve blood levels after single day injections. Infusions can be conducted either by external pumps connected to the animals vein or by internal pumps implanted subcutaneously. This mode of administration requires special equipments and great experience by the technicians. A major concern is to avoid infections of the implantation site. These types of studies should be confined to special laboratories with the necessary equipment and experience because under certain circumstances the time of infusion is 24 hours per day.

Exposure by inhalation is necessary when the clinical use is by inhalation (e.g. anti-asthmatics, propellants as excipients in metered dose inhalers). For testing pharmaceutical products, the "nose only" exposure is the preferred method. Whole body exposure can result in dermal absorption of the drug substance and oral uptake by licking the skin which is contaminated with drug particles. Small rodents are put into cylindrical holders made from Plexiglas fitted with conical head pieces, in such a manner that only the nose is exposed to the drug substance. These holders are fixed into a cylindrical container which is connected to the dust generator and to flow meters. The air flow in relation to the dust generated or real gases, determines the concentration of the substance to be tested. Inhaled concentrations have to be analyzed carefully because the "calculated" dose never corresponds to the concentration to which the animals are exposed. The analysis of the inhaled concentrations should be measured in the inhalation chamber at the same level where the noses of the rat are located.

For testing aerosols the determination of the particle size distribution because large particles ($> 0.3\ \mu m$) do not reach the site of major absorption. For inhalation studies it is very important to measure internal exposure.

In case of non-rodents the drug is administered via "gas masks".

Dose Levels

The selection of dose levels is a very critical step. Although relatively precisely described in international toxicology guidelines, the selection requires experience and the capability to understand the phar-

macodynamic mode of action and the kinetic behavior of the drug (see examples below).

In general, three dose levels should be selected. In addition, negative control group should be treated in parallel with the vehicle only. The high dose level should produce clear signs of toxicity (e.g. ideally mortality of about 10 % of the animals). The mid dose levels should provoke only mild sings of toxicity and the low dose level should result in no toxicity, i.e. the low dose level should enable the toxicologist to determine the "No Observable Adverse Effect level" (NOAEL). This principle sounds simple, however to fulfil it requires many years of experience. In case of drug substances with low toxicity the upper limit dose is 2000 mg/kg body weight.

Vehicles

No problem exists if the drug substance is water-soluble. However, considerations on the osmolarity should be conducted, especially if the drug is administered by the i.v. route because hemolysis can be induced. This can be an issue mostly in small rodents because the small diameter of veins inhibits a rapid dissolution of the administered drug solution in the blood especially when the injection volume is high or high concentrations of the drug substance can cause irritation and phlebitis. Therefore, it is recommended to perform pilot studies in order to avoid the risk of hemolysis or venous intolerance. Other vehicles for parenteral administration are solutions of low molecular weight polyethylene glycol (PEG 400), myglyol etc.

If the drug is insoluble in water, suspensions have to be administered. In such cases vehicles like sesame oil, hydroxy-methyl-cellulose or hydroxy-ethyl-cellulose can be used. The selection of the vehicle is a critical issue because of inherent toxicity of the vehicle used frequently for kinetic and metabolism studies (e.g. vehicles which form metabolically oxalic acid). Natural oils such as sesame or corn oil can be used. However, it should be checked whether the use of oily vehicles alters the gastrointestinal absorption due to their laxative properties and whether oily vehicles can cause "oil granulomas" (reaction to oily vehicles in the reticulo-endothelial system) in case of parenteral administration.

A constant volume of the vehicle should be administered. 10 ml/kg body weight is considered as the maximum volume.

The stability of the drug substance needs to be determined prior to the beginning of the study. In case of application of suspensions, the homogenous distribution of the particles in the vehicles must be investigated. Homogeneity of a suspension can be achieved by filling the syringe directly from a glass container put on a stirrer. Immediate administration to the test animals is required.

Time and Duration of Administration

The time of the daily administrations should be standardized, i.e. the administrations should occur in a defined time interval. Rats are dosed usually in the morning. Larger non-rodent species should be fasted before administration occurs. After administration food can be offered. This procedure avoids the influence of "food effects". In special circumstances the time of dosing can be adjusted to the conditions which cause optimal bioavailability of the drug and its metabolites.

The duration of treatment in toxicology studies depends on the duration of clinical studies or on the therapeutic treatment schedule. This is outlined in the following table (Fed. Reg. 1999).

Table 2 also reflects the marketing recommendations in the three regions except that a chronic non-rodent study is recommended for clinical use $>$ 1 month.

Observations

The test animals should be maintained in the testing facility before the administration starts. In the case of rodents, a one week acclimatization period is necessary. In the case of the use of non-rodents at least 4 weeks are necessary. For animals imported from other (tropic or sub-tropic) countries, e.g. monkeys, longer periods are necessary for stabilization and required by veterinary regulations (acclimatization and quarantine). The comparison of data from pre-tests with results after certain periods of exposure can provide information with regard to time of development of symptoms.

Before the treatment period starts, behavioral, hematological, clinical chemistry, and urinary parameters have to be determined in order to assure a proper health status of the animals. If one of these parameters is out of the normal range the animal is useless for toxicology studies or even contributes to a wrong interpretation of results. Further, an acclimatization period should guarantee the adaptation to the new environment in the testing facility.

Determination of General Condition and Behavior (Non-invasive Methods)

To achieve a continuous and correct description of symptoms it is advisable to develop a simple glossary for clinical symptoms. The glossary should be adapted to the degree of diagnostic capabilities of the animal

Table 1

Duration of clinical trials	Minimum duration of repeated dose studies	
	Rodents	Non-rodents
Single dose	2–4 weeks[2]	2 weeks
Up to 2 weeks	2–4 weeks[2]	2 weeks
Up to 1 month	1 month	1 month
Up to 3 months	3 months	3 months
Up to 6 months	6 months	6 months
> 6 months	6 months	Chronic[3]

[1] In Japan, if there are no phase II clinical trials of equivalent duration to the planned phase III trials, conduct of longer duration toxicity studies should be considered as shown in the next table.
[2] In the EU and the USA, two week studies are the minimum duration. In Japan, 2 week non-rodent and 4 week non-rodent studies are needed. In the USA, as an alternative to 2-week studies, single dose toxicity studies with extended examinations (hematology, clinical chemistry, urinalysis, macroscopic and microscopic pathology) can support single dose human trials.
[3] Data from 6 months of administration in non-rodents should be available before the initiation of clinical trials longer than 3 months. Alternatively, if applicable, data from a 9-month non-rodent study should be available before the treatment duration exceeds that which is supported by the available toxicity studies.
The following table describes the duration of repeat toxicity studies to support phase III clinical trials in the EU and Marketing in all regions[1].

Table 2

Duration of clinical trials	Minimum duration of repeated dose studies	
	Rodents	Non-rodents
Up to 2 weeks	1 month	1 month
Up to 1 month	3 months	3 months
Up to 3 months	6 months	3 months
> 3 months	6 months	Chronic

laboratory staff. Further, a glossary facilitates the interpretation of results with regard to reporting. A uniform glossary is also recommended for drug developments which occur on different sites. Today, outsourcing of chronic studies is a standard procedure also for large drug companies. Therefore, an identical glossary should be agreed between the sponsor and the Contract Research Organization (CRO). This procedure facilitates considerably the interpretation of the results to be prepared for the submissions to international authorities. Therefore, the large CROs and drug companies should co-operate with companies developing and using remote data acquisition software.

Diagnosis of general appearance and symptoms should be made on a daily basis. If symptoms are recorded, they should be described in dependence to time of appearance after dosing and disappearance. It is the task of the study director to perform special training of the laboratory staff in order to guarantee proper diagnostic skills and to check at intervals the correctness of the diagnoses. Ideally, after administration of the test material and in the late afternoon, observations should be conducted and recorded at least twice a day.

The observations should include the following organs/tissues/functions:

- skin (color, temperature, turgor)
- fur (hair loss, piloerrection, color)
- eyes (myosis, mydriasis, reflex to light, cornea and lens for opacity/turbidity, color of the retina)
- mucous membranes (color, ulcers, moisture)
- respiratory function (tachypnoe and changes of the respiratory rhythm)
- circulatory system (heart rate, blood pressure and ECG in large animals)
- autonomic and central nervous system (e.g. irritability, somnolence, reduced/enhanced motility, drowsiness, appearance of the faeces and urine)
- and general behavior (anything which deviates from normal behavior).

Body Weight

The body weight determination is a simple but important parameter in toxicity testing. Experience shows that the influence on body weight development is one of the most sensitive indicators for the disturbance of the homeostasis. Body weights should be measured at least once a week.

Feed Consumption

In case of rodent studies the feed consumption should be determined at least at weekly intervals. In feeding studies the drug substance is mixed with the rodent diet. From that information is provided on the dose taken by the animal. However, dietary administration is not the preferred method of administration. "Wasting" of food is a well known phenomenon in rodents. Therefore, an accurate determination of the food and drug intake is not possible. Drug agencies and other scientists working in experimental drug toxicology are not in favor of dietary studies, they prefer the use of intra-oesophageal or intra-gastric syringes. By this method a dose or volume can be administered with very great accuracy. On the other hand, it simulates better the mode of administration of most pharmaceuticals. Feeding studies are the preferred method to study possible effects of direct and indirect food additives because it simulates in a better way the uptake conditions. Whenever food intake occurs, the test animal is exposed to the food additive. The different modes of administration naturally have effects on the kinetics. In case of gavage administration, blood level peaks are observed at a certain interval after administration. In contrast, under conditions of a study with dietary administration, the drug is taken up in a more "continuous" manner and peaks in the blood level curve are lower or do not exist. This can significantly influence the result of a toxicity study if its toxicity is more determined by maximum blood concentrations (C_{max}) rather than by its "Area Under the Curve" values (AUCs).

The relation of body weight development: food intake is an important parameter in toxicology studies. "Normal" feed uptake connected with lower body weight body development can indicate an effect on feed utilization (metabolic disorders e.g. caused by hyper-thyreoidism) or *vica versa* hypo-thyreoidism in case of normal food intake but faster body weight development.

In larger non-rodent species feed is offered in a fixed quantity, i.e. a dog feed ratio is about 800–1000 grams per day. Consumption is determined by checking whether the offered amount was consumed or, if not, the weight of the feed not consumed is measured and recorded.

Ophthalmology

Investigations should be performed by experienced and board certified veterinary ophthalmologists before treatment starts and at the end of the treatment period. The investigations should focus on the cornea, lens and retina.

Clinical Pathology (Invasive Methods, Hematology, Clinical Chemistry and Urinalysis)

For determination of clinical pathology parameters blood samples have to be taken. In the rodents, esp. in the mouse it can be a difficult procedure. That is why in the mouse blood samples are frequently taken at the end of the treatment period when the mouse is sacrificed. Also "satellite" animals can be treated in parallel to the "toxicity study" animals which are sacrificed at the time when blood levels should be determined without sacrificing the "toxicology study" animals. The blood sample in killed animals can be collected from a large vein (e.g. *Vena cava caudalis*) or directly by puncture of a cardiac chamber. In the rat blood samples are taken by puncture of the retro-orbital vein plexus or from the caudal (tail) vein. In case the sample is taken from the retro-orbital vein, anesthetic (ether) and experienced technicians or veterinarians are required. In larger non-rodent species blood is collected from large veins e.g. the V. cephalica antebrachii which is easily accessible. The volume of a blood sample necessary is no longer an issue. Formerly, blood samples had to have a volume of about 2 ml. Today, with modern auto-analyzers, the volume was reduced to about 100 micro-liters in order to determine the parameters which are of toxicological significance.

Problems can come about if due to technical difficulties a second blood sample has to be taken within a short period after the first, failing withdrawal. In a small rodent the loss of blood is counteracted by rapid erythropoesis in the bone marrow, and the spleen. During the histopathological evaluation increased erythropoesis is seen in the bone marrow and the spleen (extra-medullary hematopoiesis).

Urine can be collected in small rodents by the use of metabolism cages, in large non-rodents by catheterization.

The frequency of investigation depends on the duration of the study. In the case of a one month study, blood samples are taken at the end of the treatment period or in case, at the end of the recovery period. In longer term

studies, blood is collected after 1 month or after 3 and 6 months in case of a chronic study.

In a rodent study blood is collected from 10 animals per sex and dose level. In non-rodents blood is sampled from all animals.

With the modern technology of auto-analyzers whole blood can be used to determine the toxicologically important parameters. For the measurement of the clinical chemistry values, serum is used.

Hematological parameters routinely determined are:

- Hematocrit
- Hemoglobin
- Erythrocyte count
- Total leukocyte count
- Differential leukocyte count
- Prothrombine time
- Reticulocyte count.

In addition, quotients like Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV) can be calculated. In case anaemia was induced by the testing material, its nature can be characterized: macrocytic, microcytic, hypo- or hyper-chromic. The type of the anaemia provides information on the mechanism of induction of anaemia.

Clinical chemistry parameters are determined in serum derived from blood sampling. Routine parameters are:

- Glucose
- Urea nitrogen
- Creatinine
- Total protein
- Albumin
- Globulin
- Albumin/Globulin ratio
- Calcium
- Sodium
- Potassium
- Inorganic chloride
- Bilirubin (total)
- Cholesterol
- Alkaline phosphatase (AP)
- Aspartate aminotransferase
- Alanine aminotransferase
- Gammy glutamyltransferase
- Ornithin carbamyltransferase
- Creatin kinase.

The determination of these clinical chemistry parameters gives information about possible target organs (kidney, liver, pancreas, muscles) of toxicity. It can not be the aim of this book to describe toxicological syndromes in which these parameters show deviations from the normal. The interpretation of changes has to be performed with other deviations, mainly with finding in the histopathological evaluation.

Parameters determined routinely in the urine are:

- Volume
- Specific gravity
- pH value
- Glucose
- Blood
- Microscopic examination of urinary sediment.

For the biochemical urinary parameters frequently tests sticks are used. This method has to be used with care because most of them have been developed as a diagnostic tool for human clinical use.

Post-mortem Evaluations

At the end of the studies, all animals are killed (rodents by exsanguination after carbon dioxide narcosis or by an overdose of barbiturates) and necropsied and organs have to undergo a thorough macroscopic evaluation. Weights of parenchymatic organs are determined (absolute organ weights) and relative organ weights (in relation to total body weight) are calculated. An alternative method is to put the brain weight in relation to the weights of the other organs. Brain weight is very stable compared to total body weight. Therefore, it can offer a more precise analysis of changes of organ weights. There is however no regulatory preference which method to be used. For the interpretation of changes, either macroscopically or microscopically, both the body relation to the body weight and the brain may give useful information with regard to the cause of changes.

The degree of the bleeding plays an important role with regard to organ weights. Residual blood in the heart, spleen and lungs can influence the weight of these organs considerably and are frequently influenced by the method of euthanasia. As a rule, the time between the induction the narcosis and bleeding should be constant, and narcotics causing shock like symptoms with considerable blood amounts in certain organs (e.g. lung, spleen, liver) should be avoided.

In the rodent, frequently a dose-related increase of the liver weight paralleled by hepatocytic hypertrophy occurs. This phenomenon is often due to induction of hepatic drug metabolizing enzymes. It is obviously over-expressed in the rodent species with the aim to faster metabolise xenobiotics and also degradation

products of metabolism of physiological constituents (e.g. thyroid hormones). This results in increased secretion of thyroid-stimulating hormone (TSH) which stimulates the thyroid for the synthesis of thyroxin.

After the determination of organ weights and macroscopic examinations the tissues are processed for histopathological evaluations. For this they are fixed in formalin or equivalent solutions, or they are frozen (important for the diagnosis of increased fat content in a tissue, e.g. in the liver, organic solvents would dissolved the fat) trimmed to small parts, put into paraffin blocks, cut with a microtome, and stained (hematoxylin-eosin, or special staining for fat and/or collagen etc.).

A wide variety of tissues has to be investigated microscopically.

Microscopic slides of about 50 tissues/organs have to be evaluated by board certified animal veterinary pathologists specialized on the most commonly used species.

In order to save time, histopathological diagnoses can be confined to the high dose group animals and the control animals. However, some pathologists criticize this procedure. As described in the section "dose selection" it is the aim of repeated dose studies to identify ideally a dose which causes clear sings of toxicity, an intermediate dose level which causes no or mild effects, and a dose which does not induce any changes. The procedure to check only organs from the high dose group are only of advantage if no microscopically changes were induced, a situation which is not in line with requirements of regulatory agencies. In cases, as required by the guidelines, where changes were seen, the tissues from animals of the next lower dose have to be processed and investigated microscopically. This situation can cause prolonged development times with the consequence of delays of approval by regulatory authorities.

To assure a uniform picture of morphological changes induced by a testing material it is recommended to develop a glossary for morphological deviations from the normal. Therefore, the need for uniform glossaries is underlined again.

The Role of Historical Controls

Historical control values are often used to determine the biological significance of changes not only in relation to the actual control animals but also in relation to the values determined in control animals' age from other studies. This procedure is not well accepted by international authorities; however, in case of studies with low sample numbers (e.g. one month studies) it can become necessary to use data from control animals from previous studies to interpret the results of the current experiment. This procedure is only justified if test conditions, e.g. animal strains, age of the animals, conditions of housing, etc. are the same.

Statistical Analysis of Results

The description of statistical analysis of results is not part of this text. However, some principles should are should be considered. First, data should be checked for normal distribution, sample size and continuity. Secondly, according to these conditions, a statistical method should be used which is the most adequate statistical method for the parameter to compare. Further, performing statistical analyses on parameters which are highly correlated, e.g. erythrocytes, hematocrit, MCV, etc. should be avoided. This principle should also apply for the evaluation of organ weights, such as absolute and relative organ weights. In general, statistical analysis is an absolute requirement for studies with relatively large sample sizes such as in rodent studies; however, in animal studies with low sample sizes in large non-rodent species, the evaluation of individual animals is preferred. Data from male and female animals can be pooled to increase the sample size, though, only for parameters which do not show any sex dependency.

Recent Regulations Regarding Chronic Studies

In 1999, the FDA commented on the ICH guidance document about the duration of studies in non-rodents. The FDA considers 9-month chronic studies, as recommended by ICH, as acceptable for most drug development programs. Shorter studies may be equally acceptable in some circumstances and longer studies may be more appropriate in others, as follows:

Six months studies may be acceptable for indications of chronic conditions associated with short-term, intermittent drug exposure, such as bacterial infections, migraine, erectile dysfunction, and herpes.

Six month studies may be acceptable for drugs intended for treatment of life-threatening diseases for which substantial long-term human clinical data are available, such as cancer chemotherapy in advanced disease or in adjuvant use.

Twelve month studies may be more appropriate for chronically used drugs to be approved on the basis of short-term clinical trials employing efficacy surrogate markers where safety data from humans are limited to short-term exposure, such as some acquired immunodeficiency (AIDS) therapies.

Twelve months studies may be more appropriate for new molecular entities acting at new molecular targets

where post-marketing experience is not available (Fed. Reg. 1999).

Other End Points

Recently, additional endpoints in short-term and sub-chronic studies have been proposed in order to save resources and costs of development. However, there are limitations due to possible interference with the main purpose of studies.

Genotoxicity

The principle techniques of the micronucleus assay can be applied at the end of a subacute/sub chronic study in the bone marrow of rodents (princliples are described in the section on genotoxicity elsewhere in this book). Usually, only bone marrow from one femur is used for histopathology, thus, the marrow from the second femur can be used for the micronucleus assay. It is not recommended to perform chromosomal analysis in animals from short-term or sub-chronic studies because this technique requires the in vivo treatment with anti-mitotic agents to arrest the mitotic stage of cell division.

Other assays for geno-toxicity can be performed, e.g. the in vivo/in vitro unscheduled DNA-synthesis in isolated hepatocytes. As this assay is not compatible with routine histopathology, satellite animals have to be used for the fresh isolation of hepatocytes.

Neurotoxicity

The investigation of neuro-toxicity in short-term studies has been proposed. Especially cage site observations, physical conditions and behavioral observations are of value.

The FDA has established criteria for neuro-toxicity screens as a component of short-term and sub-chronic studies (FDA/FDCA 1993):

- Histopathological evaluation of tissues (brain, spinal cord, peripheral nervous system)
- General appearance
- Body posture
- Seizures (incidence and severity)
- Tremors (incidence and severity)
- Motor activity and arousal
- Reaction to stimuli
- Motor co-ordination
- Grip strength
- Change in gait
- Response to primary sensory stimuli
- Lacrimation and salivation
- Piloerrection
- Diarrhoea
- Polyuria, anuria, oliguria
- Ptosis
- Other signs of neuro toxicity.

Immuno-toxicity

Immunosuppression can cause a reduction of the animal's resistance to infection and, under certain conditions, also to an increased susceptibility to tumorigenesis (tumor-promotion). In contrast, hyperactivity of the immune system can result in autoimmune diseases or increase the sensitivity to allergies. While it is difficult to identify the mechanisms of the effect(s) of immuno-toxicity it is relatively easy to detect such properties. Information can be provided by investigating special parameters from the rat used in short-term or sub chronic rodent studies (type I tests) (FDA/FDCA 1993). The other category of tests uses in vitro techniques (type II tests).

FDA recommended investigating certain parameters, part of them are a constituent of the usual endpoints studies in routine testing:

Blood constituents:

- White blood cell count
- Differential white blood cell count
- Numbers of lymphocytes
- Numbers of eosinophiles.
- Total Serum Protein
- Albumin
- Albumin/globulin ratio
- Serum transaminases (ALAT, ASAT)
- Globulins with quantification of Gamma globulin fraction (IgG, IgM, IgA, IgE)
- Complement
- Cytokines (IL-2, IL-1, Gamma interferon)
- Auto-antibodies.

Histological Endpoints

- Spleen
- Lymph nodes
- Thymus
- Peyer's patches
- Bone marrow
- Cytology (activated macrophages, tissue prevalence of lymphocytes)
- B- and T-cell germinal centres
- Necroses of proliferation of lymphoid tissues.

Reporting and Evaluation

International authorities have recommended standardizing the reporting of studies. In brief, the study reports should be organized in the following manner:

- Summary
- Description of the test article based on evaluation of analytical departments (physical properties, solubility in different solvents, particle seize, pH, characterization of impurities)
- Description of the methodologies for the determination of the parameters (timing of measurements food consumption, body weights, behavior, hematology, clinical chemistry, urinalysis, macroscopic organ findings, histopathology, statistical methods)
- Description of results (same order)
- Interpretation of results
- Conclusion
- Summarizing tables (same order)
- Tables with single values.

There is an ongoing discussion as to what degree the data from a single study should be finally interpreted. A comprehensive picture of the toxicological properties is optimal if all data from all studies necessary for submission are available. Therefore, it is recommended to conduct a final evaluation in the expert report only. Otherwise, interpretation of results from single studies needs to be revised.

III.C.1 Subacute and Chronic Studies for Recombinant Proteins

In contrast to small molecules, toxicity testing of recombinant proteins for life-threatening diseases can be conducted using more streamlined development strategies, i.e. testing can be confined to one animal species, usually a non-human primate. Further, teratology studies are not strictly required. Genotoxicity can be confined to tests using mammalian cells and carcinogenicity studies may not be a strict requirement. In principle, the testing of recombinant proteins requires special considerations. Special attention should be devoted to the production of neutralizing antibodies which can cause the inactivation of the pharmacodynamic active protein. Such assays should be conducted prior to the initiation of multiple dose studies.

In case of recombinant proteins for non life-threatening diseases a similar toxicological development strategy should be applied as described for small molecules. For the elaboration of a toxicological development program it is advisable to work in close connection with other biological development departments and with international agencies, such as EMEA (Europe) FDA (USA) and MHW (Japan) (Zbinden 1990).

III.C.1.1 Carcinogenicity Testing

PURPOSE AND RATIONALE

Neoplastic diseases are, after cardiovascular diseases, the most frequent cause of death in the western population. Neoplastic diseases are very often connected with a dramatic change of the quality of life and suffering. Therefore, testing of carcinogenicity for the therapy of chronic diseases is the most important constituent of the toxicology development program. It is believed that the classical bioassays in rodents (mice and rats) can predict tumorigenic properties of heterocyclic chemicals with the highest degree of accuracy, compared to other assays. Positive results in bioassays have to be put into perspective with the result from genotoxicity studies. In the case of positive assays for genotoxicity which are conducted early in the value chain of a product, the development is stopped at an early stage. Therefore, today the likelihood of developing a "genotoxic carcinogen" is very remote. However, the situation of negative results in assays for genotoxicity and positive results (tumorigenicity) in a bioassay is a frequent and challenging situation. The findings with some industrial chemicals can help to explain this. Almost all chemicals causing cancer in humans (e.g. vinyl chloride, chromium compounds, asbestos, bis-choro-methyl ester) (DFG 2004) have caused tumors in animal carcinogenicity studies. However, it is not known whether chemicals showing carcinogenic effects in animal studies are carcinogenic in humans because carcinogenic effects shown in animals (especially in the rat) are in many cases prohibitive for use in humans. Exceptions are of course drugs for cancer chemotherapy (e.g. clastogenic cancer drugs).

The performance of carcinogenicity studies in rodents contributes significantly to the costs for the toxicological development program. However, compared to the overall development (especially long term clinical trials) for a drug for the treatment of chronic disease, they are considered insignificant. In spite of this, carcinogenicity studies should be performed only if the "Proof of Concept" in patients was positive and the target product profile was achieved.

The timing of the start of carcinogenicity has to be planned very carefully together with project management and clinical development. Optimally, first result from macroscopic observations at the end of the study

should be available, before long term (Phase III) clinical studies are started. With the information about the outcome of the studies for genotoxicity (which are conducted in very early phases) the risk for carcinogenic effects is minimized.

PROCEDURE

Drug Substance

The exact determination of the analytical quality of a drug substance is an absolute need. The production of the drug substance is at an advanced stage in this stage of development. However, a comprehensive analytical certificate is required. It should provide information about:

- the chemical identity of the drug batch by batch
- that the nature of the impurities (qualitative and quantitative) is similar to the drug which has been used in shorter term studies and is planned to be used in the long term clinical trials and for marketing
- the homogeneity
- the drug substance shows is in similar particle size distribution (if particles/crystals are too large milling of the drug substance should be considered, however, after milling stability has to tested again)
- The stability of the active ingredient. Special storage conditions should be applied to guarantee stability over the treatment period.

When carcinogenicity studies are planned, relatively large quantities of the drug substance are needed due to the long duration of the treatment period a large number of animals to be treated. If the production capacity is not available in this period of development, different batches have to be produced. In this case it has to be shown that no significant differences exist with regard to the characteristics mentioned above.

Animals

For world-wide approval of drug, carcinogenicity studies in two rodent species, usually the rat and the mouse, should be conducted. This is especially a requirement of the US FDA. In Europe, only one species, in most cases the rat, is recommended. Long time experience demonstrates that in case of a new therapeutic principle or a significant improvement of a known principle, marketing strategies change and therefore carcinogenicity studies in two rodent species become necessary when the USA becomes part of the marketing territory.

If one of these rodent species demonstrates a striking difference of metabolism in comparison to humans, other rodent species can be taken into consideration. However, experience has shown that the choice of other species is difficult due to lack of experience with other rodent species. The hamster could be considered, but there are only a few studies conducted or published in this species with the consequence of little information about spontaneous tumour incidences.

For the mouse, usually NMRI, B6C3F1, or CD1 strains, and for the rat, or Wistar, Sprague Dawley or Fisher rats are recommended. However, Fisher rats show a very high spontaneous incidence (up to 100 %) of testicular tumors and are therefore not the rat strain of choice. Another example is the B6C3F1 mouse which is well known for the high spontaneous incidence of liver tumors.

Another important factor is the body weight of animals, especially the rat. There are rat strains available with "physiologically" high body weights. The males of these strains show body weights of close to 1 kg compared to other strains with about 500 g maximum body weight. The body weight has of course a consequence on the total amount of the test compound needed, because dosing is done on the basis of mg/kg body weight. The costs of the test material is high, especially at this stage of development, so by using smaller strains the costs can be significantly reduced. If the test article is tolerated well the cost savings can be several hundred thousand or US $.

The choice of strains is highly influenced by the availability of "historical controls" (data from parameters in control animals). Although some authorities do not rely on comparisons of controls from other studies, the investigator should try to use a strain for which a profound basis for comparisons with results from other studies is available. In case of the availability of data from "historical controls", the investigator can identify trends due to genetic shifts and use this information for the interpretation of the actual study.

At the beginning of the study an adequate acclimatisation is required (see subacute and chronic studies). The animals should be of an age of 6–8 weeks when the first dose is administered.

The minimal requirement with regard to animal numbers is 50 animals per dose level and sex. However, it is advisable to use more animals for certain purposes (e.g. determination of kinetics after chronic treatment).

Diet

Diet from a certified producer only should be used. The composition of the diet should be described in an analytical certificate with regard to "nutritional constituents" (carbohydrates, fat, protein, vitamins, minerals) and for contaminants (nitrosamines, afla-

toxins, chlorinated hydrocarbons etc.) (McConnel 1983). The analytical certificates for each batch should become an addendum to the test report.

The debate about feeding conditions in rodents has been going on for decades. Offering feed "ad libitum" is the usual method, but with the result that this condition leads to higher mortality, also mortality induced by malignancies within a certain period of time compared to restriction of food.

Conditions of Maintenance

There are no major differences compared to the conditions of the environment described in the section "Subacute to chronic studies". The only difference should consist in the strict and regular control of the environment in the animal room. Events like break down of the climatization equipment or bacterial infections due to bad conditions in the animal room can influence the results of a carcinogenicity study considerable up to the total damage of the study due to chronic infections (e.g. chronic respiratory disease) (Sinkeldam 1991).

Usually rodents are kept in single cages, so that cannibalism can be avoided, and therefore feed consumption can be determined individually. If animals are kept in groups of 5, separation is necessary when the male animals grow up to sexual maturity and fighting puts the study at risk. In addition, the risk of transmission of infectious diseases is lower in the case of single caging.

Rats and mice can be kept either in plastic cages (Macrolon®) or in steel wire cages. In general, Macrolon cages are preferred because in steel wire cages old animals with high body weights suffer often from "hind-limb weakness" which can influence their mobility in the wire cages.

Mode of Administration

The mode of administration should be the same as that used clinically. In case of drug substances (e.g. proteins) incorporated into polymers, the drug substances is injected sub-cutaneously. However, special care has to be taken with regard to the skin area. If the drug is administered continuously to the same site, e.g. the neck, chronic local irritation / inflammation can induce sarcomas at the site of administration. To avoid this, the site of administration should be changed day by day (see also subacute to chronic toxicity) (Stammberger et al. 2002).

For inhalation carcinogenicity the same principles are valid as described in the previous section (sub-acute to sub-chronic studies).

Dose Levels

As stated in the section sub-chronic to chronic toxicity, the selection of dose levels needs a careful analysis which requires a lot of experience. However, in the case of carcinogenicity studies, the issue is not as large as in the case of sub-chronic to chronic studies. In general, three dose levels have to be tested plus a negative control group.

The dose levels should be selected according to the following principles:

- Low dose: should not induce any changes.
- High dose: should be the Maximum Tolerated Dose (MTD).
- Middle dose: should represent the mean (geometric or logarithmic) between low and high dose.

This rule sounds simple, but the selection of dose levels requires experience. In practice it is recommended to discuss the selection of dose levels to be chosen with the regulatory agencies FDA, EMEA and MHW and to find a compromise between the different requirements. As a rule, the highest dose level should provoke significant toxicity without influencing the survival of the test animals. This requirement sounds easy, but to fulfil it is difficult, because predictions have to be made from the 6-month toxicity study, the longest study conducted before the carcinogenicity study begins.

As described, the lowest dose level should guarantee that the test animals are exposed to a dose level which is a multiple in comparison to human exposure.

In the case where a drug substance is untoxic, a dose level should be tested which causes blood levels in animals 25 times higher than that achieved by the maximum dose used for treatment of humans. Other facts have considerable influence on the selection of the high dose level.

Other considerations can influence the selection of the highest dose level. Rats and mice are relatively insensitive to sulfo-ureas used for the treatment of diabetes. Rats counteract the hypoglycaemic effect of these drugs by secretion of glucagon and 25-hydroxycortisol and no hypoglycaemia occurs at the maximum dose level (> 5 000 mg/kg). In one case of a novel antidiabetic sulfonylurea it was demonstrated that absorption becomes saturated at relatively low oral dose (ca. 1000 mg/kg). This observation convinced the FDA that testing of the maximum tolerated dose (probably not determinable) is not necessary. By that the demand of test material could be reduced at least by a factor of 5.

Vehicles

The same holds true as described for sub-acute to chronic studies.

Duration of Studies

Studies for carcinogenicity in rats and mice should last at least 24 months. This rule avoids problems of acceptance at the international health authorities. There are other aspects which can influence the duration of a carcinogenicity study, e.g. survival rate. In case survival after a certain time of treatment is below 25 % health authorities should be informed with the request to sacrifice the remaining animals. In other cases survival will be higher than 75 % after 24 months of treatment. With this result, it can be considered to prolong the exposure period. The duration (and therefore the age of the animals) of a carcinogenicity study has a definite influence on the tumor incidence in the animals. The interpretation of the results with regard to tumor incidences has to be corrected for the age of the test animals.

Observations

A similar pattern of observations with regard to general condition, body weight development, feed consumption, ophthalmology, urinalysis and clinical chemistry is necessary as described for sub-chronic to chronic studies. However, hematological, urinalysis and clinical chemistry parameters can be measured in larger intervals, e.g. after 6, 12, and 24 months.

The main focus in carcinogenicity studies should be devoted to post mortem observations such as organ weights, macroscopic and microscopic observations.

In the following table organs and tissues are listed which have to be examined:

- Adrenals
- Brain
- Caecum
- Colon
- Costochondral junction, rib
- Duodenum
- Oesophagus
- Eyes
- Gall bladder
- All macroscopic observable lesions
- Ileum
- Jejunum
- Kidneys
- Larynx
- Liver
- Lungs and bronchi
- Mammary gland
- Mandibular lymph node
- Mesenteric lymph node
- Nasal cavity (in case of inhalation studies at three levels of the nasal cavity)
- Ovaries
- Pancreas
- Parathyroid
- Pituitary
- Prostate
- Rectum
- Salivary gland
- Sciatic nerve
- Seminal vesicles
- Skin
- Spinal cord
- Spleen
- Sternebrae, vertebrae, and femur including bone marrow
- Stomach
- Testes
- Thigh muscle
- Tissues masses or suspected tumours and regional lymph nodes
- Thyroid
- Thymus
- Urinary bladder
- Uterus.

Study Report and Interpretation of Findings

The report of a carcinogenicity study is composed in a similar manner as described for sub-acute to chronic studies. Methodologies, results in a summarized form, individual results have to be the content of a study report which consists of several thousand pages.

For the description of morphological findings it is of special importance to use a "common" nomenclature as outlined in the section "Subacute to chronic toxicity". In the case of carcinogenicity studies this requirement is of greatest importance because the occurrence of findings, neo-plastic and non-neo-plastic has to be analysed statistically.

The most important part is the "interpretation of findings" section where all results are discussed and interpreted. In this section results should be evaluated with regard to the pharmaco-dynamic mechanism of action. In order to illustrate that, examples are given:

Glimepiride, a novel sulfonylurea-urea for the treatment of type II diabetes, caused adenomas of the β-cells of the pancreas in rat and mouse carcinogenicity studies. This was due to the long lasting and powerful effect of glimepiride on the β-cells. The long lasting stimulating effect of glimepiride on the β-cells of the pan-

creas to produce insulin caused a proliferation on the β-cells which after two years of treatment resulted in benign pancreatic tumors. Another example exists in the carcinogenic effect of H2-blockers or proton-pump inhibitors in the stomach which lower the pH value of the gastric juice and stimulate by that the gastrin secretion.

About twenty years ago another very interesting and dramatic situation existed. Spironolacton, a drug used for the treatment of hyper-aldosteronism, exhibited tumorigenic properties in endocrine dependent tissues (thyroid, Leydig-cells). Potassium canrenon, a water-soluble derivative of spironlactone, induced tumors at other sites (myeloic leukaemia, mammary glands). For the benign tumours induced by Spironolactone induction of metabolizing enzymes (including enzymes for the degradation of thyroxin and 3-jodo-thyronine, accelerated degradation of testosterone) could be made responsible (hormonal non-genotoxic mechanism). However, no explanation existed for the mechanism for the induction of malignant tumors induced by potassium canrenoate. In investigations it was shown, that potassium canrenoate is metabolized in a different manner. Canrenoate forms a metabolite, the 3-hydroxy-6, 7 β-epoxy canrenone, a potent mutagenic agent. With spironolactone this metabolite can not be formed. The sulphur-containing metabolites in the 7-position of the molecule inhibited the formation of a mutagenic and carcinogenic metabolite (Mayer et al. 1988; Oppermann et al. 1988). As a consequence, spironolactone is still used for the treatment of hyperaldosteronism in contrast to potassium canrenon which is restricted to certain life-threatening forms of this disease which requires i.v. treatment.

In summary, carcinogenicity can be induced by genotoxic mechanisms which alter the genetic code or by epi-genetic mechanisms which act e.g. via disturbance of hormonal mechanisms.

In the case of genotoxicity accompanied by carcinogenicity, a drug is only approvable for the treatment of life threatening diseases. For non-genotoxic mechanisms a drug is approvable if it can be shown that the mechanism of tumorigenicity is epigenetic and that a safety margin exists based on observations in clinical studies. Enzyme induction is compared to the rat a rather rare phenomenon in man.

III.C.2 Testing for Skin Irritation

Several methods have been described and more or less significant modifications have been developed. The test which is used most frequently is the "Draize Test" on which other experimental methods are based.

III.C.2.1 Draize Test

PURPOSE AND RATIONALE

Dermal tests are conducted for drugs which are applied to the skin with the aim of discovering acute irritating properties. This method was developed by Draize (Draize et al. 1944) 60 years ago. The test is mainly suitable for industrial chemicals but less for pharmaceutical products which are administered to the skin.

PROCEDURE

The test article is administered as solution or suspension to the back of six albino rabbits. The test material is administered to two sites onto the rabbits' skin where the skin has to be clipped. On one site the skin remains intact, on the other the skin is mechanically abraded in order to remove the outer layer of the skin, the stratum corneum. The clipped areas should have a surface of about 1 square inch onto which the test material is administered. The dose level to be administered to the skin is 0,5 ml in case of liquids or 0,5 g in case of solid materials. The treated skin areas are covered with surgical gaze and fixed with a non-irritation tape. A second layer consisting of an occlusive material is wrapped over the entire trunk of the rabbits in order to avoid or retard the evaporation of the test material from the treated skin areas. After 24 hours, the wrapping is removed from the animals and the treated skin sites are evaluated for erythema and oedema, the classical signs of inflammation.

Observations

Draize also developed a scoring system which allows classifying the test material with regard to their irritation potential 24 and 72 hours after administration. See Table 3.

Reporting and Evaluation

According to Draize, a primary irritation index was calculated by adding the results from the 6 animals 24 and 72 hours after administration and dividing the score by 12 (6 animals × 2 observations) of the mean of grades from both the intact skin site and the braded site. He graded the irritation index into the following irritation indices:

- < 2: non- to mildly irritating
- 2–5: moderately irritating
- > 5: severely irritating.

Table 3

Nature of change		Score assigned
Erythema and eschar formation	No erythema	0
	Very slight erythema	1
	Well defined erythema	2
	Moderate to severe erythema	3
	Severe erythema	4
Oedema	No oedema	0
	Very slight oedema	1
	Slight oedema (edges are visibly elevated)	2
	Moderate oedema (raised skin area of about 1 mm)	3
	Severe oedema (Raised skin of more than 1 mm)	4

Today the Draize test no longer playing a significant role for testing pharmaceuticals, because toxicological testing of dermatic drugs covers the potency of skin irritation more precisely, especially with regard to duration of treatment.

Alternative Methods

Several in vitro methods have been developed which use different end points to measure skin irritation (e.g. measurement of inflammation mediator, effects on survival rates of special cell lines, etc.) However, as stated for the Draize test they do not play a significant role.

III.C.2.2 Testing for Irritation of Mucosal Membranes

PURPOSE AND RATIONALE

Drugs developed for local application to mucosal membranes of the eye, mouth, membranes of the penis, vagina and rectum have to be tested for local tolerance as well. However, for testing of locally irritating effects findings from toxicology studies with repeated administration to mucosal membranes are more suitable than acute studies developed mainly for testing of industrial chemicals.

Before testing a drug substance or a formulated drug in excipients, the pH should be determined in order to avoid severe irritation and pain in the test animals.

The test described below is the most frequently used, mainly for industrial chemicals but also for drug with an application to the conjunctiva/cornea.

PROCEDURE

A volume of 0.1 ml of the drug in a non-irritating solvent is inoculated into the conjunctival sack of each on rabbit out of six rabbits or directly on the cornea. The eye lid is kept close by using two fingers for some seconds to assure close contact with the cornea. Effects occurring in the eye media (cornea, iris, nictating membrane and conjunctiva) are graded. The product of the scores for the cornea with regard to the degree of opacity and the area of cornea involved is multiplied by a factor of 5. This value represents the "weighted" corneal score.

For the diagnosis of the iris, 2 scores can be allocated, and for the conjunctivae 2–3 (see Table 4).

EVALUATION

According to the score calculated, drugs can be categorized with regard to their irritating effects (not irritant, mild, intermediate, and severe).

As stated already, this test is of limited value for drug development. However, this method is very useful for testing starting materials for synthesis of new drugs and for intermediate products to identify possible risks to workers.

MODIFICATION OF THE METHOD

Modifications are possible and described with regard to the use of corneal anaesthetics, irrigation, observation period and classification of findings using the scores (Draize 1959).

III.C.2.3 Testing for Dermal Sensitization

PURPOSE AND RATIONALE

Although sensitising properties can be also detected in the classical development program of a drug substance, the need for dermal sensitization studies is of importance for the development of certain drugs. The increasing importance of trans-dermal formulations demonstrate the need of testing for dermal sensitization on the one side, on the other side, there are classical examples which cause dermal sensitization (e.g. neomycin, procaine, sulphonamides).

Table 4 The findings are interpreted in detail.

		Lesion	Score
CORNEA	A. Opacity: Degree of density	Scattered or diffuse area, details of the retina still clearly visible Early discernable translucent areas Details of iris clearly visible Opalescent areas, no details of the iris visible, size of pupil barely discernable Opaque, iris not visible	1 2 3 4
	B. Area of cornea involved:	One quarter of (or less) Greater than one quarter, but less than one half Greater than one half, but less than three quarters Greater than three quarters up to whole area Score equals A × B × 5 Total maximum = 80	1 2 3 4
IRIS	A. Values	Folds above normal, congested Swelling, circum-corneal injection Iris still reacting to light No reaction to light, hemorrhage Gross destruction Score equals A × 5 Total possible maximum = 10	1 2
CONJUNCTIVE	A. Redness (palpebral conjunctivae)	Vessels definitely injected above normal More diffuse deeper crimson red Individual vessels not easily discernable Diffuse beefy red	1 2 3
	B. Chemosis	Any swelling above normal Obvious swelling with partial eversion Of the lid Swelling with lids half closed	1 2 3
	Discharge	Any amount of different from normal Discharge with moistening of the lid and hairs adjacent to the lids Discharge with moistening of the lids and considerable area around the lid Score (A + B + C) × 2 Total maximum = 20	1 2 3

The "Optimization Test" is suitable for the detection of sensitizing properties of a drug.

PROCEDURE

The skin of 20–25 guinea pigs is shaved in the shoulder region. Two sets of 0.1 ml intra-dermal injections of a 1:1 FCA (Freund's Complete Adjuvance/water) and the same dose of the drug sample in the FCA vehicle are put on filter paper and placed over the injection site. The sites of administration are covered with a non-irritating surgical tape. The injections are performed daily. In case the test material does not penetrate the skin, 10 % of sodium lauryl sulfate can be added to increase skin absorption. Control animals receive the vehicles only. 48 hours after the last administration, the skin covering is removed and the animals are challenged with the highest, non-irritating concentration of the drug sample (and the vehicle).

The results are evaluated in comparison to controls with regard to induction of erythema and skin oedema. A similar scoring system is used as in the case of dermal irritation. The signs of local dermal inflammation determine the degree of the allergenic potential (Draize 1944; OECD 1981; Klecak 1983).

EVALUATION

The method has the advantage of a relatively high predictive value. It was applied to develop a new local aesthetic for dentists. The dentists suffered from increased incidences of dermal sensitization towards local anaesthetics. With the introduction of articain, which was selected for development as a local anaesthetic upon the results of the dermal sensitization potential (and of its high analgesic efficacy) this product became very successful.

MODIFICATION OF THE METHOD

Methods without using Adjuvans (Klecak 1983; Buehler 1965) are less sensitive and allow only a "crude" classification of the allergenic potential.

III.C.2.4 Photo Toxicity

PURPOSE AND RATIONALE

Since tetracyclines were introduced into pharmacotherapy, the phenomenon of photo-allergy/photosensitization is well known. Another group of highly effective and relatively modern anti-infectives are the quinolones (pefloxacin, ofloxacin, norfloxacin,

ciprofloxacin etc.) which require during therapeutic use special precautions by the patient: protection against UV-irradiation (avoidance of exposure to sun light). Put simply the principle of induction of photo toxicity of a drug substance is the absorption photo-energy and the resulting formation of a toxic compound.

PROCEDURE

Albino guinea pigs or albino mice are depilated. The test compound is administered to the hairless skin in a concentration which does not cause skin irritation. Two hours after administration irradiation is performed in a dose which does not cause skin inflammation (erythema, oedema). The spectrum of the UV light should cover a wave length pattern of both UV-A and UV-B, i.e. in the range of 280–320 nm and 320–400 nm.

EVALUATION

Skin reactions by using a similar scoring system as described for dermal irritation are measured 24, 48 and 72 hours after administration of the drug (FDA 2003).

MODIFICATION OF THE METHOD

In order to detect also phototoxic properties of compounds which show a slow skin penetration, repeated UV-irradiation over a period of 5 days can be used.

III.C.2.5 Photosensitization

PURPOSE AND RATIONALE

By absorption of irradiation energy, a drug substance can be modified to a molecule which has sensitizing properties.

PROCEDURE

Albino guinea pigs are treated at a depilated skin area for about 3 weeks with the drug substance. After that period, animals are irradiated with UV-A and UV-B at a level which causes a weak erythema only. After that a 2 weeks "latency" period follows without any treatment. Thereafter animals are irradiated again with UV-A and UV-B at a dose level not causing erythema.

For this assay a positive control compound is recommended: 3,3,4,5-tetrachlorosalicyanilide.

EVALUATION

Signs of erythema and oedema are scored in a similar manner as comprehensively described.

MODIFICATION OF THE METHOD

Modification should be considered according to the pharmaco-kinetic behavior of the drug substance.

III.C.2.6 Local Tolerance Testing for Parenteral Drugs

PURPOSE AND RATIONALE

The number of intravenous drugs is quite high, therefore the testing of intravenous tolerability plays an important role. Intravenous studies are also required as a precondition for testing the absolute bioavailability in humans. In the case of i.v. administered drugs almost all classical studies of the toxicological testing program are conducted by the clinical route. Acute i.v. tolerance studies in animals are the first step for testing.

PROCEDURE

For testing the V. marginalis of the rabbit ear is used. Only water soluble drug substances should be administered. Each animal receives a volume of 0.2–0.5 ml of the solution into the mechanically congested ear vein. The ear on the other side is treated with the vehicle only. An alternative method is to use extra animals for treatment with the vehicle only. The speed of injection (volume per time) should be similar to the human clinical situation. During the injection and after the animals have to be observed for clinical signs. In total the animals have to be checked for a period of 14 days. The site of injection has to be checked for signs of local reactions.

In case of testing of infusion solutions, larger animals should be used e.g. the dog. Newer technologies with small injection pumps allow also the use of rats.

In the case of special drug applications, para-venous testing has to be conducted to check for possible risks of drugs being erroneously administered into the paravenous tissue. The animals receive an acute administration lateral and medial of the V. jugularis sinistra of each 0.2 ml. A control group with the same number of animals receives physiological saline only. For testing of reversibility one group of animals is necropsied 6 hours after administration and another 24 hours after administration.

The injection side is checked from 0–6 hours after injection and in the second group 8 and 23 hours after i.v. application (Stötzer 1989). See Table 5.

CRITICAL ASSESSMENT OF THE METHOD

As stated earlier, this method has lost importance because for the development of a drug to be clinically administered i.v. the toxicological testing program should

Table 5

Evaluation criteria		
Erythema	0	no abnormal finding
Blue reddish discoloration	1	minor change
Swelling	2	medium change
Skin necrosis	3	severe change

use the same route of administration, i.e. the i.v. route. At the end of any in vivo experiment in rats and non-rodents the site of repeated i.v. injections is very carefully evaluated clinically and histologically and therefore provides more information than after an acute administration.

III.C.2.6.1
Intra-Arterial Testing

PURPOSE AND RATIONALE
There is only a very limited number of drugs (e.g. some diagnostics) which are administered i.a. In addition, i.v. drugs can be administered i.a. by error.

PROCEDURE
The test is conducted by using the mechanically congested A. centralis of the rabbit ear. The injection is directed into the distal direction. A 7,5 % solution of thiopental can serve as a positive control because it induces severe inflammatory reactions. The further procedure includes evaluation scores being used as for the i.v. injections. To eliminate the species-specific influence of small rodents (small artery) it is also recommended to use non-rodents like the dog.

CRITICAL ASSESSMENT OF THE METHOD
In contrast to the above described i.v. administration more important is to perform an i.a. tolerance. This is because cases where i.v. drugs have been erroneously administered by i.a. administration have increased during recent years, and liability issues have become a problem if such testing is not performed (Stötzer 1989).

III.C.2.6.2
Intramuscular Testing

PURPOSE AND RATIONALE
The interest in the availability of i.m. injectable drugs is increasing. The solutions for i.m. injections must be absolutely sterile and should be isotonic, have a physiological pH (7,4) and have the same osmotic pressure in order to avoid unspecific muscular tissue reactions.

PROCEDURE
The test is preferably conducted by injections into the M. sacrospialis on the dorsum or into the M. vastus lateralis on the femur of the rabbits. Alternatively, in the rat the injections can be performed into the M. quadriceps femoris. Also dogs could be used, however for animal wellfare reasons, smaller animals are proferred. Injection of the vehicle is performed on the contra-lateral side of the animal. The injection volume should be 1 ml for smaller animals and 2 ml for the dog.

The procedure can be complemented by measuring the creatine-kinase MM (CK-MM) in the blood serum of the test animals. CK-MM is the muscle-specific enzyme which leaks out of a skeletal muscle if muscle damage has occurred. Determination of CK-MM should be conducted in the blood serum 24 hours after administration.

The evaluation of the muscle damage can be done macroscopically and microscopically. The degree of the histological changes is evaluated by morphometrical methods (Stötzer 1989).

CRITICAL ASSESSMENT OF THE METHOD
In case of drugs which are administered i.m., the preclinical i.m. toxicological is an important assay in the early phases of development. Often sings of i.m. local intolerance can be avoided or local tolerance can be improved by changes of the vehicle.

III.C.2.6.3
Sub-cutaneous Testing

PURPOSE AND RATIONALE
Subcutaneous administration testing plays an increasing role in drug development because biopharmaceuticals such as peptides, antibodies etc. are used more and more frequently for therapy. They only stay active if they are not exposed to and “digested” in the environment of the gastro-intestinal tract. Further, an increasing number of drugs is formulated in such a manner that they are slowly but continuously released from a subcutaneous depot.

PROCEDURE
The drug is administered into the subcutis of test animals (mouse, rat, rabbit, or dog) at the latero-dorsal area of the thoracic wall. The injection volume has to be adjusted to the size of the animal. The overall evaluation of findings is done according to the scoring described for intramuscular tolerance testing (Stötzer 1989).

EVALUATION

As stated already, the test serves today mainly for kinetic reasons i.e. to find a formulation with retarded drug substance release and good s. c. tolerability. The vehicle of a drug and the drug itself to be developed for s. c. administration has to exhibit a profile which guarantees continuous delivery for an optimal time period and no or tolerable local reactions to the subcutis.

III.C.2.7 Toxicological Testing of Biotechnologically Produced Drugs

PURPOSE AND RATIONALE

It is not the intent of this section to describe the methodologies in detail, rather it should provide some guidance on how the testing strategy is designed.

The purpose of preclinical testing is to define toxicological endpoints not only prior to initiation but throughout clinical development.

Biopharmaceuticals comparable to a product for which there is wide experience in clinical practice may need less intensive testing.

Biological activity can be evaluated by using in vitro techniques to determine which effects of the product are related to clinical activity. Due to species specificity of biotechnology derived products, it is necessary to select relevant species for testing. Mammalian cell lines can be used to predict in vivo activity and the relative sensitivity of various species including man. Such studies are useful to determine receptor occupancy, receptor affinity pharmacological aspects, and for the selection of adequate animal species for toxicity testing.

PROCEDURE

The safety evaluation should use relevant animal species. The relevant species is the species in which the test material is pharmacologically active due to expression of the receptor or an epitope. Several techniques can be used to identify the relevant species. Relevant are those that express the desired epitope and demonstrate a similar tissue cross reactivity profile as humans.

Safety evaluation should be conducted in two species but can be limited to only one if only one can be identified or if the biological activity is well understood.

In case no relevant species is available, the use of transgenic animals expressing the human receptor or the use of homologous proteins is recommended. Where it is not possible to use transgenic animals or homologous proteins, it may still make sense to assess some aspects of potential toxicity in a limited toxicity study in a single species (e.g. 14 days duration) that includes an evaluation of important functional endpoints (e.g. cardio-vascular, respiratory).

In recent years studies have been developed in spontaneous disease models, gene knock out models and transgenic animals. These models provide information on the pharmacological action, pharmaco-kinetics and tolerability of a biotech products.

The route of administration should be the same or as close as possible to that proposed for clinical use. As in the case of heterocyclic chemicals, pharmaco-kinetic behaviour and bioavailability in the test species should be comparable to humans. In case the product is cleared faster from the test animals than in humans, the frequency of administration in the animals can be increased.

Dose levels should be chosen in a manner that a dose-response relationship can be established including a toxic dose and a no observed adverse effect level (NOAEL). The selection of dose levels should be justified by using pharmacological/physiological effects. Availability of suitable test material can be also an important and accepted factor for the limitation of the dose regimen especially with regard to the determination of the high dose level.

The immunogenic effects of biotech products intended for use in humans are often a problem in animals. The measurement of antibodies against the biotech product is therefore a key element for toxicity testing. Antibody formation has to be characterized by titer, number of responding animals, neutralizing or non-neutralizing, and their appearance has to be correlated with any pharmacological or toxicological changes. Special attention should be devoted to the evaluation of possible pathological changes related to immune complex formation and deposition. The detection of antibodies must not be a criterion for the early termination of a toxicology study unless the immune response neutralizes the pharmacodynamic or toxicological effects of the bio-pharmaceutical product in the larger portion of animals because the immune response to biopharmaceutical products is variable.

The induction of antibodies in animal studies does not predict their formation in humans. Humans developing antibodies against humanized proteins do not predict the loss of the therapeutic activity. In addition, the occurrence of severe anaphylactic responses to recombinant proteins is rare in humans. Therefore, the result of guinea pig tests for anaphylaxis is of little value for the evaluation of these products.

Single dose studies describe the relationship of dose to systemic and local toxicity. Further, the results are the basis for the selection of dose levels in longer term

studies. The incorporation of pharmacological end points should be considered.

In multiple dose studies the same route and a similar dosing regimen are used as in clinics. A recovery period can provide information about reversal or worsening of effects and the can help to discover delayed toxicity.

The duration of studies is in general 1–3 months or in case of short term administration in humans and for acute life-threatening indications up to two weeks. For chronic indications, 6 months studies are recommended.

Carcinogenicity studies are not considered appropriate. However, product specific properties may necessitate testing (e.g. growth factors, immunosuppressive properties). Further, the ability to stimulate growth of normal or malignant cells expressing the receptor should be taken into consideration.

If other studies have not provided information to allow the assessment of a carcinogenic potential, the utility of testing in a single rodent species can be considered.

Local tolerance studies should be considered for the formulation intended for marketing.

The need for reproductive performance and developmental studies depends on the indication and patient population. The design of such studies is adjusted to the species specificity, immunogenicity, and elimination half-life.

Standard genotoxicity studies are in general not considered necessary. Also testing of production process dependent contaminants is not considered necessary (ICH 1997).

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Chapter III.D
In Silico Methods

Alexander Amberg

III.D.1 Introduction

In drug development it is very important to evaluate the potential toxicological risk of a drug candidate as early as possible to reduce costs and time in drug development process. In the traditional way the toxicological risk of a compound is investigated with the help of a battery of in vivo and in vitro methods. Since the late 1970s many different in silico methods for the prediction of toxicity have been developed. The term "in silico" stems from the computer component silicium, in silico methods therefore refers to methods or predictions using computational approaches. In silico methods have the advantage that they can make fast predictions for a large set of compounds in a high-throughput mode. Another advantage is that in silico methods make their prediction based on the structure of a compound even before it has been synthesized. In silico methods can therefore be used at a very early stage in the drug development process, for compounds planned to be synthesized, for which no or only little compound is available, or also for impurities or degradation products later in the drug development process, for which no synthesis is available. However, good predictivity of an in silico method is crucial if the method is to be introduced into the drug development process.

The two major independent in silico methods for the prediction of toxicity are quantitative-structure-activity-relationship (QSAR) and expert systems (e.g. DEREK, MultiCASE). QSAR means the quantitative relationship between a chemical structure and its biological/ toxicological activity with the help of chemical descriptors that are generated from the molecular structure. The descriptors are statistically analyzed for the development of a model describing the specified activity, such as toxicity (Durham and Pearl 2001).

Another approach to predict toxicity is basing on structure-activity-relationship (SAR), which means the qualitative relationship between a specific chemical structure and their biological/toxicological activity, e.g. the expert system DEREK is based on SAR prediction. In SAR the occurrence of specific substructures in a molecule are correlated to be responsible and necessary for a biological/toxicological activity.

Expert systems have been defined as "any formal systems", which make predictions about the toxicity of chemicals. All expert systems for the prediction of toxicity are built on experimental data and/or rules derived from such data (Dearden 2003). The expert systems can be further divided into two subclasses based on the method of generating rules. The one method is a knowledge- or rule-based expert system, for which experts/toxicologists create rules based on a list of structural features that have been related to a specified toxicity (Durham and Pearl 2001). An example of a typical knowledge- or rule-based system is DEREK, which will be described later.

The other method is a computer- or statistically-based expert system, for which a large training data set of compounds with known toxicity is needed to derive structural features that are highly correlated to the specified toxicity (Durham and Pearl 2001). An example of a typical computer- or statistically-based expert system is MultiCASE, which will also be described later.

There are a number of commercial expert in silico systems available for the prediction of toxicity. Examples of knowledge-based expert systems are DEREK (Lhasa, Leeds, UK), Hazard Expert (ComGenex, San Francisco, CA, USA), Oncologic (San Francisco, CA, USA) and COMPACT (University of Surrey, Guilford, UK). Examples of statistically-based systems are MultiCASE (MultiCase, Beachwood, OH, USA),

CASE (MultiCase, Beachwood, OH, USA), QSAR-ES (Cooperative R and D agreement between MultiCASE and the U.S. Food and Drug Administration [U.S. FDA]), TOPKAT (Accelrys, San Diego, CA, USA), ADAPT (Jurs Research Group, Pennsylvania State University, Philadelphia, PA, USA) and common reactivity pattern (CORPEA, University A. Zlatarov, Bourgas, Bulgaria). Additionally, expert systems could be further classified according to their level of molecular structure assessment. Two-dimensional structure systems include DEREK and MultiCASE, whereas three-dimensional systems include COMPACT and COREPA. These systems are further described by Patlewicz et al. (2003). In the following chapters the QSAR method and the two expert systems DEREK and MultiCASE will be described in details.

III.D.2 QSAR (Quantitative Structure Activity Relationship)

PURPOSE AND RATIONALE

Quantitative Structure Activity Relationship (QSAR) is a method that makes predictions by the quantitative description of molecular properties with the use of descriptors of the chemical structure (Dearden 2003). This means QSAR models describe the quantitative or calculated relationship between a chemical structure and their biological activity (e.g. toxicity) with the help of chemical descriptors that are generated from the molecular structure (Durham and Pearl 2001). This relationship is described in from of a mathematical equation (e.g. log 1/C = a π + b σ + . . . + const). QSAR models generally show better predictivity if all compounds of a dataset involved in the prediction are derived from a congeneric series of compounds, that means they should all act by the same mechanism of action, since the physico-chemical and structural descriptors used in the QSAR reflect the same mechanism of action. Sometimes it is difficult to determine the mechanism of action, so series of compounds involved in a QSAR model are often restricted to a given chemical class in the hope that this will ensure a single mechanism of action (Dearden 2003).

Most published QSAR models have been developed from congeneric series. But with the recent use of large, diverse chemical libraries, there is an increasing interest for QSAR models for a heterogeneous collection of compounds, a non-congeneric series of compounds. It is often possible to develop QSAR models for non-congeneric series to be used as classification models, for example for a classification into high, moderate or low toxicity (Dearden 2003).

QSAR models exist for a wide range of biological and toxicological endpoints, e.g. published QSAR models for toxicity cover over 30 different endpoints, from carcinogenicity, mutagenicity, skin sensitization, eye irritation, neurotoxicity to gastric irritancy etc. Dearden (2003) and Barratt and Rodford (2001) review in their publications some of the recent QSAR models for various endpoints.

For the prediction of toxicity there are also QSAR-based expert systems commercially available, such as TOPKAT (TOxicity Prediction by Komputer Assisted Technology, Accelrys, San Diego, CA, USA). This system already contains developed and predefined prediction models and covers up to 16 different toxicological endpoints including mutagenicity, carcinogenicity, developmental toxicity, skin sensitization, eye irritation and rat oral LD_{50} etc. The QSAR models from TOPKAT are built on large, heterogeneous databases with carefully selected data and descriptors (Lemont and Lowell 1999).

PROCEDURE

QSAR modelling generally involves three steps. The first step is the collection of a training set of compounds. The second step is the selection of descriptors that can properly relate chemical structure to biological/toxicological activity. At least the third step is the application of statistical methods, which correlate changes in structure with changes in biological/toxicological activity (Perkins et al. 2003).

The statistical methods of QSAR modelling are based on the correlation of changes in biological/toxicological activity ($\Delta\Phi$) resulting from certain chemical modifications (ΔC), either directly by structural parameters, called Free Wilson-type relationships, or by the corresponding changes of molecular properties, called Hansch-type analyses (Kubinyi 2002):

$$\Delta\Phi = f\Delta C$$

The classical QSAR methodology started 1964 with the publications of Hansch and Fujita (1964) and Free and Wilson (1964) and the statement of Hansch (1969) resulted from a proposal by Fujita. They proposed to combine several physiochemical parameters (π, σ), also called descriptors, in a quantitative model. This Hansch-type analysis is very flexible and describes many different kinds of biological activities, e.g. in vitro data such as enzyme inhibition (Kubinyi 2002):

Log 1/C = a π + b σ + . . . + const (C is a molar dose that produces a certain biological response).

In an independent publication, Free and Wilson (1964) formulated their mathematical model. This model describes the biological activity in a logarithmic scale as the sum of the biological activities of the reference compound and the group contributions of all substituents that are attached to various positions of this molecule (Kubinyi 2002):

Log 1/C = $\Sigma a_i + \mu$ (a_i are the group contribution of the individual substituents X_i to the biological activity values and μ is the calculated biological activity of a reference compound, most often the unsubstituted analogue).

Free Wilson analysis is easy to apply. No physiochemical properties are needed to describe biological activity, just values of 1 or 0, to indicate the presence or absence of a certain position.

On the other hand, Free Wilson analysis is much more restricted than Hansch analysis, because of its many parameters and the corresponding decrease on the number of degrees of freedom of the statistical analysis (Kubinyi 2002).

In 1979 3D QSARs were developed, which correlate spatially localized features across a chemical series with biological activity. The two primary types are 3D QSARs using lattice-based or surface-based descriptors. Among the lattice-based methods, Comparative Molecular Field Analysis (CoMFA) is the most used 3D QSAR method. In a CoMFA model all molecules of a training set are aligned together regarding to similarities in their structure and embedded in a 3D grid. 3D arrangements of molecular features are then correlated with the biological activity (Perkins et al. 2003).

The molecular QSAR descriptors can be grouped into three categories, 2D descriptors (e.g. molecular connectivity), 3D descriptors (e.g. molecular surface area) and physicochemical properties (e.g. log P). They can also be categorized according to their nature as well as calculation method, such as constitutional, topological, geometrical, electrostatic, quantum chemical and thermodynamic descriptors (Perkins et al. 2003). In the "Handbook of Molecular Descriptors" from Todeschini and Consonni (2000), an encyclopaedic collection and description is available for all molecular descriptors from the beginning (about 2000 of different definitions). A number of different commercial systems are available for the development of different kinds of QSAR models from a dataset. Examples are Cerius, Moe, Sybyl, Golpe, Pipelinepilot (Scitegic) and DRAGON etc.

In the expert system TOPKAT predefined QSAR models are integrated in the system with the mainly use of topological, sub-structural and electronic descriptors from the 2-D Kier and Hall type, which are developed from a large, heterogeneous databases with carefully selected data (Lemont and Lowell 1999). Thus no own development of QSAR models is necessary, the structures can immediately be entered into the system and the prediction can be started for the different available toxicity endpoints. Continuous endpoints, such as LD_{50}, are modelled using multiple linear regression QSARs to generate different value predictions (e.g. different LD_{50} values). Other endpoints like carcinogenicity, where the endpoint is either positive or negative, are modelled using two-group linear discriminant regression functions (Greene 2002). The program also provides an estimate of confidence in the prediction that the user can examine the most similar compounds in the training set based upon their chemical descriptors. The program also provides an optimum prediction space (OPS) measurement that determines if the compound being investigated is well represented in the training set (Durham and Pearl 2001).

EVALUATION

To date a number of different QSAR models have been published, for the prediction of drug toxicity QSAR models cover over 30 different endpoints. It is not within the scope of this review to cover all of them here. Many authors discussed and evaluated different QSAR models in the literature (Dearden 2003; Patlewicz et al. 2003; Tuppurainen 1999).

Good examples of QSAR models can be received for the prediction of mutagenicity with the Ames test (Ames et al. 1973). The Ames test is a suitable assay to develop a robust QSAR model because it is standardized, delivers a substantial amount of data within a short period of time, can be used for different chemical classes, covers a similar mechanism of toxicity for all compounds etc. The mechanism of mutagenicity is the result of cell membrane penetration, bioactivation, interaction and modification of DNA together with various error-free and error-prone DNA repair processes. Many different types of mutagenic compounds follow this mechanism, which is reflected in the great diversity of chemical structures that have been associated with mutagenicity. Most QSAR studies of mutagenic activity have been based on Hansch-type regression models (Patlewicz et al. 2003). Tuppurainen (1999) reviewed some QSAR models for different congeneric series of mutagenic

compounds like aromatic hydrocarbons, aromatic amines, nitrosamine, epoxides etc. One example of these QSAR models is described from Debnath et al. (1992). He developed a QSAR model with a dataset containing the mutagenic activity of 67 aromatic and heterocyclic amines acting on the Ames stain TA100 after metabolic activation with S9-mix. In this model the mutagenic activity (log TA100 in revertants/nmol) is linear dependent on hydrophobicity (log P), the energy of the highest occupied molecular orbital (ϵ_{HOMO}) and the energy of the lowest unoccupied molecular orbital (ϵ_{LUMO}). The QSAR model representing the relationship between these parameters and activity is given in the following equation:

$$\log TA100 = 0.92(\pm 0.23)\log P + 1.17(\pm 0.83)\epsilon_{HOMO} - 1.18(\pm 0.44)\epsilon_{LUMO} + 7.35(\pm 6.90)$$

This relationship is interpreted in the way that the toxicological activity is dependent on log P, describing the ability of a compound to penetrate cell membranes to reach the target DNA, and on the molecular orbital energies (ϵ_{HOMO}, ϵ_{LUMO}), describing the ability of the metabolic bioactivation to the electrophilic, reactive intermediate nitrenium-ion, which reacts with DNA resulting in a mutation and an increase of the revertants number in the Ames strain TA100.

The result of the internal evaluation of this QSAR model is shown in Figure 1. In this figure the calculated log TA100 values from the upper equation are plotted against the observed logTA100 values from the Ames tests for each of the 67 compounds. The line represents the regression and the results of the evaluation is expressed by the correlation coefficient r = 0.887 and the standard deviation from the regression s = 0.708. These data indicate the high potential of this QSAR model for the prediction of the mutagenic activity of aromatic and heterocyclic amines in the Ames stain TA100.

The expert system TOPKAT has been evaluated in several studies (Snyder et al. 2004; Dearden 2003; Patlewicz et al. 2003; Cariello et al. 2002; Durham and Pearl 2001; Richard 1998; Benfenati and Gini 1997; Enslein et al. 1994). In these evaluations all statistical parameters like sensitivity, specificity, positive and negative predictivity and the overall concordance range from 40 % to 73 % for the various endpoints.

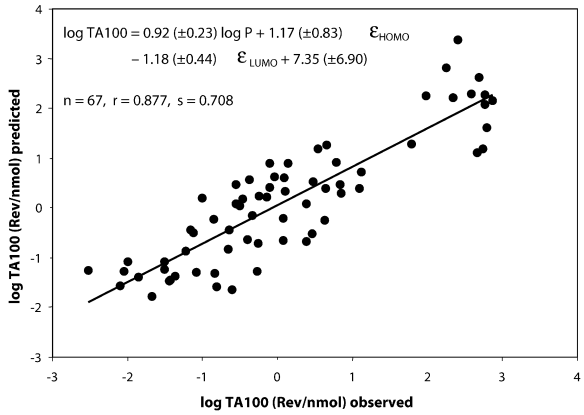

Fig. 1. QSAR model for the prediction of the mutagenic activity (in revertants/nmol) of aromatic and heterocyclic amines in the Ames strain TA100. The representing mathematical equation of the QSAR model is shown in the top left corner. In the graph the calculated log TA100 values from the QSAR model are plotted against the observed logTA100 values from the Ames tests for each of the 67 compounds of the data set. The line represents the regression given by the equation. The model is further described in the paragraph evaluation. Abbreviations: log TA100 = mutagenic activity in revertants/nmol, log P = hydrophobicity, ϵ_{HOMO} = energy of the highest occupied molecular orbital, ϵ_{LUMO} = the energy of the lowest unoccupied molecular orbital, n = number of compounds from the dataset, r = correlation coefficient, s = standard deviation (Debnath et al. 1992).

CRITICAL ASSESSMENT OF THE METHOD

The development of a QSAR model with high quality or predictivity depends on many factors. One important point is the quality of the dataset, bad data points will corrupt the model. In a qualitative good dataset the number of chemicals should be sufficiently large to ensure statistical stability (at least 15–20 chemicals), the activity range should span two or more orders of magnitude and should be evenly distributed, the dose-response relationship should be smooth and the chemicals should possess enough structural diversity. For robustness the right selection of descriptors is very important. The more is known about the biological mechanism, the better the descriptors can be selected to best encode the variation of activity with the structure. And finally, it is also very important to select the right QSAR method, which best describes the correlation between the structure and the biological/toxicological activity to give a QSAR model with a good prediction (Perkins et al. 2003).

Many different types of QSAR models and chemical descriptors for a wide range of endpoints are developed and published over the years. This makes QSAR a very flexible technique to be adapted for many different situations and a quite powerful technique that can provide a wealth of information. This has also a great potential for new QSAR models with every new experimental data.

One restriction of QSAR is that in most cases a good predictivity of a model is limited to a congeneric series or a specific class of compounds, which

all act by the same mechanism of action. However, even for non-congeneric series it is often possible to develop classification QSAR models, for example for a classification into high, moderate or low toxicity. So sometimes the QSAR models are not related to a specific mechanism. Therefore it is not always possible to propose structural changes that will remove toxicity from the compound (Dearden 2003).

For the development of good QSAR models, experience with the method and time is needed, except when using the QSAR-based experts system TOPKAT, since QSAR models are already integrated in the system. TOPKAT has also the advantage that predictions are not limited to a single mechanism or class of chemicals, the program automatically supplies training sets of compounds for different endpoints. An important limitation of TOPKAT is that it is a closed system that does not allows users to expand the training set or to modify the method (Durham and Pearl 2001).

MODIFICATIONS OF THE METHOD

In the theory, it is possible to create new QSAR models with almost all datasets of compounds with known biological/toxicological activity. But practically it is a question of the quality and predictivity of a QSAR model to be applied in prediction of biological/toxicological activity. For this reason evaluation of each QSAR model is extremely important. The evaluation of a QSAR model can be preformed either by internal validation (cross validation) or external validation (use of a test-set). External validation is preferred, but is not always possible, e.g. because of the small size of a dataset (Dearden, 2003).

Unfortunately no modification of the training set or the prediction models is possible for the QSAR-based expert system TOPKAT.

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EXAMPLE

An example of a QSAR model for the prediction of the mutagenic activity of aromatic and heterocyclic amines in the Ames strain TA100 is shown in Figure 1.

III.D.3 DEREK for Windows (DfW)

PURPOSE AND RATIONALE

DEREK for Windows (DfW, Deductive Estimation of Risk from Existing Knowledge) is a knowledge-based expert computer system for the prediction of toxicity. The prediction of toxicity is based on structure-activity relationship (SAR) analysis of a chemical structure. It provides a qualitative, not quantitative, prediction. The knowledge is stored as rules in the computer system. When the software analyzes a compound, the rules identify features within the structure that have been shown to be responsible for toxicological activity when they have been present in other chemicals. DfW highlights these substructures and give structural alerts for this compound. These toxicological active substructures within the structures are called toxicophores in DfW (LHASA Limited homepage). Since substructures can exist in a variety of molecules, the rules are not chemical-specific, but rather serve as broad generalizations with regard to the chemical structure (e.g. alkylating agent, acid or halogen-containing molecule, Cariello et al. 2002).

The knowledge is based on researching literature data or internal company data with an emphasis on the understanding of mechanisms of toxicity and metabolism required for activation of a compound to a toxicological intermediate (Patlewicz at al. 2003). It covers a wide variety of toxicological endpoints, which include genotoxicity, carcinogenicity, irritation, skin and respiratory sensitization, thyroid toxicity, and other miscellaneous endpoints (LHASA Limited homepage). Its main strengths lie in the prediction of carcinogenicity, mutagenicity and skin sensitization (Greene 2002).

The latest versions of the DfW software incorporate a reasoning engine that uses log P (calculated by a C log P plug-in) and molecular mass to predict the likelihood that a chemical will express its potential toxicity regarding a specific endpoint in the selected species. This feature is, for example, used in the evaluation of the skin permeability for the prediction of skin sensitization and photoallergenicity in humans (Patlewicz at al. 2003). This proprietary reasoning engine combines both numerical and non-numerical statements (like selected species, log P, endpoint, toxicophores etc.) to reach a conclusion about a given event. It is based upon the mathematical framework of the logic of argumentation. The reasoning engine can be adapted to any toxicological endpoint within the DfW system (Greene 2002). The result of the reasoning is that the likelihood of a structure being toxic is then expressed in one of the following terms: Certain, Probable, Plausible, Equivocal, Doubted, Improbable, Impossible, Open and Contradicted.

The rules are written and maintained by experts from LHASA Limited. LHASA Limited is a non-profit organisation that facilitates collaborations between scientists from a wide range of educational, regulatory and commercial institutions. The rules are regularly updated by LHASA Limited and new versions of the computer software are available every year. Three times a year international collaborative user group meetings are organized with representatives from pharmaceutical, agrochemicals and regulatory organizations. At these user group meetings changes in computer software and knowledge base developments are presented and the users provide feedback about the improvements. Users suggestions for improvements to knowledge base and software are discussed during the wish list session, and new rules and toxicological information derived from internal company data is presented from the users. This unique system encourages the sharing of toxicological information and knowledge for the benefit of all, without organisations compromising the confidentiality of their proprietary data (Patlewicz et al. 2003).

PROCEDURE

The new versions of DfW are compatible with Microsoft Windows. For the prediction of toxicity the chemical structure of a compound is input into the system with the use of ISIS/Draw (from MDL Information Systems) or by importing a mol-file or sd-file (Figure 2a). After the system has the structure, pushing the “process” button starts the prediction. During processing, the system searches and identifies all known toxicophores in the structure from all the different toxicological endpoints within seconds. After completion of the analysis, the system opens a result window. The result window contains: the imported structure, a list of toxicological endpoints that the system has made a prediction for, the number and name of

Table 1 DfW mutagenicity predictions (version 7.0) and Ames results for 1441 Aventis proprietary structures.

Ames Results	DfW prediction (version 7.0) Mutagenicity alert	No alert	Total	
Positive	141	84	225	Sensitivity[1] 141/225 = 63%
Negative	263	953	1216	Specificity[2] 953/1216 = 78%
Total	404	1037	1441	Concordance[3] (141 + 953)/1441 = 76%

[1] Sensitivity is the percentage of correctly predicted positive compounds
[2] Specificity is the percentage of correctly predicted negative compounds
[3] Concordance is the percentage of correctly predicted positive and negative compounds

the toxicophores found in the structure (the position of the toxicophores in the structure is highlighted in red), and the level of likelihood for each endpoint in each species (Figure 2b). For each prediction in the result window additional information is available including a reasoning report, an alert description, supporting examples, and processing constraint details. For example by choosing the "Alert Description" button, additional information and a description of the alert including information about the SAR, references, endpoint and comments are displayed in an extra window for a specific alert (Figure 2c). From the "Alert Description" window users can choose the "Example" button, this opens a new window which displays toxicity data for a number of chemicals which are pertinent to the alert including information about the structure, name, CAS number, and toxic activity. References and comments can be displayed in an extra window (Figure 2d). It is also possible to process a big list of many different structures in the mol-file or sd-file format with one button click by using the Auto DEREK function, making batch-processing possible with DfW.

All the results described above can be exported from DfW to other applications by generating a "DEREK for Windows report" in either rich text (rtf), tab delimited text, or modified sd file format (Figure 3).

EVALUATION

Richardt and Benigni (2002) stated in their paper that there are two common approaches to assess the predictive performance of computer programs for the prediction of toxicity: beta-testing and prospective prediction. In beta-testing, the available data set is divided into a training and test set. The model is built upon the training set and the performance is then measured for the test set. In prospective prediction, the model is applied to a novel group of chemical structures where there is often little consistency with training set used to generate the model. A good way to use the prospective evaluation method is for pharmaceutical companies to use their historical, unpublished toxicity data of internal compounds (unavailable to the model developers) to evaluate the prediction systems (Greene 2002).

At Aventis Pharma a prospective evaluation of DfW predictions for the mutagenicity endpoint was performed. The Ames test (Ames 1973) is a well established and fast assay for the prediction of mutagenicity of drug candidates in the pharmaceutical industry. The result of the Ames test is also necessary for approval of a drug. The consequence is that every year a lot of unpublished propriety Ames results are produced in every company and added to each company's historical toxicity database. In 2004 an evaluation of DfW version 7.0 was performed with 1441 proprietary structures from the Aventis historical toxicity database, for which results in mutagenicity assay Ames were available. Of these structures 225 compounds (16 %) were positive and 1216 (84 %) were negative in Ames test. The results of this exercise are shown in Table 1.

The results of this evaluation are summarized with the statistical parameters sensitivity, the correctly predicted positive compounds, specificity, the correctly predicted negative compounds, and concordance, the correctly predicted positive and negative compounds. In this evaluation 141 out of 225 Ames positive compounds were correctly predicted, which gives a sensitivity of 63 % for the system. On the other hand 953 out of 1216 Ames negative compounds were correctly predicted, which gives a specificity of 78 % for the system. The overall concordance, correct positive and correct negative predictions, was 76 %.

These results are in good agreement with those found by other authors (Snyder et al. 2004; Cariello et al. 2002; Greene 2002; Durham and Pearl 2001; Greene et al. 1999; Benfenati and Gini 1997; Sanderson and Earnshaw 1991).

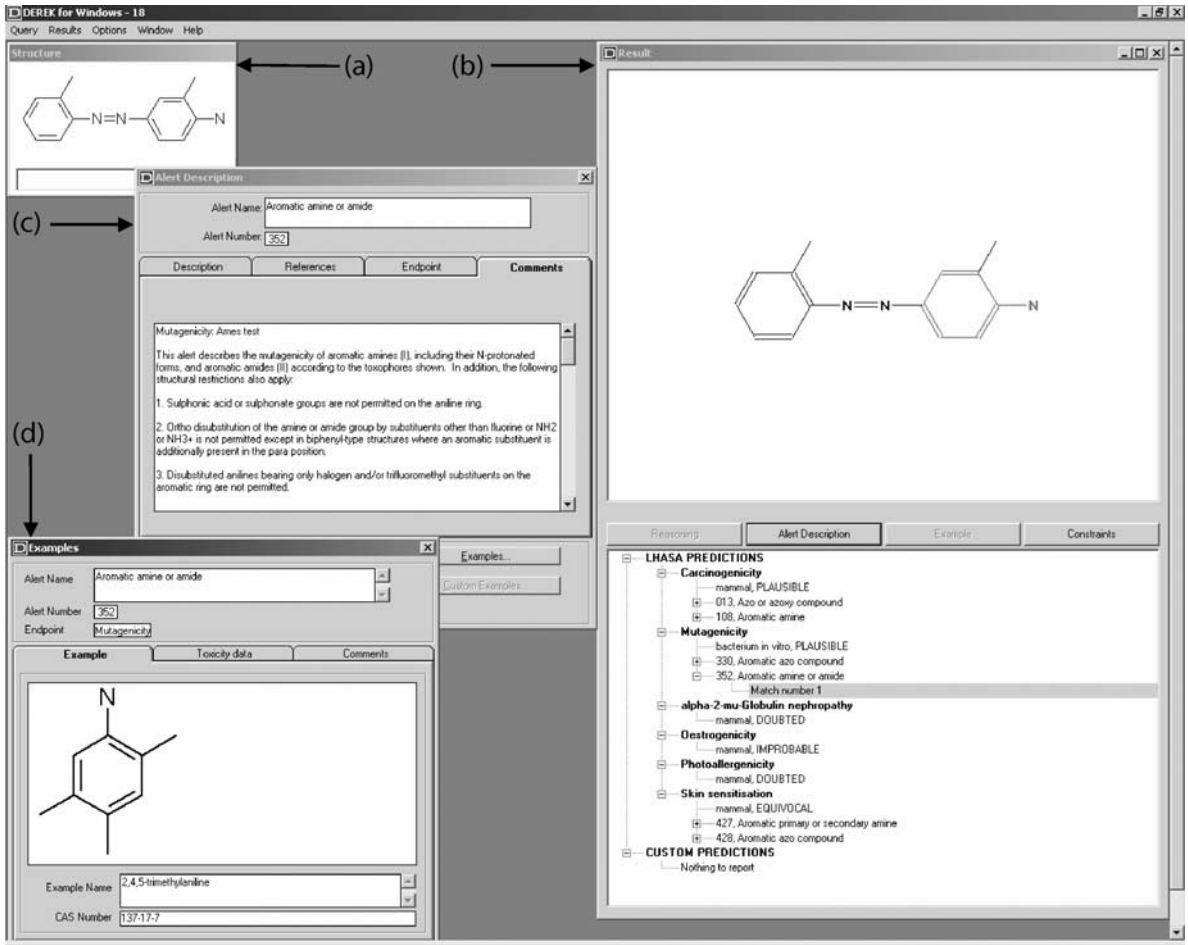
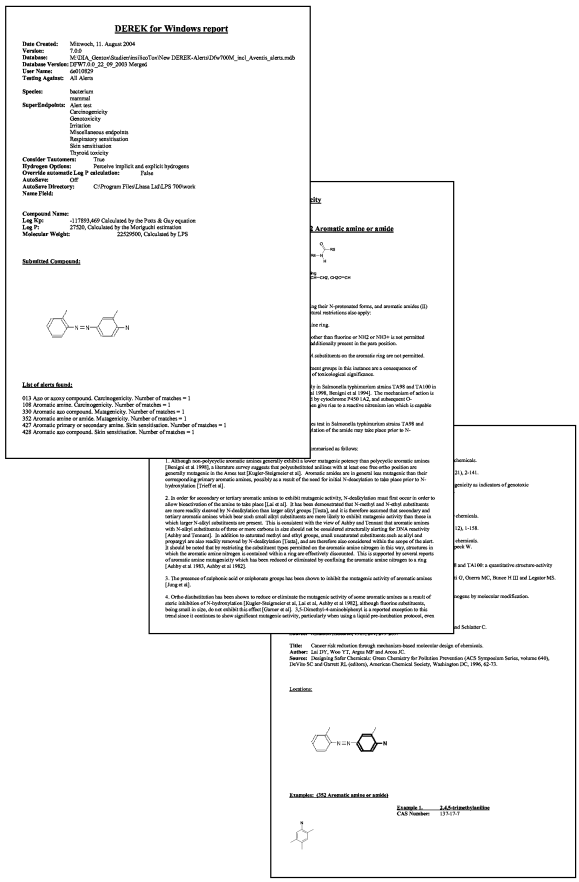


Fig. 2. Screen-print from the DEREK for Windows prediction for the compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine: (a) imported structure, (b) prediction results window, (c) alert description window, (d) example compounds window

CRITICAL ASSESSMENT OF THE METHOD

Greene (2002) listed some strengths and limitations of DfW in his review article. One of the strengths highlighted in this review is that the rules from DfW are regularly updated by LHASA Limited and peer reviewed by the users. This unique system of collaboration between LHASA Limited and the users is also important for Patlewicz et al. (2003) specifically because it encourages the sharing of toxicological information and knowledge for the benefit of all without organizations compromising the confidentiality of their proprietary data. Another strength for Greene (2002) and Durham and Pearl (2001) is that DfW summarizes, on one screen or with one report, the full list of alerts, the highlighted toxicophores and the justification for its prediction including the alert description, published references and example compounds credited for the generation of this alert. It is also very easy with DfW to add new company internal rules to the system together with an alert description, literature and example compounds related to this alert. For Greene (2002) additional strengths are that with the batch processing feature it is possible to make high-throughput predictions for a large number of compounds for both testing and validation. However a written script/program is needed to transform the output data into a useful table format (Durham and Pearl 2001). At least one of the most important strengths of DfW is that the rules are based on scientific knowledge of structure toxicity relationships and mechanisms Greene (2002).

For Greene (2002) one of the limitations of DfW is that only a few physicochemical parameters are used, he suggested that their use should be extended to include other 2D and 3D parameters and also their use in predicting other toxicological endpoints should be explored further. Another point is that the activating and detoxification effects of metabolism need to be explored in more detail. At least with the help of links



DEREK for Windows report

Data Created: Mittwoch, 11. August 2004
Version: 7.0.0
Database: M:\DIA_Genzor\Studien\ames\IsoTan\New DEREK-Alerts\DfW700M_incl_Aventis_alerts.mdb
Database Version: DfW7.0.0_22_09_2003 Merged
User Name: 6601/0820
Testing Against: All Alerts

Species: bacterium
mammal
SuperEndpoints: Alert test
Carcinogenicity
Genotoxicity
Irritation
Miscellaneous endpoints
Respiratory sensitisation
Skin sensitisation
Thyroid toxicity
Consider Tautomers: True
Hydrogen Options: Perceive implicit and explicit hydrogens
Override automatic Log P calculation: False
AutoServe: Off
AutoServe Directory: C:\Program Files\Lhasa Ltd\LPS 700\work
Name Field:

Compound Name:
Log Kp: -11789,669 Calculated by the Potts & Guy equation
Log P: 27520, Calculated by the Mortgudt extraction
Molecular Weight: 22529500, Calculated by LPS

Submitted Compound:

List of alerts found:

613 Azo or azoxy compound. Carcinogenicity. Number of matches = 1
108 Aromatic amine. Carcinogenicity. Number of matches = 1
330 Aromatic azo compound. Mutagenicity. Number of matches = 1
332 Aromatic amine or amide. Mutagenicity. Number of matches = 1
427 Aromatic primary or secondary amine. Skin sensitisation. Number of matches = 1
428 Aromatic azo compound. Skin sensitisation. Number of matches = 1

Fig. 3. Report from the DEREK for Windows prediction for the compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine

to public or commercial toxicity databases, structure searching would be possible and would therefore lead to greater confidence in the predictions made by the program (Greene 2002). For Patlewicz et al. (2003) the primary limitations of DfW relate to the inadequate number of rules for a number of toxicity endpoints and chemical classes reflecting knowledge gaps of the developers and user groups. For Durham and Pearl (2001) the system is limited in that DfW only identifies activation fragments, so the negative prediction is solely based on the lack of positive toxicophores. Therefore the program is extremely biased towards the prediction of positive compounds. Consequently, DfW has a low percentage of false negative predictions. The program automatically screens for some complex rules, e.g. identifying structural exceptions to a rule, but not for other complex rules, e.g. presence of toxicity related to the presence of two or more unconnected toxicophores, even though the program is capable of storing these kind of rules (Durham and Pearl 2001).

MODIFICATIONS OF THE METHOD

Beside the possibility of using the general knowledge base of DfW offered by LHASA Limited, it is also possible to create an internal knowledge base by adding alerts, reasoning rules and supporting data examples and references in-house. For the creation of new rules a knowledge base editor is available that enables the users to define one or more "patterns" for a new alert. These patterns include the SAR for a new rule and the definition for alerting a fragment within a molecule. The original rules offered by LHASA Limited cannot be modified, but it is possible to duplicate the LHASA Limited rules and to modify these copies to create new rules.

To get a more specific prediction for a special study design it is possible to change the processing constraints in DfW. For example by choosing a species DfW will make a species specific prediction. The different species that can be selected are human, primate, mammal, rodent, rat, mouse, guinea pig, hamster, and bacterium incl. Salmonella typhimurium. The other processing constraints that can be changed include: consider tautomers or not, perform a DEREK prediction for a single alert etc.

DfW predictions include information about the mechanism of activity, for example metabolic steps necessary for toxicity. For a more detailed analysis of the toxicity of a compound and its metabolites DfW can be used in combination with an in silico program for the prediction of metabolites. LHASA Limited offers such a program with the name METEOR. For the combination of these two systems, first the metabolites of a compound are predicted by METEOR. These metabolites are then sent to DfW for the prediction of toxicity of the compound and its predicted metabolites.

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EXAMPLE

The Figures 2 and 3 show the DEREK for Windows prediction for the example compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine (CAS-No.: 97-56-3). For this compound many positive mutagenicity and carcinogenicity results are reported in the literature (results from the CCRIS database search). In agreement with the literature, this compound was predicted by DEREK for Windows to be mutagenic (mutagenicity alerts 013: Azo or azoxy compound, 108: Aromatic amine) and carcinogenic (carcinogenicity alerts 330: Aromatic azo compound, 352: Aromatic amine or amide). Figure 2 shows the screen-print and Figure 3 shows the report from the DfW prediction for this compound.

III.D.4 MultiCASE

PURPOSE AND RATIONALE

MultiCASE (Multiple Computer Automated Structure Evaluating, MultiCase, Beachwood, OH, USA) is a statistically-based expert computer system for the prediction of toxicity. It consists of a computer system to perform the prediction and different databases (modules). Each database contains a series of diverse non-congeneric chemical structures and their observed activity (quantitative or qualitative) for specific toxicological endpoints, including toxicological active and inactive compounds. Some authors also classify MultiCASE as a hybrid QSAR and artificial expert structure-based program. The QSAR portion of the program is based upon 2-D chemical descriptors that utilize proprietary statistical analysis developed by Klopman (1984). The artificial expert-structure based program is based upon the identification of atom fragments that are present in active and inactive molecules and that have a high probability of being relevant or responsible for the observed toxicological activity (Durham and Pearl 2001).

For the prediction of toxicity in a first step each molecule of a database is broken down from MultiCASE into all possible fragments from two to ten heavy (non-hydrogen) atoms including also overlapping fragments. These are then classified statistically as “biophores”, fragments associated with toxicity, and “biophobes”, fragments not associated with toxicity. In addition to utilizing molecular fragments, MultiCASE also identifies relevant two-dimensional distances between atoms within a chemical structure. MultiCASE then creates organized dictionaries of these biophores and biophobes and develops ad hoc local QSAR correlations that can be used to predict the activity of unknown molecules. The results of this first prediction step are saved and identified biophores are visible for the users (MultiCASE homepage).

In the second step of the prediction a new molecule is entered into MultiCASE, then the program evaluates this molecule against this organized dictionary and the appropriate QSARs it has created and makes a prediction of the toxicological activity of the molecule for the corresponding endpoint. To do this, MultiCASE identifies all relevant biophores and biophobes of the unknown molecule, combines these into an equation and calculates the toxicological activity expressed in

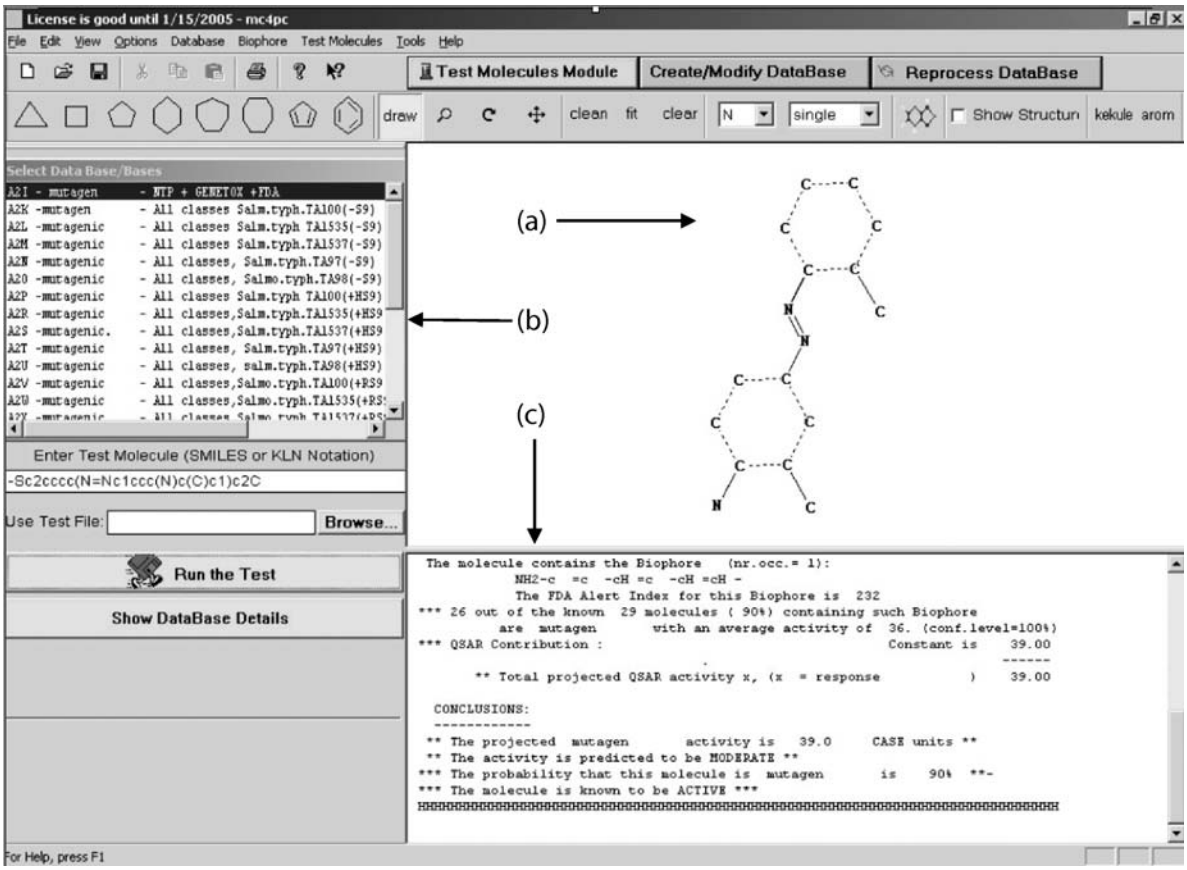

Fig. 4. Screen-print from the MultiCASE prediction for the compound 2-methyl-4-[(2-methylphenyl)azo]benzenamine: (a) imported structure, (b) chosen databases for the prediction, (c) prediction results window

CASE units with the help of the following equation (Dearden 2003):

CASE units = constant + a (fragment 1) + b (fragment 2) + . . .

The scale of CASE units has linear range from 10-99 and normally chemicals with an assigned value of 10–19 are inactive, 20–29 have marginal activity and 30–99 are moderate active, active, very active and extremely active. The system is also capable for identifying fragments that act as modifiers to the activity of each biophores class (Greene 2002).

Currently MultiCASE covers different toxicological endpoints like mutagenicity, carcinogenicity, irritation, teratogenesis, miscellaneous toxicity, short-term genotoxicity assays, biodegradation and rodent hepatocarcinogenicity. For each of these endpoints one or more databases (modules) containing active and inactive molecules are separately available. The number of compounds varies from 70 up to 1300 per module (MultiCASE homepage).

There are also other systems available based on the MultiCASE technology. CASE (Computer Automated Structure Evaluating) was first developed before the MultiCASE system. It uses the same technology but differs in some ways. The major algorithmic difference in MultiCASE is the use of hierarchy in the selection of descriptors, leading to the concept of biophores and modulators. Another important difference is that only with MultiCASE new internal proprietary data can be used to create new databases.

PROCEDURE

The new versions of MultiCASE (MC4PC) run on Windows 9X/NT/2000 computers. For the prediction of toxicity, the structures of a compound can be easily entered into MultiCASE by using an internal graphical interface from the system (Figure 4a). It is also possible to import structures in mol-file format or to run a set of compounds in an sd-file format. For MultiCASE prediction, one or more databases must be chosen, which should be used for the prediction (Figure 4b). After starting the prediction, MultiCASE utilizes the set of statistically significant fragments and/or distance to find the biophores that has the highest probability

of being responsible for the observed toxicological activity. The presence of biophores determines the likelihood of a compound to exhibit toxicity. The prediction then consists of the identification of the biophores responsible for toxicity and the probability (in percent) of a compound being toxicologically active. A compound is presumed to be inactive if it contains no biophores. Within each group of compounds containing a particular biophores, MultiCASE also performs a local QSAR in order to identify molecular features, which controls the degree of activity. These features, termed modulators, are selected from the pool of molecular fragments, 2-D distance descriptors, calculated electronic indices (molecular orbital energies, charge densities) and calculated transport parameters (octanol/water participation coefficient, water solubility). These local QSARs are utilized to predict the potency of chemicals containing specific biophore. At least for an overall prediction, the four individual predictions corresponding to the probability of activity and QSAR potencies are combined using Bayes' theorem. For that purpose the predictivity of each of the four SAR models are determined by analysis of the sensitivity and specificity of the model for chemicals not in the database (Rosenkranz et al. 1999).

During the MultiCASE prediction, the output of the prediction is displayed in a text window on the screen (Figure 4c). This text will be automatically saved in an ASCII-file with a dat-file format. In parallel a second ASCII-file with a dat-file format will be saved when the prediction for a batch of molecules is performed, containing a short summary of all molecules (Figure 5).

File D00.DAT Version 1.54 Date is : 06-APR-04 14:55:44

	A2P	A2R	A2S	A2T	A2U	A2V	A2W	A2X	A2Y	A2Z
COMPOUND04900	-	-	-	inc	-	-	-	-	inc	-
COMPOUND1242A0	inc	-	inc	inc	inc	(+)	inc	inc	inc	inc
COMPOUND1987D	-	inc	inc	inc	-	-	inc	inc	inc	inc
COMPOUND2225D	-	inc	inc	inc	-	-	inc	inc	inc	inc
COMPOUND2347D	-	inc	-	inc	-	-	inc	-	inc	-
COMPOUND2516D	(+)	+	inc	-	(+)	inc	(+)	inc	inc	-
COMPOUND2534D	-	-	+	inc	-	-	-	-	inc	-
COMPOUND2647A0	inc	(+)	inc	inc	inc	-	inc	inc	inc	inc
COMPOUND2941D	+	inc	inc	+	-	+	inc	inc	+	inc
COMPOUND3296D	-	-	inc	inc	inc	inc	inc	inc	inc	inc
COMPOUND3625D	-	inc	inc	inc	-	inc	inc	inc	inc	-
COMPOUND3700A0	inc	(+)	inc	inc	-	(+)	inc	inc	inc	inc
COMPOUND3711D	-	inc	inc	inc	-	-	inc	inc	inc	-
COMPOUND3828A0	-	(+)	inc	inc	-	-	inc	inc	inc	-
COMPOUND3930D	-	inc	inc	inc	-	inc	inc	inc	inc	-
COMPOUND4002A0	-	(+)	-	inc	-	-	inc	-	inc	-
COMPOUND4026A0	inc	(+)	inc	inc	-	-	inc	inc	inc	inc
COMPOUND4607D	inc	inc	inc	inc	inc	inc	inc	inc	inc	-
COMPOUND4777D	-	inc	inc	inc	inc	inc	inc	inc	inc	-
COMPOUND4864D	inc	inc	inc	inc	inc	-	-	inc	inc	inc
COMPOUND6804D	-	-	-	inc	-	-	-	-	inc	-
COMPOUND8010D	-	inc	inc	inc	-	-	inc	inc	inc	inc
COMPOUND8010A0	-	inc	inc	inc	-	-	inc	inc	inc	inc
COMPOUND8074D	-	-	-	inc	-	-	-	inc	-	-
COMPOUND8074A0	-	-	-	inc	-	-	-	inc	-	-
COMPOUND8154D	-	-	-	inc	-	-	-	inc	-	-
COMPOUND8176D	+	inc	inc	inc	+	-	inc	inc	inc	+
COMPOUND8306D	-	-	-	inc	-	-	-	inc	-	-
COMPOUND8418D	inc	(+)	inc	(+)	-	-	inc	inc	inc	+

Fig. 5. Example of a MultiCASE report for a dataset of compounds

EVALUATION

Analogue to the evaluation of the expert system DEREK, a prospective evaluation was also performed with MultiCASE with the use of the historical, unpublished toxicity data of internal Aventis compounds, which were therefore unavailable for the developers of MultiCASE. This evaluation was performed for the endpoint mutagenicity with results of the assay for mutagenicity, the Ames test (Ames 1973). The prediction was performed using version 1.55 of MultiCASE with the identical 1441 proprietary compounds from the Aventis historical database, which were also used for the DEREK evaluation. 225 of these compounds (16 %) were positive and 1216 (84 %) were negative in the Ames test. The selected modules for the prediction were the "single strains mutagenicity for salmonella (A2K-A2Z)", which contain compounds with Ames results for the tested Ames strains TA98, TA100, TA1535 and TA1537 each without metabolic activation and with metabolic activation with human and rat S9-mix, resulting in 12 independent modules. Each module contains 438 up to 1409 compounds. The MultiCASE prediction was defined as positive when the compound was predicted as active in at least one of these modules with a CASE-Unit more than 35. The results of this MultiCASE prediction are shown in Table 2.

The results of this evaluation are summarized with the help of the statistical parameters sensitivity, the correctly predicted positive compounds, specificity, the correctly predicted negative compounds, and concordance, the correctly predicted positive and negative compounds. In this evaluation 131 out of 225 Ames positive compounds were predicted correctly, which results in a sensitivity of the system of 58 %. On the other hand 755 out of 1216 Ames negative compounds were correctly predicted, which gives a specificity of the system of 62 %. The overall concordance, correct positive and correct negative predictions, was 61 %.

Compared to the evaluation of these data with DEREK, the sensitivity of MultiCASE is in the same range as DEREK but the specificity is lower in MultiCASE compared to DEREK. The reason for this may be that in the MultiCASE evaluation, the prediction was performed against 12 independent modules and the compound was defined as inactive only if no activity was predicted in all modules, while the prediction with DEREK was only against one database. The same is true for the concordance, which was also lower in the MultiCASE evaluation compared to the DEREK

Table 2 MultiCASE predictions (version 1.55) using the "single strains mutagenicity for salmonella (A2K-A2Z)" modules for 1441 Aventis proprietary structures.

Ames Results	MultiCase prediction Positive	Negative	Total	
Positive	131	94	225	Sensitivity[1] $131/248 = 58\%$
Negative	461	755	1216	Specificity[2] $755/1216 = 62\%$
Total	592	849	1441	Concordance[3] $(131 + 755)/1216 = 61\%$

[1] Sensitivity is the percentage of correctly predicted positive compounds
[2] Specificity is the percentage of correctly predicted negative compounds
[3] Concordance is the percentage of correctly predicted positive and negative compounds

evaluation. The regular updates of the modules will possibly improve these evaluation results.

These results of the MultiCASE evaluation are in good agreement with those found by other authors (Snyder et al. 2004; Klopman et al. 2003; Greene 2002; Durham and Pearl 2001; Rosenkranz et al. 1999; Cunningham et al. 1998; Benfenati and Gini 1997).

CRITICAL ASSESSMENT OF THE METHOD

In his review Greene (2002) pointed out some strength and limitations of MultiCASE. One of the strength of MultiCASE is that it is capable of generating predictive models without knowing the mechanisms of toxicity. The program also utilizes a number of physiochemical properties to modulate its predictions. Finally another strength is that due to the input of FDA data, MultiCASE has substantially increased the applicability to the prediction of rodent carcinogenicity for pharmaceutical type compounds. For Durham and Pearl (2001) some other strength of MultiCASE compared to a knowledge base system like DEREK is that it searches for both activating and deactivating fragments for the toxicity prediction. Another advantage is that it is very easy to add new proprietary data to the databases in order to increase the chemical space and to achieve a more specific prediction for the internal proprietary compounds.

The limitations for Greene (2002) are that the quality of the predictions made by the system is closely linked to the quality of the data used in the training set. The output from the program is also often ambiguous and can lead to misinterpretation of the predictions. Moreover the system often fails to distinguish between molecules containing several small chains within one complex fragment from other molecules containing the same fragments distributed separately.

MODIFICATIONS OF THE METHOD

To perform a prediction with MultiCASE different commercially available databases from the developer of the program can be used. They are updated regularly by adding new compounds to the databases. However, it is also possible to modify the commercial databases as well as to create new databases using internal company data. For this a dat-file first has to be created, containing all compounds for the new database together with their structure code and biological activity. By screening this dat-file with MultiCASE, statistical algorithms are used to create a model capable of predicting the biological activity of the new compounds. A chart-view is available to adjust the indicated properties. After that the dat-file can be stored and other fields for chemical properties calculated by the program such as boiling point, molecular weight, water solubility and Log P are added. The next step is the creation of the new database. The database description must be entered after which MultiCASE starts to calculate the biophores and biophobes of the new database.

There is also a separate expert system for the combination with MultiCASE, which predicts the possible metabolites, formed of a compound. This system is known as META, which was developed to identify molecular sites susceptible to metabolic transformation. The metabolism dictionary associated with META is able to recognize 663 enzyme-catalyzed reaction rules, which have been categorized into 29 enzyme-reaction classes and 286 spontaneous reactions (Klopman and Rosenkranz 1994).

The U.S. Food and Drug Administration (FDA) has adapted the MultiCASE technology and developed a new system known as MultiCASE QSAR-ES (quantitative structure-activity relationships expert system). This system uses the MultiCASE program and new database modules that were developed under a Cooperative Research and Development Agreement

(CRADA) between the U.S. FDA and MultiCASE. It was designed to improve the prediction of the carcinogenic potential of pharmaceuticals. Matthews and Contrera (1998) performed a beta-test evaluation utilizing 126 compounds not included in the test database with 934 compounds. The results demonstrated an improvement in all statistical parameters for the prediction of carcinogenicity compared to the MultiCASE prediction with an overall concordance of 75 % (Patlewicz et al. 2003).

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EXAMPLE

The Figures 4 and 5 show the MultiCASE prediction for the example compound 2-methyl-4-[(2-methylphenyl)- azo]benzenamine (CAS-No.: 97-56-3). For this compound many positive mutagenicity and carcinogenicity results are reported in the literature (results from the CCRIS database search). In agreement with the literature, this compound was predicted by MultiCASE to be moderate mutagenic with a calculated activity of 39 case units. Figure 4 shows the screen-print from the MultiCASE prediction for this compound and Figure 5 shows an example of a MultiCASE report for a dataset of compounds.

Chapter III.E
Alternative Methods for Carcinogenity Testing

Alberta Argentino-Storino
John Brightwell

III.E.1
Hemizygous p53 +/– Knockout Mouse

PURPOSE AND RATIONALE

The transgenic hemizygous p53 +/– mouse has been proposed as a shorter-term alternative model to the conventional 18 to 24 month bioassay in mice. The performance of this model has been reported by Storer et al. (2001) through a review of the data from 31 studies with 21 compounds, selected by the Alternatives to Carcinogenicity Testing (ACT) Committee as part of the project sponsored by ILSI/HESI (International Life Science Institute/Health and Environmental Sciences Institute). The p53 +/– mouse line derives from the wild-type C57BL/6, carrying a knockout allele of the p53 gene, which is critical for cell cycle control and DNA repair. The functional hemizygous condition should increase the probability for either loss of p53 tumor suppressor function or gain of transforming activity by requiring only a single mutation, as described by Tennant (1993) and Tennant et al. (1996). This model can demonstrate 1) rapid induction of treatment-related tumors, 2) multiple, carcinogen-specific target organs and 3) carcinogen-induced loss of heterozygosity involving the p53 gene. Demonstration of loss of heterozygosity involving the p53 locus is consistent with a common finding in human cancer and supports extrapolation between this rodent model and humans, as reported by French et al. (2001).

PROCEDURE

C57BL/6TacfBR-[KO] p53 N5 heterozygous mice and C57BL/6TacfBR-[KO] p53 N5 wild type mice were supplied by Taconic Farms, Inc., Germantown, NY, USA. Mice were approximately 6 to 10 weeks old at the beginning of the studies. Standard rodent diet and ad libitum drinking water were supplied. Standardised protocols for the 4-week dose-range finding studies and for the 26-week carcinogenicity assays were used, as described by Robinson (1998). Genotype analysis was recommended prior to assignment to dose groups. Animals were generally single caged. All 21 selected compounds were tested. p-Cresidine at 400 mg/kg/day by gavage in corn oil (volume of 10 mL/kg), or benzene at 100 mg/kg/day by gavage in corn oil (volume of 5 mL/kg) were recommended as positive controls. Groups of 15 mice/sex/group received the vehicle alone, the positive control item and 3 dose levels of each test compound. Two additional groups of 15 wild-type mice/sex/group received the vehicle and the high dosage of the test compound. During the in-life phase, animals were observed for mortality, clinical signs, body weight, food consumption and dietary intake of test item (for diet studies). At necropsy, detailed macroscopic observations were performed and the required tissues were weighed and collected. The histopathological examination was performed on control, positive control and high dose groups, as well as on all macroscopic abnormalities, including all masses. Target organs were examined in the low and mid-dose groups. Appropriate statistical analyses were performed.

EVALUATION

A total of 31 of the 32 studies planned for the 21 compounds chosen for evaluation were completed. The results were reported by Storer et al. (2001). The overall spontaneous tumor incidence was low, 2.8 % for males and 6 % for females in studies without transponders (microchips for animal identification) and 8 % for males and 11.3 % for females in studies

with transponders. The most frequent spontaneous tumors were subcutaneous sarcomas, lymphomas and osteosarcomas, as previously reported by Mahler et al. (1998). p-Cresidine was the most commonly used positive control item. A positive response was defined as either a statistically significantly increased incidence of tumors in the urinary bladder or an increase judged to be significant by the rare tumor criteria in at least one sex, as described by Popp (2001). Benzene was used in two studies only. The recommended dose level of 100 mg/kg/day did not produce any statistically-significant increases in tumor incidence. A higher dosage of 200 mg/kg/day has been reported to give positive results, based on the available data presented by French (2001). The overall results for the 21 ILSI ACT test compounds were discussed by the Committee on Carcinogenicity (COC, 2002), which confirmed that the p53 +/– mouse model could identify some genotoxic carcinogens. The fact that results from some compounds (phenacetin, diethylstilbestrol, oestradiol, chloroform, DEHP) differed from expectation, as well as inconsistent or inadequate results with the positive control groups could suggest that the assay is not very reproducible.

CRITICAL ASSESSMENT OF THE METHOD

When reviewing the draft reports of these studies, the p53 AWG (Assay Working Group) discussed the rationale and adequacy of the selected dosages and tumor incidence data and statistics. One of the major issues was to agree on general guidelines regarding the nomenclature and the classification of sarcomas, which appear to be the most important spontaneous neoplastic lesion in p53 +/– mice. The consensus opinion was to analyse tumor incidence for individual tumor sites, although most pathologists considered that some grouping of sarcoma types was justified. Another point of discussion was the confounding effect of transponder implants, which could induce subcutaneous sarcomas, thus increasing the difficulty of identifying treatment-related tumors. The inclusion of positive controls in the experimental designs, which was also recommended by regulatory agencies, obliged testing facilities to manipulate known carcinogens. Confirmation of the genotype of the mice should be a protocol requirement, if the positive control is not included. The use of wild-type mice, where no tumors are normally induced, confirms the role of the p53 gene in accelerating tumorigenesis. Comparison of the views, expressed at the ILSI HESI ACT Workshop (2003) by U.S., European and Japanese agencies, indicated that the European CPMP-SWP (Committee for Proprietary Medicinal Products-Safety Working Party) considers the p53 +/– model acceptable for regulatory purposes and does not limit its use for genotoxic compounds only. U.S. FDA (United States Food and Drug Administration) and Japanese MHLW (Ministry of Health, Labor and Welfare) indicate this as an appropriate model when dealing with compounds that are clearly or equivocally genotoxic only.

MODIFICATION OF THE METHOD

In order to minimise possible variations depending on the experimental design, the agreed standard protocols were used in the laboratories involved in the program. In some studies, female mice were group caged, blood samples were taken and hematology and clinical chemistry parameters were also measured. In most of the short-term carcinogenicity studies toxicokinetic analyses were performed. The 26-week study duration appeared to be appropriate for detecting potent agents, but may not be satisfactory for less active substances. The possibility of enhancing the sensitivity and specificity of the assay by increasing the duration of the studies to 8–9 months and/or increasing the number of mice per group to 20 or 25 was discussed. However, this would impede the comparison to existing control databases and would increase the time required to conduct studies as well as the cost.

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III.E.2
Tg.AC (v-Ha-ras) Transgenic Mouse

PURPOSE AND RATIONALE

The Tg.AC (v-Ha-ras) transgenic mouse is one of the alternative short-term in vivo assays, selected to detect the carcinogenic potential of chemicals and evaluated by the Alternative to Carcinogenicity Testing (ACT) Committee of the international project, sponsored by ILSI/HESI (International Life Science Institute/Health and Environmental Sciences Institute). The utility of this model as an early indicator of potential carcinogens is based on the hypothesis that skin papillomas, induced at the site of application (SOA), could represent a reporter phenotype of tumors induced by either genotoxic or non-genotoxic carcinogens, as described by Tennant et al. (1995). This mouse line was produced in FVB/N mice by pronuclear injection of a v-Ha-ras oncogene, linked to a foetal zeta-globin promoter and an SV40 polyadenylation/splice sequence, as reported by Leder et al. (1990). The transgene, with up to 20 copies in tandem per allele, is located on chromosome 11. Thompson et al. (1998) showed that the presence of two copies, head to head in a palindromic configuration, is required for transgene expression. Bos (1998) confirmed that the Tg.AC mouse line could be considered to be an appropriate short-term carcinogenesis model because the overexpression or mutation in members of the ras gene family is present in some human tumors as well as in spontaneous and induced tumors, commonly seen in conventional carcinogenicity studies in rodents. With the exception of the bone marrow, the transgene is not detected in the tissues of young and adult mice, it remains silent until activation by wounding, as described by Cannon et al. (1997), UV light, as reported by Trempus et al. (1998) or specific chemicals, as observed by Spalding et al. (1993, 1999, 2000).

PROCEDURE

Tennant et al. (1999) defined the standard protocol to be followed for conducting topical application studies in Tg.AC mice. Oral studies were performed according to standard operating procedures applied at each laboratory. Of the 21 chemicals selected in the ILSI/HESI project, 14 were administered by the topical and oral (gavage and/or diet) routes in this model. Male and female Tg.AC and parental strain (FVB/N) mice were obtained from Taconic Farms, Inc, Germantown, NY. The animals were either hemi- or homozygous for the v-Ha-ras oncogene, fused to the promoter of the zeta-globin gene. Mice were approximately 8–9 weeks old at initiation of dosing. Animals were individually housed with free access to rodent standard diet and drinking water. Groups of 15 mice/sex/dose were randomly assigned to the study groups. The vehicles used for drugs and positive control agents were acetone, ethanol or DMSO. TPA (12-o-tetradecanoylphorbol-13-acetate) was the positive control compound used in topical studies, and was administered three times/week at dosages of 1.25 or 2.5 μg/dose. The site of application was the clipped dorsal skin over an area of approximately 8 cm^2 from the intrascapular region to the base of the tail. In studies in which the drugs were administered orally, DMVC (Dimethyl-vinyl-chloride) was employed as a positive control. For both oral and topical studies, the duration of the in-life phase was 26 weeks. The dosages of the test compounds were selected either on the basis of range finding studies performed on Tg.AC and FVB/N mice, or on chronic studies conducted with different strains of mice. The Maximum Tolerated Dose (MTD) or the Maximum Feasible Dose (MFD) in relation to solubility or other limitations, were administered as highest doses. Survival was checked daily; food consumption and body weight were registered weekly; clinical signs, palpable masses and, for topical studies, cutaneous tumors were recorded. Necropsies were performed on all moribund and terminal kill animals. Terminal body weight, body weight gain and absolute and relative organ weights were registered. All neoplastic and nonneoplastic lesions and 12 to 40 target organs were examined microscopically.

EVALUATION

In dermal studies, criteria for grading skin tumors were fixed, as described by Tennant (2001).

Skin tumor data, collected during the in-life clinical observations at SOA, were classified considering a) percent of animals with tumors; b) weeks to initial tumor; c) number of tumors per affected mouse and d) number of weeks until maximal tumor yield was achieved (Eastin et al., 2001). The statistical method applied is described by Dunson et al. (2000). Tumors which were noted distant to the SOA, were reported in an additional section of the summary tables. The Tg.AC AWG (Assay Working Group) agreed that the following elements were to be taken into account when evaluating tumors at the SOA in any control or treated groups:

- the incidence of tumors in treatment and control groups;
- the mean latency period, that is the time between start of treatment and the onset of the first papilloma;
- the maximum multiplicity of papillomas that occurred during the treatment period;
- the time required for maximum papilloma multiplicity to occur.

In oral studies, the SOA was considered the upper alimentary tract and the forestomach, which were observed at necropsy. The statistical methods used were the Poly-k-test or Fischer exact test, as described by Piegorsch et al. (1997), Portier et al. (1989) and Gart et al. (1979).

The results of the studies conducted on the 14 chemicals selected by the ILSI project were summarised by Eastin et al. (2001). Most studies confirmed the hypothesis that the dermal Tg.AC model responds to both mutagenic and nonmutagenic carcinogens. However, data from these studies suggested that the mechanism of action could be fundamental in obtaining the expected positive response. For example, topical application of ethinyl oestradiol, clofibrate and diethylstilbestrol gave clear positive results, whereas cyclosporin A was positive only in females and melphalan, phenacetin and cyclophosphamide did not produce any papillomas at SOA. The lack of receptor sites in the skin for these last compounds or selectivity between tissues for proliferative activity of some chemicals, could explain the negative responses.

Oral administration of the test compounds in this model did not add value to the results from the topical route of administration.

CRITICAL ASSESSMENT OF THE METHOD

Hemizygous and homozygous Tg.AC mice were both tested in early studies and they gave similar responses. Therefore, the ILSI Committee recommended the use of hemizygous mice in the first instance. In 1997, however, when the results on positive controls (TPA) from studies performed in different laboratories were compared, a high incidence of nonresponding mice was discovered. In order to lower the frequency of nonresponders, the ILSI Committee recommended the use of homozygous mice. The possible cause of this event was identified as a genetic instability of the model, which showed a deletion of the transgene sequence, as reported by Thompson et al. (1998). As a result, some of the studies were conducted on hemizygous and some on homozygous mice. Different onset time and incidence level of papillomas were evident in the two different mouse lines. The incidence of spontaneous tumors was found to be significantly higher in the ILSI sponsored studies compared to previous investigations. This model appears not to respond to many chemicals that show strain- or species-specificity in the conventional bioassays and to liver tumor inducers. The available data showed that there were problems in identifying human carcinogens using the Tg.AC model. For all of the above reasons, it was concluded that further investigations were necessary to optimise the methods used, to provide a larger database of results and to more fully understand the different mechanisms of tumor induction in dermal and oral studies. Regulatory perspectives presented at the ILSI HESI ACT Workshop (2003) by U.S., European and Japanese agencies indicated that 1) the Tg.AC model is apparently unable to distinguish nongenotoxic promoters from complete genotoxic carcinogens; 2) it should be used for dermally applied products only; 3) there is some concern on the phenotype stability. Based on the responses obtained, the Tg.AC model could have utility as a test in additional to the battery of standard toxicity studies, performed for carcinogenic risk assessment.

MODIFICATION OF THE METHOD

Regarding the experimental design applied in the ILSI studies, Tennant et al. (2001) illustrated some considerations on the basis of the available data. In order to obtain a greater confidence in the presence of a negative response, it is possible to use doses higher than the MTD. No dose should be used that could cause injury to the skin, as wounding itself could induce papillomas. The group size (15/sex/dose) could be increased in order to obtain greater statistical significance. Positive control groups are always required when the Tg.AC model is used as a regulatory toxicity study. Toxicokinetic studies could be conducted in order to provide additional information on the penetration of the dermally applied test agent and on potential systemic exposure. In several studies, peripheral blood was collected for micronuclei determination, as described by Witt et al. (2000). When agents known to be peroxisome proliferators were tested, liver samples were taken for peroxisomal enzyme analyses, according to methods described by Lazarow (1981) and Small et al. (1985) and for cell cycle biomarker evaluation, as reported by Rininger et al. (1997).

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III.E.3
rasH2 Transgenic Mouse

PURPOSE AND RATIONALE

The rasH2 mouse is one of the gene-modified animal models proposed to detect the carcinogenic potential of pharmaceutical compounds by evaluation of data obtained from short-term carcinogenicity studies. Most of the tests were conducted as part of the ILSI-HESI (International Life Science Institute-Health and Environmental Science Institute) project. In addition, results from other studies not performed in the context of the ILSI program, but using similar protocols, were included in the evaluation of this model. As described by Tamaoky (2001), the rasH2 mouse is a hemizygous transgenic strain, carrying the human prototype c-Ha-ras gene with its own promoter/enhancer. Point mutations in some genes, among them the ras gene family, are expressed in some human cancers, such as those in the pancreas, colon, lung and in melanomas and leukemias. The genetic background is F1 of Tg male C57BL/6J and normal female BALB/cByJ mice, screened for the presence of human prototype c-Ha-ras gene. Approximately 5 to 6 copies of the gene are integrated into chromosome 15 in a tandem array. Transgenes are expressed in normal tissues and in tumors. The encoded product, p21 protein, is 2–3 times higher in Tg mice than in wild type animals. No mutations of the transgene are detected in normal tissues of Tg mice. The phenotypic characteristics of the transgenic animals include smaller body weights, as reported by Yamamoto et al. (1998). The life span is reduced, due to the early onset of neoplastic lesions. The incidence of spontaneous tumors is low up to 30 weeks, then approximately 50 % of mice develop tumors such as lung adenoma/adenocarcinomas, forestomach and skin papillomas, Harderian gland

adenomas, spleen haemangiomas/sarcomas and lymphomas.

PROCEDURE

CB6F1-rasH2 transgenic mice and their wild type CB6F1 mice were obtained from the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan). Mice were approximately 6 to 8 weeks old at the start of dosing. As reported by Usui (2001), ILSI standardised protocols for the 4-week dose-range finding studies and for the 26-week carcinogenicity assays were used, with the exception of melphalan, phenacetin and cyclosporin, for which CIEA protocols were used. In these protocols, the dose levels used in the NTP (National Toxicology Program) 2-year carcinogenicity studies were utilised as the high dose. N-methyl-N-nitrosourea (MNU), administered as a single intraperitoneal dose of 75 mg/kg in citrate-buffered saline at pH 4.5, was used as a positive control. Three groups of 15 mice/sex/group received 3 different dose levels of the test compound. A negative control group received the vehicle alone. Two additional groups of 15 wild-type mice/sex/group received the vehicle or the high dose level of the test compound. During the in-life period, mortality was evaluated and clinical observations, physical examination and measurement of body weight and food consumption were performed. At the conclusion of the dosing phase, organ weight analysis, necropsy and histopathological examination were carried out. Hematological and blood chemical values were analysed if required. Satellite groups for determination of exposure of the test compound were added in some studies. Appropriate statistical analyses were performed when deemed necessary.

EVALUATION

The ILSI study participants completed 19 of the 20 planned studies. All data were evaluated according to the criteria developed by the ILSI HESI Alternative to Carcinogenicity Testing (ACT) Committee, as described by Popp (2001). Usui et al. (2001) reported incidences and timing of spontaneous tumors, which were observed up to 8 months after birth. Common spontaneous tumors were defined as those showing an incidence greater than 1.0 %. They were represented by hepatocellular adenomas and skin squamous papillomas in males; forestomach squamous papillomas, malignant lymphomas and Harderian gland adenomas in females; spleen haemangiosarcomas and lung adenomas in animals of both sexes. The positive control item, MNU, gave reproducible results between laboratories. Incidences of squamous cell tumors in the stomach, skin and vagina, Harderian gland carcinomas, lung adenomas and lymphomas were seen in positive control rasH2 mice. The results of the evaluation performed by the Assay Working Group (AWG) were discussed by the Committee on Carcinogenicity (COC, 2002), which noted that the immunosuppressive human carcinogen cyclosporin A and the hormonal human carcinogen oestradiol were negative in this model. Members also considered that further explanation of the positive results with the peroxisome proliferators clofibrate and DEHP was required.

CRITICAL ASSESSMENT OF THE METHOD

Usui et al. (2001) presented an overview of data available from the short-term oncogenicity studies performed on the rasH2 transgenic model. It was concluded that this model responded to a spectrum of weakly to strongly genotoxic compounds, and that it was more sensitive than wild type mice to non-mutagenic human carcinogens. However, it did not appear to be sensitive to most rodent carcinogens which act through non-genotoxic mechanisms. Based on the results obtained from studies conducted with carcinogenic compounds, inducing different mechanisms of DNA repair, Tamaoky (2001) suggested several possibilities for explaining the enhanced tumorigenic response in rasH2 mice: 1) mutation of the c-Ha-ras gene; 2) mutation of the endogenous mouse ras gene(s); 3) mutation of other oncogenes/suppressor genes; 4) overexpression of the transgene. After evaluation of the potential roles of the above mentioned mechanisms, it was concluded that mutation of the transgene is not fundamental in tumor development. Overexpression, followed by mutation of the ras transgene, is the most probable mechanism for the earlier tumor response. However, although this mechanism could explain the response to genotoxic compounds, the mechanism for tumor induction by nongenotoxic carcinogens remaining to be elucidated. The Committee on Carcinogenicity (COC, 2002) agreed that very little weight could be given to results from this model, considering the proposed, most probable, mechanism of carcinogenicity. As reported by MacDonald et al. (2004) U.S., European and Japanese regulatory agencies considered this alternative model to be appropriate to support the tumorigenic risk assessment for both genotoxic and nongenotoxic compounds. However, it is recommended that further nongenotoxic chemicals be tested.

MODIFICATION OF THE METHOD

Morton et al. (2002) indicated that 25 animals per group could provide statistical power generally equiv-

alent to that of a standard two-year mouse bioassay. The positive control, administered a single dose of MNU, was generally considered adequate, but it was proposed that the supplier or breeder could establish a phenotypic testing scheme, in order to verify the genetic integrity and stability of the model.

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III.E.4
Xpa–/– and Xpa/p53 +/– Knockout Mouse

PURPOSE AND RATIONALE

DNA repair-deficient Xpa–/– and Xpa/p53 +/– knockout mouse models have been proposed as alternative assays for detecting the carcinogenic potential of pharmaceuticals in the collaborative research program, coordinated by the ILSI-HESI (International Life Science Institute-Health and Environmental Science Institute). As explained by Van Kreijl et al. (2001), the suitability of a genetically modified model for short term carcinogenicity testing will depend on the expression pattern of oncogenes or gene knockout in the different tissues and on their involvement in the various types of human and/or rodent tumors. Considering the general role of H-ras and p53 genes in carcinogenesis, it might be expected that these models could exhibit some tissue restriction. In fact, oncogenes or tumor suppressor genes are not normally expressed in all tissues. In order to overcome this limitation, it was postulated that DNA repair-deficient mice could represent a promising third type of model for short-term carcinogenicity evaluation. The rationale for the selection of this model was based mainly on the importance of spontaneous or induced DNA damage in tumor induction, but also on the assertion that a defect in DNA repair could increase the tumor incidence, as recognised in human patients with xeroderma pigmentosum (XP), who show a more than 1000-fold increased risk to develop skin cancer. NER (Nucleotide Excision Repair) is one of the most important pathways by which DNA damage is repaired. As described by De Vries et al. (1995), the Xpa–/– homozygous knockout model, which is almost completely NER-defective, was created in 1995. It is also referred to as the XPA model. When compared with wild-type animals, it showed a low spontaneous tumor incidence even at an advanced age, but responded with higher tumor induction and shorter tumor latency periods after exposure to several genotoxic carcinogens, by both the dermal and oral routes. In order to further improve the sensitivity of this model, Xpa–/– mice were crossed with p53 +/– heterozygous knockout mice. As postulated by Van Oostrom et al. (1999), the resulting model, termed the XPA/p53 model, should display an enhanced tumor response, due to the reduction of cell cycle arrest and/or apoptotic processes.

PROCEDURE

According to the standardised protocols proposed by the ILSI-HESI Committee, C57Bl/6 wild type, C5BL/6 Xpa–/– knockout mice and double transgenic Xpa–/–. p53 +/– mice were obtained from the RIVM (National Institute of Public Health and the Environment), Bilthoven, The Netherlands. At the start of the studies, the mice were from 9 to 13 weeks old. The protocols indicated a duration of 9 months (39 weeks) for all studies, with one exception. Fifteen animals/sex/group were used. The Xpa–/– animals were dosed with 3 dose levels of the test compounds and 2 additional groups received either the vehicle or the positive control item. The positive controls received either benzo[a]pyrene (gavage, 20 mg/kg, 3 ×/week), p-cresidine or 2-acetamidofluorene (dietary, 2500 ppm and 300 ppm, respectively). As reported by Van Kreijl et al. (2001), no positive control was included in the phenobarbital study, but extensive genotyping was performed. Dose levels were selected on the basis of the results obtained in dose range finding studies, in which the actual transgenic mice, instead of wild-type animals, were used. The XPA/p53 and wild-type mice

were treated with vehicle and the high dose level of the test items only.

The animals were housed under conventional conditions, generally with males being single-caged and females group-housed. Food and drinking water were supplied ad-libitum. During the treatment phase, body weight and food consumption were recorded once weekly until week 13, thereafter every 2 weeks until necropsy. Daily observations were performed for mortality and clinical signs. At necropsy, terminal body weights, organ weights for 8 organs and macroscopic changes were recorded. Samples of 40 tissues/organs were taken and preserved for histopathological examination. Non-neoplastic and neoplastic lesions, including toxic alterations were analysed and evaluated according to the indications recommended by the Statistics Subcommittee, as described by Popp (2001). The tumors were defined as "common" when they showed an incidence of $\geq 1\,\%$ and "rare" when observed with an incidence of $< 1\,\%$. The presence of ≥ 2 tumors in a group was considered as positive if no similar neoplastic lesion was observed in control groups and other relevant proliferative lesions were observed in a number of treated animals.

EVALUATION

From the selected 21 ILSI compounds, 13 were tested in the XPA model and wild-type mice and 10 in the XPA/p53 model. Van Kreijl et al. (2001) presented an overview of the available data. Due to the lack of historical tumor data for these new models, the tumor incidence observed in the control groups of the ILSI studies was taken into account to define a tumor as common or rare. After 9 months of treatment, when the animals were approximately 11 months old, the incidence of spontaneous tumors was 5 to 7 % for the XPA model. The number of neoplastic lesions in 15 control groups was seen to be none or 1, with the exception of one group showing 3 spontaneous tumors. The "common" tumors were lymphomas and adrenal pheochromocytomas in females and lung bronchioalveolar adenomas and adrenal cortical adenomas in males. It was noted that, after the 9-month experimental phase, the overall spontaneous tumor incidence in controls was similar in wild-type animals and in XPA mice. The only major differences were represented by the small intestine tumors observed in wild-type males (1.7 %) and the pheochromocytomas in XPA females (1.1 %). The XPA/p53 genotype showed a slightly higher incidence of spontaneous tumors, ranging from 9 % in males to 13 % in females. The background of common tumors in XPA/p53 mice was similar to that observed in the XPA and wild-type strains, but mammary adenocarcinomas and hemangiosarcomas were also found in females and males, respectively. In addition, instances of various types of sarcomas were reported in females, confirming the general higher incidence of spontaneous neoplastic lesions in this sex. The tumor response of the 3 positive controls was satisfactory. Benzo[a]pyrene induced malignant lymphomas, papillomas/carcinomas in the forestomach and intestinal adenomas/carcinomas. Liver, bladder and kidney tumors were induced by p-cresidine, while liver and bladder were seen to be the target organs for 2-acetamidofluorene. Considering the results obtained with the ILSI compounds, phenacetin was the only genotoxic human carcinogen tested. Negative results were obtained in all the three genotypes in both sexes. Only karyomegaly and hyperplastic changes were observed in XPA and XPA/p53 models, representing an effect clearly linked to the status of DNA repair deficiency. Only 1 renal adenoma was reported in an XPA/p53 female. A positive response was reported for the immunosuppressive human carcinogen cyclosporine A in XPA and XPA/p53 models, but also in the wild type strain. Oestradiol and DES (diethylstilbestrol) were seen to be positive in XPA/p53 mice, while only DES was positive in the XPA model. With the exception of the peroxisome proliferator WY-14,643, seen to be positive in XPA mice, nongenotoxic rodent carcinogens and non-carcinogen compounds gave negative responses. In summary, 6 out of 7 of the genotoxic human and/or rodent carcinogens were positive in the XPA model and 3 out of 4 in the XPA/p53 mice.

CRITICAL ASSESSMENT OF THE METHOD

Considering the expectations, Van Kreijl et al. (2001) commented that the results obtained from the studies performed on these models were interesting and intriguing. The positive results obtained with 4 nongenotoxic ILSI compounds (cyclosporin A, DES, WY-14,643 and oestradiol) could be evidence of other carcinogenic mechanisms being involved, but could also indicate some doubt about the true non-genotoxic nature of these compounds. In general, XPA and XPA/p53 models appeared to be more sensitive to carcinogens than wild-type mice. A higher toxicity of some carcinogens in Xpa–/– mice could have interfered with tumor induction. Klain et al. (2001) demonstrated that this toxicity is most likely due to the lack of transcription-coupled repair in active genes. XPA/p53 was seen to give a more robust response, when compared to the XPA model. This increased sensitivity is considered to be due to the synergistic

effect deriving from the DNA repair deficiency and the condition of heterozygous p53 deficiency. The members of the Committee on Carcinogenicity (COC, 2002) agreed that there was no mechanistic rationale for producing a transgenic animal model which was deficient for Xpa and heterozygous for the p53 gene, other than maximising the predisposition to detection of specific categories of genotoxic carcinogens. As reported by MacDonald et al. (2004), the European CPMP (Committee for Proprietary Products, 2003) expressed the view that these models, while promising, require further development. Neither U.S. FDA (Food and Drug Administration) nor NIHS (Japanese National Institute of Health Sciences) appeared to have experience with this model.

MODIFICATION OF THE METHOD

As previously mentioned, the duration of 9 months (39 weeks) was used instead of the 26-week period fixed in the standardised ILSI protocols for other transgenic models. Some hematological and biochemical blood parameters were measured in a proportion of the studies. In many, but not all carcinogenicity studies, the exposure to the test compound was evaluated by toxicokinetic analysis. For all studies performed at the RIVM, two positive-control studies were performed concurrently. The RIVM study protocol was less extensive than the standard ILSI protocol, lacking histopathological examination of non-target organs, toxicokinetics, hematology and blood biochemistry.

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III.E.5 The Neonatal Mouse

PURPOSE AND RATIONALE

The neonatal mouse is one of the alternative in vivo models, proposed by the ILSI-HESI (International Life Science Institute-Health and Environmental Science Institute) for detecting the carcinogenic potential of pharmaceuticals. This is in agreement with the suggestions of the International Conference of Harmonisation (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, which allows the use of one alternative study in place of one of the 2-year carcinogenicity studies. This model has been used in various forms to test chemicals since 1959, when it was described by Pietra et al. (1959). Different terminology has been used to describe this model. When treatment begins within the first 24 hours of life, the study design is described as "newborn mouse". The "neonatal mouse" includes test item administration at different timepoints from birth to three weeks of age. Fujii (1991) reported that the neonatal mouse assay showed a sensitivity of 85 % and a positive prediction rate of 96 % compared to the results of the adult mouse 2-year carcinogenicity study. Flammang et al. (1997) considered this model to have high sensitivity and specificity to detect genotoxic carcinogens as well as presenting advantages such as reduced test article requirements, decreased animal numbers and costs and a reduced completion time. It does not respond to chemicals acting via epigenetic mechanisms. In addition, McClain et al. (2001) reported that neonatal mice have been shown to have a reduced time for tumor induction, a higher multiplicity of induced tumors, a lower spontaneous tumor rate and an equivalent or higher sensitivity to carcinogens when compared to adult mice. This model also responds to a wide range of structurally dissimilar genotoxic compounds. Additionally, the neonatal mouse possesses the majority of the phase I and II biotransformation liver enzymes involved in the processes of activation and

detoxification of carcinogens from different chemical classes.

PROCEDURE

Standardised protocols developed by the ILSI-HESI Committee have been used in the studies performed with the object of evaluating and validating the neonatal mouse as a model to predict the carcinogenic potential of drugs. CD-1 mice were obtained from Charles River Breeding Laboratory Inc., approximately 10 to 12 weeks of age. Mice were bred to provide a sufficient number of litters, culled to 4/sex/group. Mice were caged with 5 females per male and examined each day for the presence of a vaginal copulation plug. Females were isolated until delivery, 6 litters with 4 neonates/sex/litter were assigned to each group during the first week after birth. Alternatively, pregnant mice could be obtained from the supplier. Three or four dose levels, a vehicle and possibly a positive control were used. Groups consisted of 24 animals/sex/group. They were dosed on the basis of their average bodyweight, on days 8 and 15 of age, using dose volumes of up to 100 and 200 µl, respectively. Dose levels were selected on the basis of the results obtained in dose range finding studies, in which the MTD (Maximum Tolerated Dose) or the MFD (Maximum Feasible Dose) for neonatal mice, were determined. The pups were weaned around 22 days of age, housed 4/sex/cage and then maintained until 1 year of age, when they were sacrificed. DEN (diethylnitrosamine) at a dosage of 2 mg/kg dissolved in water was used as the positive control. During the in-life phase, body weight and food consumption were measured every 2 weeks and clinical observations were registered daily. At necropsy, terminal body weights, organ weights and macroscopic lesions including masses were recorded. A full histopathological examination was performed on controls and high dose group animals and on all masses, macroscopic lesions and target organs from the remaining groups. Standard tumor terminology and appropriate statistical analyses were applied as necessary. McClain et al. (2001) reported that compounds were traditionally administered via the intraperitoneal route in neonatal mice. This procedure was seen to be limiting the amount of material, due to the low dosing volumes, which could be administered. In the ILSI project, the model was modified for oral administration in order to solve the above mentioned problem, but also because most pharmaceuticals use the oral route of administration. In studies performed to evaluate the feasibility of oral administration to neonates, it was observed that the volume which could be administered was 10-fold more than that by intraperitoneal administration. In addition, the ability to dose drugs in suspension was verified.

EVALUATION

McClain et al. (2001) reported that, due to the extensive evaluation performed over many years, a vast database outside the ILSI collaborative project existed for the neonatal mouse assay. A total of over 150 chemicals have been evaluated in this model. Flammang et al. (1997) considered a number of issues regarding the neonatal mouse as an alternative model to the conventional 2-year carcinogenicity assays, such as differences between young and old animals regarding metabolic patterns, immunological reactivity, target tissue sensitivity based on physiological parameters of developing organs and the earlier onset time of tumors. In the ILSI program, 13 out of the 21 selected chemicals have been tested in this model. McClain et al. (2001) reported an overview of the results obtained from 18 studies which were evaluated by the AWG (Assay Working Group) and which included duplicates for some compounds. The incidence of the three most common spontaneous tumors at 1 year of age was respectively 6.4 % and 0.3 % for hepatocellular adenomas and carcinomas, 8.1 % and 8.6 % for lung adenomas and carcinomas, 0 % and 0.6 % for Harderian gland adenomas and carcinomas. All 3 genotoxic carcinogens examined (cyclophosphamide, diethylnitrosamine and 6-nitrochrysene, the last one used as positive control) were clearly positive. The non-genotoxic rodent carcinogens, which are considered putative human non-carcinogens, two human carcinogens (phenacetin and DES), and non-genotoxic, immunosuppressant human carcinogens were all negative. However, oestradiol was positive in one of the two studies performed. This unexpected result could be explained by some genotoxic properties of the oestrogens, which could or could not be involved in some hormonally related tumors.

CRITICAL ASSESSMENT OF THE METHOD

McClain et al. (2001) states that the neonatal mouse model could add to the results obtained from classical oncogenicity studies on rats by providing mechanistic data and due to its higher sensitivity in detecting genotoxic agents. Such higher sensitivity is mainly due to the process of rapid cellular division, which also makes this model more comparable for carcinogenicity assessment in the paediatric population. This model could identify in vivo genotoxic effects and could be integrated with information obtained from the standard

genetic toxicity tests. A positive result could indicate that a chemical may be a trans-species genotoxic carcinogen. Negative results could give mechanistic information related to the lack of in vivo genotoxic activity of a drug. Additionally, negative responses for compounds that were shown to be tumorigenic in rats could indicate the involvement of epigenetic mechanisms. However, the Committee on Carcinogenicity (COC, 2002) reiterated their concern about the health status of the animals, following evidence of considerable mortality during the studies. In general, members concluded that there was no rationale for including the neonatal mouse model in carcinogenicity testing strategies. The European CPMP (Committee for Proprietary Medicinal Products) accepted some selected proposals, U.S. FDA (Food and Drug administration) suggested to limit the use of this model to specific circumstances, for compounds clearly or equivocally genotoxic. Japanese authorities only had a limited experience with this model.

MODIFICATION OF THE METHOD

Due to the high mortality in most of the experiments, extra litters or pups could be treated to allow substitutions. Neonates could be dosed based on the individual, not only average, body weights. Due to the noticeable response of DEN when administered orally, the inclusion of a positive control group was not considered to be necessary in individual experiments. The standard protocol suggests other chemicals could be used as the positive control: 6-nitrochrysene, 300 nM/mouse, or 4-aminobiphenyl, 1000 nM/mouse, both dissolved in 35 μl DMSO administered via intraperitoneal injection on days 8 and 15 of age in volumes of 10 and 20 μl, respectively. Males could be singly caged if problems of fighting arose. On evaluation of the data from the ILSI studies, the scientists concluded that it would have been more appropriate to have examined the two main target tissues, liver and lungs, in all dose groups. This procedure was recommended for future studies.

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III.E.6
The Syrian Hamster Embryo (SHE) Cell Transformation Assay

PURPOSE AND RATIONALE

The Syrian Hamster Embryo (SHE) cell transformation assay has been proposed by LeBoeuf et al. (1996) as a short-term in vitro assay, capable of predicting rodent carcinogenicity of chemicals with a high degree of concordance with 2 year bioassay results. As reported by Berwald et al. (1965) SHE cells have been used extensively with the objective to study the process of in vitro cell transformation. In contrast to other short-term in vitro assays, this model was expected to recognise both genotoxic and epigenetic carcinogens. The SHE assay does not use specific events, such as mutations or chromosomal aberrations, as endpoints to measure the carcinogenic potential of a compound, but a transformed phenotype. As cancer is recognised as a multistep process, in which multiple mechanisms eventually lead to the ultimate transforming event, this model could be of high predictive value. LeBoeuf et al. (1996) have demonstrated that SHE cells, when cultured in an environment with low pH, morphologically transform at a rate sufficient to allow application of rigorous statistical evaluation. Pienta et al. (1977) demonstrated that the SHE cells, when cryopreserved, maintain a competent metabolic system capable of activating procarcinogenic agents to their carcinogenic forms. The transformed phenotype of the SHE cells has been associated with increased neoplastic potential and the morphological endpoints can be determined in 7 to 9 days, as reported by LeBoeuf et al. (1987, 1990). The SHE assay, originally developed by Berwald et al. (1965) and modified as described by LeBoeuf et al. (1986), was included in the collaborative research program, co-ordinated by the ILSI-HESI (International Life Science Institute-Health and Environmental Science Institute).

PROCEDURE

Mauthe et al. (2001) reported the experimental procedures used in the tests, performed in agreement with the indications given by the ACT (Alternative Carcinogenicity Testing) Committee. In all experiments, benzo[a]pyrene was used as a positive control.

Complete culture medium was prepared by adding 128 mL of foetal bovine serum (FBS) and 12.8 mL of 200 mM L-glutamine to 500 mL Dulbecco's modified Eagle's medium-LeBoeuf's modification (DMEM-L). For optimised cell growth and transformation, complete medium pH should be 6.65 to 6.75 after incubation with 10 % CO_2. If needed, pH was adjusted by addition of sterile 7.5 % sodium bicarbonate or 1N HCl. Preliminary solubility experiments were performed to determine the most appropriate solvent for each test article, the order of preference being culture medium, DMSO, ethanol and acetone. Stock solutions of each organic solvent were diluted 500-fold into complete culture medium, resulting in a final solvent concentration of 0.2 %. Preliminary cytotoxicity and SHE assays were conducted according to the methods summarised by Custer et al. (2000). For each test chemical, cytotoxicity experiments were performed, prior to the transformation assay, in order to establish the appropriate dose range for testing. Each experiment for the definitive transformation assays consisted of 20 to 40 culture dishes per treatment group, including 1 vehicle control and 1 concentration of benzo[a]pyrene. The target SHE cells, in clonal growth, were exposed to at least 3 concentrations of the test article. The highest concentration was that which was seen to result in $\geq$ 50 % cytotoxicity, insolubility in the culture medium or a maximum of 5 mg/mL. In some cases, cultures were dosed with the test compound for 24 hours, then left undisturbed for a 7-day clonal expression period. In other cases, they were exposed for the entire 7 days. After the incubation period, cells were fixed with methanol and stained with 10 % buffered aqueous Giemsa, rinsed in tap water and air-dried. The total number of colonies per dish was counted and the stained dishes were screened for morphologically transformed colonies using a stereomicroscope. The criteria for defining morphological transformation were piled up cells, extensive random-oriented three-dimensional growth, criss-crossing cells with increased cytoplasmic basophilia at the perimeter of the colony, and cells with decreased cytoplasm/nucleus ratios relative to normal cells. The data were then analysed for statistically significant treatment-related effects, using pooled data from the combined experiments. A chemical was considered to be a carcinogen if it was positive with either a 24-hour or 7-day exposure.

EVALUATION

A total of 19 ILSI compounds were tested in the SHE assay. As reported by Mauthe et al. (2001), of the 3 noncarcinogenic compounds tested, 2 were negative, whereas Ampicillin tested positive. The remaining 16 compounds were rodent and/or human carcinogens. Among these chemicals, 15 gave positive results. Phenacetin, a genotoxic carcinogen, was negative. Several compounds were tested in more than one laboratory and similar results were obtained. Although rigorous inter-laboratory studies were not conducted, it can be concluded that different SHE cell preparations used in different laboratories can generate comparable data. The overall concordance of the SHE assay with the rodent bioassay was confirmed to be 89 % (17/19). However, when compared to known or predicted human carcinogenesis information, the overall concordance was reduced to 39 % (7/19).

Based on these data, it was concluded that the SHE cell transformation assay could be of some utility for predicting the results of rodent carcinogenicity tests, but lacks selectivity to distinguish between rodent and human carcinogens.

CRITICAL ASSESSMENT OF THE METHOD

The data collected were commented on by Mauthe et al. (2001). The performance of the SHE assay suggests that this model could be considered a valuable predictor of rodent carcinogenesis and possesses the ability to distinguish between subtle chemical or mechanistic differences, leading to different carcinogenic outcomes. The assay also appears to have the capacity to identify human carcinogens, but with the limitation of low specificity and, therefore, a high false-positive rate. The lack of specificity for human carcinogens is possibly a direct result of the rodent source of cells. When the Committee on Mutagenicity (COM, 2002) evaluated the results on this test, it had considerable reservations on several aspects of the ILSI studies relating to the assay system, the study design, the data obtained and the data analyses. Members also considered that the difficulties in objectively identifying transformed cells, combined with the limited number of repeat trials made the interpretation of the ILSI/HESI data particularly problematic. The Committee concluded that the SHE cell transformation assay should not be used for regulatory screening of chemicals for potential carcinogenicity. There were insufficient data on validation of the test system to justify the development of an OECD test guideline. There were considerable reservations regarding the mechanistic basis of transformation, casting doubt on the rationale for using this model to screen for chemical carcinogenesis and regarding the validity of using mor-

phological assessment alone to define transformed cell foci.

MODIFICATION OF THE METHOD

As previously mentioned, inter-laboratory modifications of the methods were mainly regarding the exposure to the test article for either 24 hours or the entire 7-day clonal expression period. More than 1 vehicle control was used in some experiments.

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Chapter III.F
Genotoxicity

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INTRODUCTION

The task of Genetic Toxicology is to use recognized test methods in order to identify the so-called genotoxic or mutagenic potential of pharmaceuticals and chemicals. The genotoxic or mutagenic potential is what we call the potential health risk of a substance with respect to its ability to cause cell mutations. Substances with this potential are known as mutagens, and genotoxicity studies – also called mutagenicity studies – help identify such substances.

Early warnings that a possible new drug may have a genotoxic effect are of immense importance for drug development. If a potential new drug is proven to have a genotoxic potential, this generally leads to termination of development of that drug, thereby preventing human exposure to drugs with mutagenic effects.

Numerous test methods are available for assessing the genotoxic potential of substances. There are essentially three main categories of genotoxic damage that can be caused by a substance:

- Gene mutations
- Chromosome aberrations and genome mutations
- DNA damage and DNA repair.

Gene mutations include base pair substitutions and frameshift mutations, where base pair substitutions arise from the substitution of one or several base pairs in the DNA, and frameshift mutations arise from an insertion or deletion involving a number of base pairs that is not a multiple of three and consequently disrupts the triplet reading frame, usually leading to the creation of a premature termination (stop) codon and resulting in a truncated protein product.

Chromosomal aberrations include both numerical and structural aberrations. Numerical aberrations are changes in the number of chromosomes of the normal number characteristic of the animals utilized (aneugenicity). Structural aberrations are classified into two types, chromosome or chromatid aberrations (clastogenicity). Chromosomal mutations and related events are the cause of many human genetic diseases and there is evidence that chromosomal mutations and related events are involved in cancer development.

DNA damage can happen in several ways. For example, energy production in cells can produce toxic molecules, such as the so-called free radicals. They can react with the bases in the DNA and modify them, thus preventing the genetic code from being used properly. Those DNA damages then need to be repaired. All cells have evolved a system of recognizing DNA errors, fixing them and in special cases repairing them. This DNA repair mechanism can be faulty, which in itself results in a genotoxic effect.

There are specific assays available to detect each of these types of genotoxic damage, and a battery of tests is often used to determine if a substance causes any or all of these 3 types of damage. An overview of which assays can be used to detect a given type of genotoxic effect is shown in the following table.

These assays are generally performed by applying a test substance to well characterized cell systems (e.g. bacterial or mammalian cell cultures) and evaluating changes in the growth and characteristics of the cells. This may involve assessing the speed with which colonies form and the size of the colonies as well as

Table 1 Overview of genotoxicity assays

Assay	Alternative name/abbreviation	Genetic endpoint tested	Specific damage detected
Bacterial reverse mutation test	Ames Test	Gene mutations	Basepair substitution, addition or deletion
L5178Y tk+/− mouse lymphoma test	MLA	Chromosome aberrations	Point mutations and structural alterations, based on mutations in the tk gene
Micronucleus test in vitro	MNT in vitro	Chromosome aberrations	Induction of micronuclei
Micronucleus test in vivo	MNT in vivo	Chromosome aberrations	Induction of micronuclei
Mammalian chromosome aberration test in vitro	CA in vitro	Chromosome aberrations	Mainly structural alterations (chromosome or chromatid)
Mammalian chromosome aberration test in vivo	CA in vivo	Chromosome aberrations	Mainly structural alterations (chromosome or chromatid) including polyploidies
Unscheduled DNA synthesis test with mammalian liver cells in vitro	UDS in vitro	DNA repair	DNA repair synthesis
Unscheduled DNA synthesis test with mammalian liver cells in vivo	UDS in vivo	DNA repair	DNA repair synthesis

using markers to label chromosome structure or to monitor DNA synthesis.

Usually, most bacteria and cell lines do not possess the full capability for metabolizing pro-mutagens and pro-cancerogens. To overcome this deficiency, substances are tested in the presence of an exogenous metabolic activation system (S9-mix) as well as in its absence. Adding the S9-mix to the cell culture allows for metabolism of the test substance by enzymes not present in the cells. The S9-mix is a postmitochondrial supernatant fraction from liver supplemented with a NADP-generating system. It is generally made from rat liver activated by treatment with Arochlor 1254. However, livers from hamsters, monkeys or other species may be used depending on the anticipated metabolism of the substance being tested. An S9-mix has been successfully used in eukaryotic in vitro systems for the metabolic activation of various compounds.

III.F.1 Bacterial Reverse Mutation Test

PURPOSE AND RATIONALE

The bacterial reverse mutation test (Ames Test) investigates the ability of chemicals and drugs to induce reverse (back) mutations in bacteria, which involves base pair substitutions additions and/or deletions (frameshift mutations) of one or a few DNA base pairs. The bacterial strains used in the test system have mutations in genes coding for enzymes required for the biosynthesis of the amino acids histidine (*Salmonella typhimurium*) and tryptophan (*Escherichia coli*). If a test substance causes a mutation that restores function to the enzyme gene, then the bacteria will be able to grow and produce colonies. An extensive database exists based on the use of this test, and it has been found that compounds producing a positive result in the bacterial reverse mutation test have a high potency to induce cancer in animal studies.

PROCEDURE

The commonly used strains for *S. typhimurium* are TA100 and TA1535 (base pair substitution), TA98 and TA1537 (frame shift mutations) and TA102 for cross-link mutations. The preferred strain of *E. coli* to detect base pair substitution mutations is WP2.

Bacteria of an overnight nutrient broth culture are mixed with the test compound or solvent (as a negative control), S9-mix or buffer, and molten top agar, are poured into a petri dish containing a layer of minimal agar. After incubation for approximately 48 hours at approx. 37 °C in the dark, colonies (representing the number of revertants) are counted by hand or an automatic colony counter. The method can be modified by including a pre-incubation step or by using a higher amount of S9-mix in the test system. Pre-incubation would involve incubating the test compound, S9-mix or buffer, and bacteria for a short period before pouring this mixture onto plates of minimal agar.

EVALUATION

To evaluate the result of a test compound, the number of revertant colonies has to be determined for each concentration used in the test system as well as for the negative (solvent or untreated) and positive control. Precip-

itation and bacterial toxicity are expressed as a thinning of the bacterial lawn or in a reduction in the number of colonies compared to the negative control, and should be taken into consideration when assessing the mutagenicity of a substance. The following criteria should be met to consider a bacterial reverse mutation test as valid.

- The number of revertant colonies on both negative (solvent or untreated) control plates should be in an historical control range described in literature or determined in the laboratory.
- The positive control should induce a significant increase in the number of revertant colonies.

A test compound is classified as inducing point mutations if it causes either of the following:

- It produces at least a two-fold increase in the mean number of revertants per plate of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control at complete bacterial background lawn.
- It induces a dose-related increase in the mean number of revertants per plate of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control in at least two to three concentrations of the test compound at complete bacterial background lawn.

If the test substance does not achieve either of the above criteria, it is not considered as showing evidence of mutagenic activity in this system.

CRITICAL ASSESSMENT OF THE METHOD

The advantages of the bacterial reverse mutation test are the ease with which it can be performed, the short amount of time required and the low cost. The test should be regarded as the first test in the GLP testing strategy for the detection of a genotoxic potential of a test compound.

MODIFICATIONS OF THE METHOD, MINI-AMES AND AMES II

The bacterial reverse mutation test does not detect all compounds with the potential to induce point mutations. For some chemical series, modifications of the test system are necessary. For example, the potential for azo compounds to induce point mutations can only be detected by using an S9-mix prepared by hamster liver. To get an indication early in the development of a test compound for its potential to induce point mutations, modified test systems of the standard bacterial reverse mutation test are performed as a screening test. These are the so called Mini-Ames test, performed on smaller agar dishes, or the Ames II test, performed with microtiter plates. Both screening tests have the same principle as the standard test, and use S. *typhimurium* strains. The advantages of both are that they need much less compound and have a higher throughput in the number of tests per week.

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III.F.2
L5178Y tk +/– Mouse Lymphoma Test

PURPOSE AND RATIONALE

The MLA is used for the detection of point mutations, structural aberrations and aneugenicity. The principle of the assay is that cells deficient in thymidine kinase (TK) due to the $tk^{+/-}$ or $tk^{/-}$ mutation are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). TK proficient cells are sensitive to TFT. This influences the cellular metabolism and leads finally to an inhibition of further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain TK, are not. The major advantage of the assay is its ability to detect a broad range of mutagenic events represented by optimal detection of both large and small colonies.

The assay was described by Clive and co-workers (Clive et al. 1972) as a mutational assay system using the TK locus in mouse lymphoma cells. In the following years, he and his collaborators undertook a large-scale of investigation of the potential and optimal conduct of the assay. This included the use of the above mentioned TFT to select tk mutants, a comparison of the hypoxanthine guanine phosphoribosyl transferase (hprt) and tk loci, an analysis of the best expression time for tk mutant selection and a description of distinct 'large' and 'small' colony tk mutants.

Originally the assay was performed mainly in soft agar plates. In 1986 a protocol variation was successfully applied using cloning cells in liquid medium in 96-well microtiter plates instead of soft agar in Petri dishes (Cole et al. 1986). As a result, a better discrimination between small and large colonies was possible. Using a variety of chemicals, Doerr and Moore conducted an extensive evaluation of the correlation between gross chromosome aberration induction in mouse lymphoma cells and small colony tk mutant induction (Moore and Doerr 1990). From these studies it was clear that chemicals that induce small colony tk mutants also induce gross aberrations in the mouse lymphoma cells. Such mutants could result from alterations that: (a) affect expression of the tk and linked loci (chromosome 11 events); (b) affect the tk and other single essential loci in the genome, i.e. multiple point mutations; (c) affect the tk locus and have a chromosome event (other than chromosome 11) that results in slow growth.

In 1998 it was demonstrated that L5178Y cells contain two mutant p53 alleles (Clark et al. 1998). It is possible that the dysfunctional p53 protein in L5178Y cells may account for the sensitivity of these cells to mutagens and for the assay's capability to detect the chromosomal rearrangements and mitotic recombination often seen in the later stages of cancer development.

A number of recommendations and comments were made on the conduct of the MLA (Sofuni et al. 1997), including heterogeneity and the use of single or duplicate cultures, cytotoxicity parameters, relative survival and relative total growth, strategies for dose range finding and the statistical analysis of MLA data. From the International Working Group of Genotoxicity Testing (IWGT) a very detailed description of the protocol, the validity criteria and the evaluation was published in 2003 (Moore et al. 2003).

PROCEDURE

The genotoxic potential of a compound in the MLA is generally evaluated with and without metabolic activation by S9 liver homogenate obtained from rats pretreated with Aroclor or Phenobarbital/ßNaphtoflavone. The addition of the S9-mix allows for metabolism of the test compound by enzymes not present in mouse lymphoma cells. The cells were exposed to the compound for 3–4 hours or 24 hours without a metabolic activation system and for 3 hours with metabolic activation. Cells were then grown for a 48 hour phenotypic expression period (two cultures per concentration). The phenotypic expression period is the time required for the mutant (TK deficient) phenotype to be expressed, i.e. loss of pre-existing TK and depletion of the pre-existing TMP pool. Cells were then cloned and incubated for 10–13 days with the selection agent (TFT) while their cloning efficiencies were checked in non-selective medium.

The ICH4 committee concluded in 1995 that it is possible to detect point mutations and most substances that induce chromosome aberrations (including aneugens). Therefore, the Chromosome aberration assays and the Mouse Lymphoma assay are currently considered interchangeable, if the Protocol recommendations of the ICH S2A and S2B documents are fulfilled. That means: (a) in the case of a negative result following 3–4 h (treatment with and without S9), a continuous treatment of 24 h without activation was considered advisable; (b) a requirement for the use of the microwell cloning protocol rather than agar cloning to better discriminate small and large colonies (c) the use of an appropriate positive control inducing a higher proportion of small colonies. However, further validity criteria were described in the OECD Guideline 476 and the ICH2b Guideline. One major point for the interpretation of the data is the fulfillment of the toxicity criteria (10–20 % relative survival or relative total growth).

EVALUATION

Small colony mutants have been shown predominantly to lack the TKb allele as a consequence of structural or numerical alterations or recombinational events, whereas large colonies are the consequence of point mutations. Based on the recommendations of the ICH2b the following points should be considered in general for the interpretation of the results:

- Is the increase in response over the negative or solvent control background regarded as a meaningful genotoxic effect for the cells?
- Is the response concentration-related?
- For weak/equivocal responses, is the effect reproducible?

- Is the positive result a consequence of an in vitro specific metabolic activation pathway/in vitro specific active metabolite?
- Can the effect be attributed to extreme culture conditions that do not occur in in vivo situations, e.g. extremes of pH; osmolality; heavy precipitates especially in cell suspensions?
- Is the effect only seen at extremely low survival levels/high cytotoxicity?

In addition, the use of statistical methods is recommended for the evaluation of the MLA. Two methods should be used, one to evaluate the statistical difference between the different groups and, more importantly, the statistical significance of the dose–response curve. For the evaluation of the results the consideration of the biological relevance is the most important. A detailed discussion of parameters indicating a biological relevance is published in the ICH2B Guideline (1997) and in the recommendation of the International Working Group of Genotoxicity Testing (Moore et al. 2003).

CRITICAL ASSESSMENT OF THE METHOD

It is apparent that the MLA has some advantages compared to other mutation assays including: (a) rapid growth in suspension culture to high cell density, which provided for the very large numbers of cells necessary for a statistically valid test; (b) the relatively short time (48 hours) required for the expression of newly induced mutants.

However, the assay is only really capable of detecting large increases in mutation frequencies. This is because very small increases in mutation frequencies can often be seen at high cytotoxicity levels. At very low survival levels in mammalian cells, mechanisms other than direct genotoxicity per se can lead to 'positive' results that are related to cytotoxicity and not genotoxicity (e.g. events associated with apoptosis, endonuclease release from lysosomes etc.). It has long been recognized for all in vitro systems that mutations induced under these circumstances would not normally occur in vivo. Thus, these responses, while perhaps statistically significant by some methods, are not considered to be biologically relevant. A careful discussion of such responses is needed.

In addition, while the spectrum of mutations detected by the assay is very broad, it should be noted that not all such events are always detected with equal efficiency following treatment with particular test substances. Especially for the detection of structural aberrations and aneugenicity the recommended conditions of the protocol should be kept in detail.

MODIFICATIONS OF THE METHOD

The test could be performed with different cell lines (e.g. TK6 cells) and different metabolic activation systems.

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III.F.3
Micronucleus Test in vitro

PURPOSE AND RATIONALE

The purpose of the micronucleus test in vitro is to evaluate the potential of compounds to induce micronuclei (formation of small membrane-bound DNA fragments) in different cell lines or primary cultures, with and without metabolic activation by S9 liver homogenate.

The test system allows discrimination between a clastogenic and aneugenic potential of a test compound by using an immunochemical labeling of the

kinetochores or staining the DNA fragments with FISH (fluorescence in situ hybridization) technique.

PROCEDURE

The cells are treated with the test article in 96-well microplates for a short treatment period with metabolic activation (e.g. 3 hours) and for a long treatment period without metabolic activation (e.g. 24 hours) and harvested 24 hours (recovery time) after the end of the treatment. Cells are then fixed and stained. The cytotoxicity of the test compound is evaluated by the relative cell growth, expressed as a percentage of the negative control. The highest evaluated concentration should produce not less than 30 % cell survival or should be the first concentration where precipitation is observed. Duplicated cultures should be performed at each dose level. A very detailed description of the protocol, the validity criteria, and the evaluation was published by the IWGT in 2003 (Kirsch-Volders et al. 2003).

EVALUATION

Structural/numerical chromosome damage is evaluated by the increase in the number of micronucleated cells, scored out of 1000 cells in three analyzable concentrations. The compound is considered positive if either:

- the increase of micronucleated cells is statistically significant compared to the negative (solvent or untreated) control, or
- the number of micronuclei is dose dependent and showed a biological relevance compared to the negative control.

The positive control must show a clear statistical significant effect compared to the negative control.

CRITICAL ASSESSMENT OF THE METHOD

The micronucleus test in vitro is easy and rapid to perform, inexpensive and needs much less test compound compared to the mammalian chromosome aberration test. In addition, no detailed training of the personnel for the light microscopical evaluation of the slides is necessary. In fact, the micronucleus test in vitro is currently the only in vitro test which allows the differentiation between a clastogenic or aneugenic effect, and is becoming ever more important in the genotoxic testing strategy.

MODIFICATIONS OF THE METHOD

An alternative method for the evaluation of the induced micronuclei is measurement by flow cytometry. This method allows the discrimination between micronuclei that contain one or several acentric chromosome fragments (clastogenic action) or one or several whole chromosomes (aneugenic action, interference with the mitotic spindle apparatus) or even a combination of both. To discriminate between the actions, an additional staining of the micronuclei with e.g. CREST antibodies is necessary.

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III.F.4 Micronucleus Test in vivo

PURPOSE AND RATIONALE

The in vivo mammalian micronucleus test is used to assess the mutagenic hazard in consideration of factors like in vivo metabolism, pharmacokinetics and DNA-repair processes, although these may vary among species and among tissues. This assay is an important part of the gentox testing battery, applied for pharmaceuticals and chemicals.

During erythropoiesis, nuclei are expelled during the formation of polychromatic erythrocytes, while micronuclei are retained in the cells. This fact is used for the detection of micronuclei. A significant increase in the number of micronucleated polychromatic erythrocytes is usually considered as indicative of structural and/or numerical chromosome damage caused by exposure to a clastogenic and/or aneugenic substance. The identification of the presence or absence of a kinetochore or centromeric DNA in the micronuclei is needed to discriminate a clastogenic effect from an aneugenic effect. The incidence of micronuclei in the polychromatic erythrocytes is scored 24 hours after the end of treatment to take into account the time interval between the last mitosis and the formation of polychromatic erythrocytes (at least eight hours) and the lifespan of polychromatic erythrocytes, which is approximately 24 hours.

PROCEDURE

The bone marrow of rodents (rats and mice) is routinely used in this test. Since polychromatic erythrocytes are produced in that tissue, it is a highly vascularised tissue and it contains a population of rapidly cycling cells that can be readily isolated and processed. The assay was developed by Schmid (1975) and modified by Salamone et al. (1980). Recent protocols and recommendations are published in the OECD Guideline 474 and Hayashi et al. 2000. The measurement of micronucleated immature (polychromatic) erythrocytes in peripheral blood is equally acceptable in any species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated.

The route of administration of the test compound should ensure a relevant target exposure and in the case of pharmaceuticals it should consider the application route in humans. Each treated and control group must include at least 5 analyzable animals per sex. It is possible to use only one gender if it could be demonstrated that no substantial differences in metabolism, toxicity and pharmacokinetics between genders was observed. Test substances could be administered as a single treatment. Repeated treatment up to 28 days is also possible. If a single treatment is used, the sampling time should be between 24 and 48 hours. If repeated treatment is used, the sampling time is 24 hours after the last treatment.

Bone marrow cells are usually obtained from the femurs or tibias immediately following sacrifice. Usually, cells are removed from femurs or tibias in a suitable medium such as fetal serum, and are prepared and stained using established methods. DNA specific stains (e.g. acridine orange) are preferred instead of conventional stains like Giemsa.

The likelihood that the test substance or its metabolites reach the general circulation or the target tissue (e.g. systemic toxicity) should be demonstrated. Preferably, experimental evidence of systemic or target tissue exposure should be presented (e.g. blood level or bone marrow concentration analysis), especially in the case of a negative result with an agent that does not induce observable toxicity.

EVALUATION

The proportion of immature among total (immature + mature) erythrocytes as measure for target organ toxicity is determined for each animal by counting a total of at least 200 erythrocytes for bone marrow and 1000 erythrocytes for peripheral blood. At least 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes to determine the clastogenic/aneugenic potential of the test compound. The automatic analysis of micronucleated erythrocytes was developed by Romagna and Staniforth (1989).

For a discussion of the result of the micronucleus assay a comparison of the data from the treatment group vs. concurrent negative control data and historical control data, as well as a statistical analysis of the experimental data using trend analysis or pair-wise comparison (treatment group versus control) need to be considered. It is also recommended to check the variance between the animals and gender. However, for the final assessment, biological relevance of the results should be considered.

CRITICAL ASSESSMENT OF THE METHOD

There are compounds for which standard in vivo tests do not provide additional useful information. This is particularly true for compounds for which data from studies on toxicokinetics or pharmacokinetics indicate that they are not systemically absorbed and therefore are not available for the target tissues. Examples of such compounds are some radioimaging agents, aluminum based antacids, and some dermally applied pharmaceuticals. In those cases other tests, systems should be considered to be more relevant.

In addition, parameters like decreased body temperature may lead to an indirect induction of micronuclei that is not biologically relevant.

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III.F.5 Mammalian Chromosome Aberration Test in vitro

PURPOSE AND RATIONALE

This in vitro cytogenetic test is a clastogenicity test system for the detection of chromosomal aberrations in cultured mammalian cells or primary cultures. Chromosomal aberrations may be either structural or numerical. However, because cytogenetic assays are usually designed to analyze cells at their first post-treatment mitosis and numerical aberrations require at least one cell division to be visualized, this type of aberration is generally not observed in a routine cytogenetic assay. The best estimate of aberration frequency is the first cell division after the start of treatment. Structural aberrations are of two types: chromosome or chromatid aberrations.

Chromosome-type aberrations are induced when a compound acts in the G_1 phase of the cell cycle. Chromatid-type aberrations are induced when a chemical acts in the S or G_2 phase of the cell cycle.

- Chromosome-type aberrations are structural chromosome damages expressed as breakage, or breakage and reunion, of both chromatids at an identical site.
- Chromatid-type aberrations are structural chromosome damages expressed as breakage of single chromatids or breakage and reunion between chromatids.

The best estimate of aberration frequency is the first cell division after the start of treatment.

PROCEDURE

In this test, cultured cells are seeded onto slides and the cells, which had been treated with and without metabolic activation for a short time period (e.g. 3 hours). Where negative or equivocal results are obtained, an independent experiment is conducted in which cells are treated for a long time period (e.g. 20 hours) in the absence of metabolic activation alone, and then sampled and examined for chromosome analysis. In both experiments the cells were sampled 20 hours after the start of treatment, as were the concurrent solvent and positive control cultures. Colcemide was added to each culture 2 hours before sampling in order to arrest cell division. Chromosome preparations were made, fixed, stained and examined. However, if clearly positive results were obtained in the first experiment, those from the second assay were not examined. If equivocal or negative results were obtained in the first experiment, modifications to the testing procedure were included in order to clarify the result.

EVALUATION

The set of chromosomes is examined for completeness and the various chromosomal aberrations are assessed and classified. The metaphases are examined for the following aberrations: chromatid gap, chromosome gap, chromatid break, chromosome break, chromatid acentric fragment, chromosome acentric fragment, chromatid deletion, chromosome deletion, chromatid exchanges including intrachanges, chromosome exchanges including intrachanges, dicentrics, pulverization and ring formation. Metaphases including 5 or more break events are scored as multiple aberrant. Furthermore, the incidence of polyploid metaphases is determined for each cell culture. The quantity of cells is determined by counting the number of cells in e.g. 10 fields of vision per slide as an indicator of toxicity. The survival of cells is expressed as a percentage. Additionally, the mitotic index should be determined by counting the number of cells undergoing mitosis in a total of e.g. 1000 cells. The mitotic index is also expressed as a percentage. For each experiment the results from the dose groups is compared with those of the control group and the positive control at each sampling time.

The assay is considered valid if both of the following criteria are met:

- the solvent control data are within the laboratory's normal control range for the number of cells carrying structural chromosomal aberrations
- the positive controls induce increases in the number of cells carrying structural aberrations which are statistically significant and within the laboratory's normal range.

A test substance is classified as non-clastogenic if either of the following are met:

- the number of induced structural chromosome aberrations in all evaluated dose groups are in the range of our historical control data
- no significant increase of the number of structural chromosome aberrations is observed.

A test substance is classified as clastogenic if both the following are met:

- the number of induced structural chromosome aberrations are not in the range of our historical control data

- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

When evaluating the findings, both biological and statistical significance should be considered together. If the criteria mentioned above for the test item are not clearly met, the classification with regard to the historical data and the biological relevance should be discussed and/or a confirmatory experiment should be performed.

CRITICAL ASSESSMENT OF THE METHOD

Care should be taken to avoid conditions (pH, high cytotoxicity, osmolality etc) that would lead to positive results but which do not reflect intrinsic mutagenicity. Mammalian carcinogens are often positive in this test. Nevertheless, there is not a perfect correlation between this test and carcinogenicity, which depends on the chemical class. Some chemicals may test positive in this test because they appear to act through other mechanism than direct DNA-damage, for example apoptosis.

MODIFICATIONS OF THE METHOD

The test could be performed with different cell lines (permanent and primary) and different metabolic activation systems.

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III.F.6
Mammalian Chromosome Aberration Test in vivo

PURPOSE AND RATIONALE

Like the in vivo micronucleus assay, the in vivo mammalian chromosome aberration assay is especially relevant for assessing the mutagenic hazard while taking into consideration factors like in vivo metabolism, pharmacokinetics and DNA-repair processes, although these may vary among species and among tissues. In the gentox testing battery this assay is mainly used for further investigation of mutagenic effects detected by an in vitro test. In addition, the assay can be used for the detection of compounds that induce polyploidies. An increase in the number of polyploid cells may indicate that a compound has the potential to induce numerical aberrations.

The induction of structural chromosome aberrations is classified in two types, chromosome or chromatid aberrations. The majority of induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosomal mutations and related events are the cause of many human genetic diseases and there is evidence that chromosomal mutations and related events are involved in cancer development.

In addition, the assay can be used for the detection of compounds that induce polyploidies. An increase in the number of polyploidy cells may indicate that a compound has the potential to induce numerical aberrations.

PROCEDURE

Rodents (rat and mice) are routinely used in this test. Although chromosome aberrations can be detected in various tissues, the most common methodologies are available for investigations of bone marrow (Preston et al. 1987), peripheral blood, and female and male germ cells (Russo 2000). Bone marrow is the target tissue normally used in this test, since it is a highly vascularised tissue, and it contains a population of rapidly cycling cells that can be readily isolated and processed (Tice et al. 1994). However, methodologies are available for investigations of other tissues and cells.

The route of administration of the test compound should ensure a relevant target exposure and in the case of pharmaceuticals it should consider the application route in humans. Each treated and control group must include at least 5 animals that can be analyzed per sex. It is possible to use only one gender if it can be demonstrated that no substantial differences in metabolism,

toxicity and pharmacokinetics between genders was observed. Test substances are preferably administered as a single treatment. However, repeated treatment up to 28 days could also be performed. If a single treatment is used two sample times (12–18 hours and 36–44 hours) should be used for bone marrow. If repeated treatment is used, the sampling time is 6–24 hours after the last treatment. If species other than rodents or other tissues are used, the sampling time must be scientifically justified.

Prior to sacrifice (3–5 hours), animals are treated with a metaphase-arresting agent (e.g. colchicine). Chromosome preparations are then made from the respective tissues and stained with an appropriate method. For a better discrimination of the chromosomes and to detect translocations, the FISH technology can be used (Natrajan and Boei 2003). Metaphase cells are microscopically analyzed for the occurrence of structural and numerical chromosome aberrations.

The likelihood that the test substance or its metabolites reach the general circulation or the target tissue (e.g. systemic toxicity) needs to be demonstrated. Preferably, experimental evidence of systemic or target tissue exposure should be presented (e.g. blood level or bone marrow concentration analysis), especially in the case of a negative result with an agent that does not induce observable toxicity.

EVALUATION

At least 100 metaphase plates per animal should be scored per animal based on the use of at least 5 animals per gender per treatment group. The minimal classes of aberrations to score and categorize would be chromosome type versus chromatid type. Within these two categories, gaps, breaks and rearrangements should be differentiated. To describe the toxicity in the target organ, the mitotic index is determined in at least 1000 nucleated cells per animal. However, for the detection of polyploidies at least 22 metaphase cells should be scored. Due to differences in the mechanism of development, endoreduplicated cells should be scored separately.

For a discussion of the result of the chromosome aberration assay the following parameters need to be considered: a) comparison of the data from the treatment group versus concurrent negative control data and historical control data b) statistical analysis of the experimental data using trend analysis or pair-wise comparison (treatment group versus control). It is also recommended to check the variance between the animals and gender. However, for the final assessment, biological relevance of the results should be considered.

CRITICAL ASSESSMENT OF THE METHOD

There are compounds for which standard in vivo tests do not provide additional useful information. This is particularly true for compounds for which data from studies on toxicokinetics or pharmacokinetics indicate that they are not systemically absorbed and therefore are not available for the target tissues. Examples of such compounds are some radioimaging agents, aluminum based antacids, and some dermally applied pharmaceuticals. In those cases other tests systems should be considered to be more relevant.

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III.F.7
Unscheduled DNA Synthesis Test with Mammalian Liver Cells in vitro

PURPOSE AND RATIONALE

The UDS test detects DNA repair synthesis after excision of damaged segments. DNA repair synthesis is demonstrated by autoradiographic measurement of tritium-labeled thymidine incorporation. Liver cells are routinely used for the UDS test because cells in S-phase are rare and easily distinguished from cells undergoing DNA repair and because hepatocytes exhibit

a high metabolic activity that enables the detection of pro-mutagens.

PROCEDURE

Liver cells are isolated by a two-step perfusion procedure from anesthetized rats.

A medio-ventral incision is made from the pubic symphisis to the thorax. A catheter is inserted into the portal vein and the liver is perfused with collagenase solution. At the end of the perfusion, the liver is taken out, the liver capsule is removed and the cells are mechanically dispersed by gentle shaking. After dispersion of the liver cells in medium, the vessels and remaining conjunctive tissue are removed and collagenase activity is neutralized. Cell viability is assessed by the percentage of refringent cells. Only cell suspensions with more than 70 % viability should be retained.

Liver cells are then exposed in vitro to the test compound and incubated with tritium-labeled thymidine for about 18 hours. At the end of the incubation, the cells are fixed on slides and prepared for autoradiography. For that the slides are first exposed to liquid photographic emulsion, air-dried and following a 7-day exposure in the dark, exposed to developing solution.

EVALUATION

Cells undergoing DNA repair are identified by the increase in the number of silver grains in the nuclei, i.e. the net nuclear grain count. Only normal-appearing nuclei are scored; occasional nuclei blackened by grains are excluded since these are nuclei undergoing replicative DNA synthesis. One hundred cells per concentration and control are analyzed. For each cell, the number of silver grains in the nucleus and the number of silver grains in three adjacent nucleus-equivalent areas on the cytoplasm are measured. For each cell, the following parameters are calculated:

- the nuclear grain count (N)
- the mean of the three cytoplasmic grain counts (C)
- the net nuclear grain count (NG), i.e. the difference between the nuclear grain count and the mean of the three cytoplasmic grain counts.

A cell is considered undergoing DNA repair if the value of the net nuclear grain count is greater than five.

For each slide, the following parameters are calculated:

- the mean and standard deviation of nuclear and cytoplasmic grain counts, and of net nuclear grain count
- the percentage of cells undergoing DNA repair and mean value of net nuclear grain count for these cells.

The test article is considered positive in the UDS assay if the mean net nuclear grain count is greater than five and if the percentage of cells undergoing DNA repair is greater than 20 %.

CRITICAL ASSESSMENT OF THE METHOD

The biological significance has to be taken into consideration for positive evaluation (i.e. cytotoxicity can artefactually lead to an increase in the value of net nuclear grain count). The positive control should induce a clear increase in the mean net nuclear grain count higher than the threshold value of five.

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III.F.8
Unscheduled DNA Synthesis Test with Mammalian Liver Cells in vivo

The principles of this method are the same as and the procedure similar to that for the above described method “Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells in vitro”. The differences between the in vivo and the in vitro method are related

to the test compound exposure. In the in vivo assay, rats are first treated in vivo with the test compound and the liver cells isolated 14 hours after treatment. Then the liver cells are incubated for four hours with a medium containing tritium-labeled thymidine, followed by 18 hours in a medium containing non-labeled thymidine. The incorporation of tritium-labeled thymidine is measured by autoradiography. Cells undergoing repair are identified by the increase in the number of silver grains in the nuclei.

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Chapter III.G
Reproduction Toxicology

Christian Baeder

III.G.1 General Considerations

Reproduction toxicity, recently also called developmental toxicity, is a special part of toxicology which comprises any effect of chemicals and active substances on mammalian reproduction. The investigations and the interpretation of the results should be related to all other pharmacological and toxicological data available to determine the risk of the test compound to humans. To allow detection of immediate and latent effects of exposure, the observations should cover one complete life cycle from the mature adult through all stages of development from conception of one generation to conception of the following generation(s).

Reproductive Cycle

The reproductive cycle of a mammalian individual consists of a sequence of various periods and inter-related events which can be defined as follows:

- Premating to conception (adult male and female reproductive functions, development and maturation of gametes, mating behavior, fertilization)
- Conception to implantation (adult female reproductive functions, preimplantation development, blastogenesis, implantation)
- Implantation to closure of the hard palate (adult female reproductive functions, embryonic development, major organ formation)
- Closure of the hard palate to the end of pregnancy (adult female reproductive functions, fetal development and growth, organ development and growth)
- Birth to weaning (adult female reproductive functions, parturition, lactation, neonate adaptation to extrauterine life, preweaning development and growth)
- Weaning to sexual maturity (postweaning development and growth, maturation, adaptation to independent life, attainment to full sexual function).

Based on this characterization of the reproductive cycle, testing strategies were developed to detect effects on reproduction. The first fundamental introduction in reproductive toxicology was given by Wilson and Warkany in 1965b. The first test guideline was published by the FDA in 1966, followed by the CSM (1974), MHW of Japan (1975) and many other

countries and temporarily ending by the ICH Harmonized Tripartite Guideline "Detection of Toxicity to Reproduction for Medicinal Products" in June 1993, which was initiated by the IFTS (International Federation of Teratology Societies), pharmaceutical industry and the health authorities of EEC, Japan and USA (Bass and Ulbrich 1991, ICH 1993). A critical assessment of the actual situation in reproductive toxicology at the time was also given by the ECETOC (1983).

Testing Strategy

The testing strategy consists of various study designs according to the state of the art covering all stages of the reproductive cycle which can be used on a case by case basis.

In planning the studies, they must reflect human exposure to the medicinal product and allow specific identification of stages at risk. Furthermore, the anticipated drug use especially in relation to reproduction, the physical nature of the test substance and route of administration or exposure should be taken into account. Also any existing data on toxicity, pharmaco-dynamics, kinetics, mechanisms of reproductive toxicity in humans or known from previously conducted studies, and similarity to other class-related compounds in structure and activity should be taken into consideration.

According to the various events during the reproductive cycle, the following most probable option of study designs has been ascertained:

- Studies for effects on fertility and early embryonic development
- Studies for effects on pre- and postnatal development
- Studies for effects on embryo-fetal development.

On principle, it is imperative to leave no gaps between the various stages, and thus to allow direct and indirect evaluation of all stages of the reproductive process. When an effect on reproduction is detected, further studies must be designed to characterize the nature of the response on a case by case basis.

Animals

In all of the reproductive toxicity studies mentioned above, mammalian species are used as these are the most relevant model for risk evaluation in humans. It is recommended to use laboratory animals of the same species, strain and quality also used in acute and chronic toxicity studies as this enables the kinetics, pharmacological and toxicological data to be taken into account. Although all species have their disadvantages, the rat is the predominant rodent animal of choice because of their easy breeding, availability, practical handling, and the great amount of experimental and background knowledge accumulated. As in the acute and repeated dose toxicity studies, also in embryo-fetal toxicity studies, the use of a second but non-rodent animal is required. Although not used in general toxicity studies, traditionally the rabbit as non-rodent – but as a lagomorph – is the second animal of choice. This is because in that species thalidomide was first found to be teratogenic. This animal is likewise easy to breed and handle and, in the meantime, an extensive amount of experimental data and background knowledge is available. Thus, kinetic studies have also to be done in the rabbit which are lacking from the general toxicity studies. If unsuitable for reproduction toxicity studies, especially in embryo-fetal toxicity studies, alternative animal species must be considered on a case by case basis. Mice are sometimes – or regionally (Japan) – used for reproduction toxicity studies, while nonhuman primates (Rhesus or Cynomolgus monkeys) are used in embryo-fetal toxicity studies. The latter are the optimal model for the detection of thalidomide teratogenicity.

Number of Animals per Group

For all but the rarest events such as malformations, abortions or total litter loss, the evaluation of between 16 and 20 litters for rodents and rabbits tend to provide consistency between studies. Below 16 litters per group for evaluation, the study results become inconsistent, whereas more than 20–24 litters per group do not enhance the consistency and precision of the study data. Therefore, and considering a certain non-pregnancy rate, generally 20–24 animals per sex are used per group.

Other Test Systems

There are many alternative in vivo mammalian and non-mammalian and in vitro test assays. But at present no alternative test can totally replace the existing reproductive toxicity tests using live mammalian animals (ECETOC 1989). In many instances, however, the alternative methods can be used to screen test compounds of known or unknown efficacy for further technical development or further testing.

Selection of Doses

In practice, the results from previous pharmacology and acute and repeated dose toxicity studies of at least

one month's duration are considered for dose selection. Additionally, preliminary or range-finding-studies are conducted using small amounts of test substance and low numbers of animals (3–10 per dose group) for justification of the doses to be tested in the main studies.

It makes sense to conduct preliminary or range-finding-studies to find out the highest non-toxic dose and the dose inducing marginal maternal, embryo-fetal or reproductive toxicity. Generally, at least three doses should be studied in the main studies. The lowest dose should represent the one (or manifold) pharmacologically active – and presumably – daily therapeutic dose for humans, or that which is active in the test animal at which no toxic effects are expected in the parent animals and conceptuses. At the highest dose, signs of marginal intolerance are expected to appear in the parent animals and conceptuses. The geometric mean between the low and high dose should be tested as intermediate dose.

The route and frequency of administration should be similar to those of the prospected human use considering the kinetic data. Kinetics should also be determined in the pregnant and lactating animal as well as in the conceptus and the litters after birth.

In each experiment, a simultaneous group of control animals is treated with the vehicle only in the same manner as the animals in the dose groups. If it is expected that the control preparation may cause effects, a group of same-treated or untreated animals should be added to the study.

Compounds that cause neither lethality at doses of 2 g/kg body weight, nor any toxicity at doses of 1 g/kg body weight in repeated dose studies, need only be tested firstly in a 2-generation study consisting of two dose groups treated with 0.5 and 1 g/kg body weight, and secondly in one control group.

Study Conditions

As with all other toxicity studies, these should be conducted according to the state of art covering Good Laboratory Practice (GLP).

Study Designs

For basic drug discovery in the first stage of development of the test compound, the study program starts with testing for effects on embryo-fetal development including teratogenicity and maternal toxicity. If no adverse effects on embryo-fetal development and maternal health are observed, the test compound is highly indicative of continuing the pharmacological and clinical development. The next step is to study the effects on fertility and early embryonic development. In the case of no adverse effects, clinical testing of the test compound also in man can be released. Testing for effects on pre- and postnatal development will be started at a later stage of preclinical and clinical development of the test compound.

Data Presentation and Statistics

Good reporting includes tabulation of all individual data in a clear concise manner. Group summary tables should be presented in a biologically plausible manner. Presentation of individual fetal and pup data, especially structural changes, should clearly identify the litters containing the abnormal fetuses and pups, and also identify the individually affected fetuses and pups. All abnormalities should be attached to the individual offspring. As far as it is biologically acceptable, all the data and the occurrence of all abnormalities should be compared statistically with the simultaneous control group data. It should also be compared with the data of the previous relevant control groups (accumulated historical control data) on a fetal/pup and litter basis using scientifically well defined methods.

III.G.2 Study for Effects on Embryo-Fetal Development

The aim of this study, often called "teratogenicity study", is to detect adverse effects of the test compound on the pregnant female and on the development of the embryo and fetus, from implantation of the blastocyte to closure of the hard palate. Adverse effects to be assessed are: enhanced toxicity to the pregnant organism in relation to the non-pregnant, death of the embryo or fetus, altered growth and structural changes in the conceptus.

PURPOSE AND RATIONALE

Young adult females are used, preferably rats and rabbits, mature for breeding and virgin. Rats in the estrus phase (observed by vaginal smears) are mated overnight with untreated sexually mature males at the ratio of one male to one female. The following morning, vaginal smears are examined for spermatozoa. Rabbits in the estrus phase – observed by slight external reddening and swelling of the vulva or provoked by hormonal induction of ovulation by injection of estrogen – are mated individually once or twice a day at an interval of approximately 5 hours with an untreated male of proven fertility. Ovulation takes place approximately 10 hours after copulation

or hormonal injection. Copulation is confirmed by detection of spermatozoa in the vaginal smear after the mating attempt. Artificial insemination after hormonal induction is also usual. The day on which spermatozoa are detected is taken as day 0 or 1 (rats) or day 0 (rabbits) of pregnancy. Pregnancy is confirmed by the detection of implantation sites or corpora lutea in the ovaries. The animals are assigned to groups simultaneously after spermatozoa detection. It is assumed that for rats and rabbits implantation occurs on day 6–7 of pregnancy and closure of the palate on day 15–18 of pregnancy. Other timing conventions of pregnancy are equally acceptable but must be defined. Therefore, the mated female rats are treated with or exposed to the prepared test compound from day 6–16 (or 7–17) and rabbits from day 6 to 18 of pregnancy. The inseminated rats are killed and delivered by caesarean section and dissected on day 20 or 21, and rabbits on day 29 of pregnancy.

Maternal toxicity, such as altered behavior, clinical symptoms, mortality and general physical condition, food consumption and body weight is continuously examined during the study. All mated animals are autopsied and checked macroscopically for outward appearance and outwardly visible organ changes, with emphasis on the uterus. The implantation sites in the uterus are counted macroscopically (rabbits) or after staining with ammonium sulphide (rats). The latter procedure, together with counting the number of corpora lutea in the ovaries, allows the detection of early embryonic death not visible in the unstained uterus.

The uterus is opened and the implants, live and dead fetuses, dead embryo-fetal primordia undergoing resorption and the respective placentas, as well as the corpora lutea in the ovaries are counted and examined macroscopically. The fetuses are assessed for signs of life, sex, outward appearance and outwardly detectable anomalies, and their body weight and, optionally, crown-rump-length are measured. Then the rat fetuses are killed by CO_2-asphyxia. The rabbit fetuses are immediately placed in an incubator for 24 hours to test their capacity to survive and then also killed by CO_2-asphyxia.

In rats, approximately half of the fetuses from each litter and all dead fetuses are fixed in alcohol, dissected for macroscopic organ abnormalities (micro-dissection or by body cross sections, according to Wilson (1965a)), eviscerated, and the carcasses are then cleared in 1 % caustic potash solution. The skeletons are stained in alizarin red S solution, then preserved in glycerol and examined for anomalies with the aid of a stereo-microscope. The skeletal examination distinguishes between 1. the stage of individual skeletal ossification indicating growth retardation (reversible), 2. minor abnormalities irreversible but without any consequences to life, and 3. major abnormalities (malformations, irreversible) with consequences to life and social status. The remaining rat fetuses of each litter can be fixed in Bouin's solution and examined in body cross-sections for visceral anomalies according to the method of Wilson (1965a). Alternatively, the fetuses may also be examined for organ abnormalities by using micro-dissection methods for example according to Barrow and Taylor. The examination of the internal organs also distinguishes between minor and major abnormalities, with or without the aforementioned consequences. All rabbit fetuses, after testing the capacity to survive during the first 24 hours following delivery, are likewise examined for abnormalities of the internal organs (by stereo-microscope) and for skeletal abnormalities (by magnifying glass) using the same procedures mentioned for the rat fetuses. Additionally, the skull cap is opened and the brain is removed and cross-sectioned, according to the method of Wilson. Separate examination of half of the rabbit fetuses per litter by cross section only is no longer usual (according to Wilson).

III.G.3 Study for Effects on Fertility and Early Embryonic Development

The aim of the study, often called "fertility study", is to assess adverse effects of the test compound on maturation of gametes, mating behavior, fertility, pre-implantation stages of the embryo and implantation. Adverse effects can be detected at the estrus cycle, tubal transport, implantation, and development of the pre-implantation stages of the embryo. In males, functional effects on libido and epididymal sperm maturation may be detected which cannot be observed by histological examination of the male reproductive organs.

PURPOSE AND RATIONALE

Young adult male and virgin female animals, preferably rats, are assigned to groups randomly by a computer-generated algorithm and are treated or exposed with the prepared test compound. The males are treated for at least 28 days and the females for at least 14 days prior to mating covering the developmental stages of the germ cells relevant for copulation, and both through the mat-

ing period at a 1:1 mating ratio. After successful mating, the females are treated continuously until day 6 or 7 of assumed pregnancy. Treatment of the males continues until killing. From the end of the pre-mating treatment period onwards, vaginal smears are taken from all females to determine the estrus cycle. If an animal is found to be proestrous or early estrous, it will be mated overnight with a male partner of the same dose group. If spermatozoa are found in the vaginal smear on the following morning, the females are considered to be on day 0 or 1 of pregnancy. In cases of no spermatozoa, the vaginal smears and mating attempts can be continued with the same partner or another male of proven fertility from the same group. The fertility of those males which did not impregnate can be checked with untreated females not belonging to the study. The investigator decides which intensity the fertility of the animals should be tested by further mating attempts will lead to a most meaningful evaluation of this parameter. Pregnancy can be confirmed by the uterine status of the females at dissection.

Females which are found to be mated are killed at or after mid-pregnancy, preferably on the same day of pregnancy to allow meaningful evaluation. After outwardly inspection the uterus is opened and examined for its contents. The live and dead conceptuses are counted. The embryo-fetal primordia undergoing resorption and the number of corpora lutea in the ovaries are also determined. The conceptuses in the amniotic sac after removal from the uterus can be placed in physiological saline and examined under a magnifying glass for gross anomalies. The uterus of those females exhibiting no conceptuses can be stained in ammonium sulphide solution in order to identify empty implantation those sites which cannot be observed in the unstained uterus wall. Pregnant females without observed insemination are either killed on day 17 or later or allowed to deliver and are killed shortly after delivery. In those females, the number of live and stillborn pups are likewise counted and the uterus contents and number of corpora lutea graviditates examined.

Parental toxicity, such as the animal's behavior, clinical symptoms, mortality and general physical condition, food consumption and body weight are also examined during the study. Killing of males should not occur before the outcome of mating is known. After killing, the males and females are dissected and the internal organs examined macroscopically for pathological changes. Organs showing conspicuous macroscopic changes are removed and, as a precaution, preserved and kept available for histo-pathological examination. Testes and epididymides of those males in which no sperm analysis could be conducted can also be weighed and kept for histopathology.

From some males in all groups and from those males which did not impregnate, a sperm sample is collected from an incised distal tubule in the cauda of one epididymis. The sample is suspended in a specially defined culture medium of a mixture of bovine serum albumin. The number of motionless, motile and progressively forward moving spermatozoa is then counted on a warmed microscopic slide or using an automatic counting system. Spermatological examination can also be conducted in males whose mated females showed an increased number of dead conceptuses. Additionally, the number of spermatozoa is counted from a part of the semen isolated from the opened cauda epididymis in a Thoma chamber.

III.G.4
Study for Effects on Pre- and Postnatal Development Including Maternal Function

The aim of this study, often also called "peri-postnatal study", is to detect adverse effects on the pregnant and lactating female, and on the development of the conceptus and the offspring following exposure of the female from implantation through weaning. Adverse effects to be assessed are: adverse toxicity relative to that of non-pregnant females, pre- and postnatal death of offspring, altered growth and development, functional deficits of offspring including behavior, maturation (puberty), and reproduction (F1-generation).

PURPOSE AND RATIONALE

Young adult virgin females, preferably rats, are mated with untreated males of proven fertility. They are then assigned to groups randomly by a computer-generated algorithm. Then they are treated or exposed with the prepared test compound from implantation (day 6 or 7 of pregnancy), through pregnancy and parturition, up to the end of the 3 week lactation period on day 20 or 21 after birth. Before implementation of the ICH Test Guideline, treatment or exposure of the animals started with closure of the hard palate of the fetuses on day 16 or 17 of pregnancy. The females are allowed to deliver spontaneously and to rear their offspring up to weaning.

As in the other studies, maternal toxicity, such as the animal's behavior, clinical symptoms, mortality and general physical condition, food consumption and body weight, is examined during the study. The duration of pregnancy, course of birth, and body

weight gain until weaning, are recorded. The number of live and dead offspring, their sex, body weight at birth, and abnormalities, are also obtained. During the lactation period, the behavior and body weight gain of the pups, and the number of surviving pups up to weaning, are evaluated. After weaning, the females are killed and dissected for macroscopic changes of the internal organs. The uterus is removed and stained in ammonium sulphide solution in order to count the number of all implantations.

In order to find out adverse effects on postnatal development of the offspring, a great battery of physical landmarks, reflex and behavioral tests, was established. Generally, the onset of the following physical landmarks is tested: Pinnae detachment, fur development, incisor eruption, eye opening, normal gait, preputial separation, testes descent, and vaginal opening. These parameters are studied in the lactation period. Sensory functions and reflexes are generally examined after weaning by surface righting, negative geotaxis, righting, cliff avoidance, olfactory orientation, startle reflex, pupillary reflex, gripping reflex, swimming development, visual placing, corneal reflex and/or pain sensitivity. Behavior is also tested after weaning by activity, open field (often studied during lactation), photophobotaxis, maze test, social interactive test, electric current avoidance, shock avoidance, passive avoidance, FOB including grip strength and/or landing food spread. Further studies are fore- or hind limb hanging, or rotarod. Learning behavior, such as trainability, memory, and re-trainability, as well as motor activity, coordination, and sense of balance, can be checked in a water-maze-test. It has to be decided which test should be used in a routine test battery and which should be used in special cases of test substances. How many pups from each litter and dose group should be examined to provide a meaningful evaluation must also be fixed.

III.G.5 Additional Study Designs

According to the ICH Harmonized Tripartite Guideline, particularly in cases of low or no toxicity, but also in cases of chronic exposure, single and two study designs in rodents are also possible and acceptable.

Apart from various deviations in the study conduct of the following OECD test guidelines from the ICH principles of drug testing, the predominating difference is the much more extended duration of exposure to the test compound in the OECD test procedures, taking into consideration the uncontrolled and longer duration of exposure of men to chemicals in contrast to the much more controlled and in most cases limited exposure of the patient to drugs. In other words, the OECD test guidelines reflect the general risk of uncontrolled exposure of the chemical in general life, whereas in drug testing, depending on the exposure of the drug, effects on stages of the reproductive cycle, consisting of several inter-related events, are of major concern. Similar conditions to the OECD concept, however, must be taken into consideration in cases of chronic exposure to drugs, which are also covered by the alternative single and two study designs.

For such cases, and due to the fact that some similarities in the study conduct of the OECD test guidelines 414, 415 and 416 for testing chemicals and the single or two study design for testing medicinal products exist, it is recommended to take notice of the OECD study protocols when reproductive toxicological testing of a new drug has to be planned.

III.G.5.1 Single or Two Study Design

These study protocols combine the design of a fertility study and of a peri- and postnatal toxicity study. The study element of an embryo-fetal toxicity study can be included. In both cases, provided clearly negative results at sufficiently high doses or exposure are achieved, no further reproduction studies in rodents are required. Such a combination of all the study designs mentioned would provide all examinations required in a most probable option using considerably fewer animals. But an embryo-fetal toxicity study in a second species is expected.

III.G.6 Critical Assessment of the Studies and Methods

The above study designs and procedures for testing medicinal products represent the state of the art in evaluating direct and indirect effects of test compounds at each stage of the reproductive process. This principle of exposure to the test compound at specific stages of development, and its evaluation, corresponds with the prospective therapy of drugs in the human. Many drugs are provided for relatively short-term therapy. Limited exposure during special stages of development also allows evaluation of primary effects, whereas longer duration of exposure or administration may affect enzymes, thereby leading to changes in the kinetics and metabolism of the test compound, and therefore

to different efficacy. There may be a difference to drugs provided for long-term therapy. In those cases, overlapping effects are taken into account. But additional kinetic investigations can help to understand the disposability and efficacy of the test compound.

When behavior studies are conducted in the pups of a pre- and postnatal toxicity study, it has to be taken into consideration that the effects may be induced primarily by the intake of the drug via the mother's milk. Up to now, its significance as a toxic or reproductive developmental toxic effect is unclear.

The lack of alternative mammalian or non-mammalian tests compared with those using laboratory mammals is that the materno-embryonic unit is missing. But prescreening of compounds representing chemical structures or groups of similar efficacy inducing enbryotoxicity and teratogenicity may be useful.

III.G.7 Different Study Designs of Other Test Guidelines

Besides the study designs described above of reproductive toxicity testing especially developed to examine medicinal products, a battery of further reproductive and developmental toxicity test guidelines was published by the OECD. The first OCED reproductive toxicity test guideline, especially developed for testing chemicals, agricultural products and pesticides, was adopted in 1981. It is in permanent competition with comparable test guidelines of the US Environmental Protection Agency (EPA) from 1979 onwards.

III.G.7.1 The Prenatal Developmental Toxicity Study Guideline 414

This is comparable with the ICH study for effects on embryo-fetal development for testing medicinal products (historically also called teratogenicity study). At present this 2001 guideline is in a draft status, serving as a proposal for updating (OECD 2001a). The difference of the study design to the test method for drugs is the exposure of the experimental animal to the chemical, not only during the period of organogenesis, but through the whole pregnancy.

III.G.7.2 The One-Generation Reproduction Toxicity Study Guideline 415

This was adopted in 1983 (OECD 1983) as a combination of the study for effects on fertility and early embryonic development (also called fertility study) and the study for effects on pre- and postnatal development including maternal function (also called peri- and postnatal study), both of the ICH test guideline for drugs. The study design also differs from the testing strategy for drugs by an extended premating exposure time to the chemical of 70 days in rats and 56 days in mice, covering at least one complete spermatogenic cycle.

III.G.7.3 The Two-Generation Reproduction Toxicity Study Guideline 416

This is likewise a combination of the study for effects on fertility and early embryonic development (also called fertility study) and the study for effects on pre- and postnatal development including maternal function (also called peri- and postnatal study), both of the ICH test guideline for drugs. In that study design, the exposure of the experimental animals to the chemical is continuously extended over two complete generations (P- and F1-gerneration), including weaning of the F2-generation. At present this guideline, published in 2001, is in a draft status serving as proposal for updating (OECD 2001b).

III.G.7.4 Developmental Neurotoxicity Study Proposed New Guideline 426

This is a draft document from 2003, serving as a Proposal for a New Guideline 426. The principle of this study design is, as in the design of study guideline 415, a combination of the study for effects on fertility and early embryonic development (also called fertility study) and the study for effects on pre- and postnatal development including maternal function (also called peri- and postnatal study), both of the ICH test guideline for drugs. Apart from all other parameters to be evaluated, the examination of the experimental animals is especially focussed on physical landmarks, reflexes, and behavior, which are also recommended in the peri- and postnatal study according to the ICH test guideline for drugs. Additionally, the brain weights have to be determined and neuropathology examination during postnatal development and adulthood is required. Incorporation of this study protocol into the perinatal developmental toxicity study 414, one-generation reproduction study 415 or two-generation reproduction toxicity study 416 of the OECD is acceptable, but the integrity of the two studies combined with each other must be preserved.

III.G.7.5 Additional OECD Study Protocols

Two screening reproduction toxicity study protocols were prepared by the OECD for initial evaluation of existing chemicals, especially of existing high production volume chemicals but also for initial exploratory tests at an early stage of assessing the toxicological properties of new chemicals or chemicals of concern.

It is considered that, due to the shortened period of exposure to the chemical, the reduced number of experimental animals involved in the study, and the reduced number of parameters to be examined ("selected end points of concern"), neither screening test procedures not provides complete information on all aspects of male and female reproduction and development, and only limited means of detecting postnatal manifestations of prenatal and postnatal exposure. The same also applies to the development of the conceptus and parturition of the experimental animal. Therefore, they are neither an alternative to, nor can they replace, the existing OECD test guidelines 414, 415 and 416. But both screening test guidelines are expected to be useful as part of the Screening Information Data Set (SIDS) of OECD for the assessment of existing and new chemicals for which little or no toxicological information is available, to decide the need and urgency of further toxicological testing.

III.G.7.5.1 The Reproduction/Developmental Toxicity Screening Test 421

This was adopted in 1995 (OECD 1995c). In principle this test is a combination of the study for effects on fertility and early embryonic development (also called fertility study) and the study for effects on embryofetal development for drugs (also called "teratogenicity study"), of the ICH test guideline for drugs. The study procedure is extended up to four days after parturition. The experimental male animals are continuously exposed to the chemical up to the end of the mating attempts and the females are continuously exposed throughout the study up to day four after parturition.

III.G.7.5.2 The Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test 422

This was adopted in 1996 (OECD 1996). This test is a combination of a repeated dose toxicity study (OECD test Guideline 407 (OECD 1995b)) and the reproduction/developmental toxicity screening test 421 described above. In this study conduct, besides other parameters, the neurological potential and basic information of immunological effects of the test compound should be identified preferably.

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Chapter III.H
Toxicogenomics and Toxicoproteomics

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III.H.1 General Considerations

In toxicology, the full range of genomics and proteomics technologies can be used in efforts to uncover the cellular and biochemical mechanisms at work in response to xenobiotic/toxin exposures. These new technologies offer several practical benefits. Due to a parallel approach, it should be possible to screen for toxic effects more rapidly than with conventional methods, such as histopathology and clinical chemistry. Since molecular changes occur prior to pathological outcomes, detection of disease and organ toxicity should be possible at earlier time-points during a pathological process. In addition, these technologies are highly sensitive, so that long-term toxic effects can potentially be detected at lower doses.

Today, genomics and proteomics applications in toxicology are mainly used for differential expression analysis (Bandara 2002; Kennedy 2002; Wetmore Merrick 2004; Ellinger-Ziegelbauer et al. 2004; Ezendam et al. 2004) – the measurement of a gene/protein expression in two samples, comparison of corresponding expression levels and subsequent identification of differentially expressed genes/proteins. Applications can be divided into two broad and partly overlapping classes: investigative studies and predictive toxicology. Investigative studies may help to identify new molecular targets for toxicants or provide novel and deeper insights into mechanisms of action (Man et al. 2002; Ruepp et al. 2002; Fella et al. 2005; Hewitt et al. 2005). The belief that different groups or classes of compounds will induce specific molecules or expression patterns provides the basis for predictive toxicology. Such single markers or gene/protein patterns can have a high degree of predictive power (Elcombe et al. 2002; Li et al. 2002; Petricoin et al. 2002; Ellinger-Ziegelbauer et al. 2004). Currently, researchers try to set up databases with expression profiles derived from known toxins. These can in the future be used to screen novel compounds in the drug discovery and pre-clinical evaluation processes.

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III.H.2 Toxicogenomics

Toxicogenomics is becoming a well-accepted technology to compliment traditional toxicology methods. The development of these new technologies represents a great opportunity to elucidate toxicological responses to pharmaceuticals, and other chemicals, at a very early stage in drug development. This has the potential to greatly impact toxicology over the next few years, and to help in the risk assessment of new drug entities. Predictive toxicity approaches will rely heavily on bioinformatics, and the tools to allow the establishment of extensive databases based on gene expression profiles will take some time to perfect. Currently, the majority of studies published relating to toxicogenomics are based on mechanistic approaches and not compound screening. The full power of toxicogenomics has yet to be realised, and there are numerous platforms available on the market. Both global expression systems (whereby all genes in a given organism are examined simultaneously) and smaller focused arrays (hypothesis-based selection of a small number of specific genes) are widely used for many different purposes. Most people involved agree that standardization of microarray experiment procedures and of genomic signatures are key to the broad acceptance and use of these data. Many journals now only accept papers that have used the MIAME (minimum information about a microarray experiment) guidelines. Therefore, this chapter will be separated into different subjects based on different levels of expression profiling: global expression arrays, focused arrays and real-time PCR analysis, which can be used for single gene expression studies. All of these technologies should complement a toxicogenomics study, and their use will be dependent upon the questions being asked. For all approaches high quality total RNA (or mRNA) has to be extracted first.

III.H.2.1 Total RNA Isolation

PURPOSE AND RATIONALE

For the investigation of expression changes due to xenobiotic/toxin exposure, total RNA must be extracted, whether from body fluids, tissues or cells. Bolton and McCarthy first published a method for RNA isolation in 1962. Since then many extraction protocols have been developed and adapted to different sample types. All protocols follow the main objective to recover high yield, high quality RNA with as little contamination by DNA and protein as possible. Sample preparation has to be performed prior to any toxicogenomics technology used for investigative studies and predictive toxicology.

PROCEDURE

Prior to total RNA extraction, sample lysis procedures have to be performed. Lysis conditions are very important for the success of the RNA extraction and depend strongly upon the sample used. Due to great diversity, the biological sample can be pulverized, homogenized, sonicated, or otherwise disrupted to yield a mixture that contains cells, subcellular components, and other biological debris in an aqueous buffer or suspension. Here is described the protocol for the Trizol method of RNA extraction.

Trizol is a mono-phasic solution of phenol and guanidine isothiocyanate, which maintains the integrity of the RNA, and is an improvement on the original single-step RNA isolation method described by Chomczynski and Sacchi (1987). After addition of chloroform, vigorous shaking for several minutes and centrifugation, the RNA exclusively remains in the aqueous phase generated. RNA is recovered by precipitation with isopropyl alcohol, after incubation and centrifugation at 12 000 g. The isolated RNA is then washed with 75 % ethanol, and centrifuged at a lower speed. This method facilitates isolation of a variety of RNA species, both of small and large molecular size.

The resulting cleaned RNA pellet should be briefly dried (air-dry, but not to complete dryness) before re-dissolving in either RNase-free water or 0.5 % SDS solution.

EVALUATION

After sample preparation, total RNA yield can be measured by optical density. Several methods are available. Typically OD at both 260 nm and 280 nm gives an indication of RNA purity and quantity (the ratio of OD 260/280 should be close to 2). The integrity of RNA can be checked rapidly by running 1 ml on an RNase-free agarose gel and staining with ethidium bromide. Strong 28S and 18S bands should be visible. Similarly, the more modern 2100 Bioanalyser from Agilent (Liu et al. 2003) performs the same task in higher throughput, and quicker time (Figure 1).

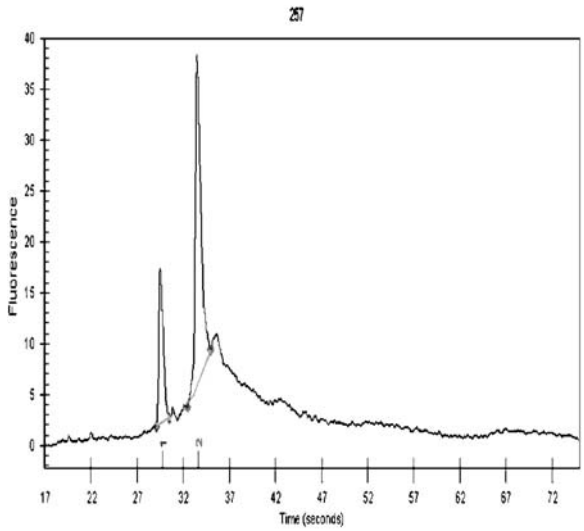

Fig. 1. Typical Bioanalyser spectra showing two distinct peaks for 18S and 28S RNA

CRITICAL ASSESSMENT OF THE METHOD

Since differential expression analysis means to compare the quantities of RNA species in two samples, every step during sample preparation has to be highly reproducible. In order to maximize reproducibility, complete total RNA extraction in a one step procedure is recommended. Care must always be taken when working with RNA, to avoid contamination with RNA'ases, which may result in RNA degradation.

MODIFICATIONS OF THE METHOD

When using very small quantities of sample ($<10^6$ cells or <10 mg tissue) 5–10 μg RNase free glycogen can be added to the aqueous phase as a carrier prior to addition of isopropyl alcohol. This aids in the visualization of such a small RNA pellet that is produced.

Additional isolation steps may be required for samples with high content of proteins, fat, polysaccharides or extracellular material.

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III.H.2.2 Global Expression Profiling

It has been said that there are no toxicologically relevant outcomes, with the possible exception of rapid onset necrosis, that do not require differential gene expression (Farr et al. 1999). Therefore, the determination of such gene expression changes due to toxic insult has become the area of intense research. These changes can be monitored by comparing the level of mRNA for each gene of interest in control and treated tissues/cells/fluids/etc. The differential giving hints to molecular changes that lead to overt toxicity.

III.H.2.2.1 Affymetrix GeneChip

PURPOSE AND RATIONALE

There are multiple platforms available that allow one to look at the gene expression of all known genes in a given organism. The aim of this chapter is not to cover all of them, but to give an overview of one such method, the Affymetrix GeneChip. Affymetrix can be considered to be the market leader in such technology. Correspondingly it is the most reproducible, robust system available and is based on single-colour analysis. Each gene is represented by 13 × 25'mers, with both match and mismatch probes (whereby the central nucleotide is changed). By comparing whole genome expression changes, we have an objective and hypothesis-free method to gain better understanding of the relationship between toxicity and gene expression. Currently there are approximately 25 genome-wide arrays commercially available from Affymetrix, covering a wide range of different organisms.

PROCEDURE

RNA is known to be different between eukaryotic and prokaryotic organisms, and as such different protocols exist for RNA labeling. The following description is relevant for eukaryotic samples.

Double stranded cDNA is synthesized from total RNA or mRNA using Reverse Transcriptase and an oligo dT_{24}-T7 promoter primer. The obtained cDNA is firstly cleaned using phenol/chloroform extraction, followed by ethanol precipitation. The cleaned cDNA is then used as a template for in vitro transcription using a Megascript kit and biotinylated nucleotides (Bio-11-CTP and Bio-16-UTP) to produce biotin-labeled cRNA (for example, One-Cycle Target Labeling Kit, Affymetrix). Additionally, a second round of mRNA amplification is possible when the starting material is too small, for example when micro-dissected tissue is used.

The purified material is then assessed for yield, purity and integrity by spectrophotometric and Agilent Bioanalyzer analysis. Fragmented (35–200 bases) in vitro transcripts (cRNAs) are purified with spin columns before hybridising overnight onto the Affymetrix GeneChip (for example the Rat Expression 2.0 array contains approximately 30 000 rat specific probe sets). The hybridised samples are stained with streptavidin-R-phycoerythrin (SAPE) and the signal amplified using a biotinylated antibody, followed by a final staining. Washing, staining and amplification are carried out using the manufacturers fluidics station. The arrays are scanned using the manufacturers fluorescent scanner (Figure 2).

EVALUATION

Most Affymetrix arrays include a set of maintenance genes to facilitate normalisation and scaling of gene expression experiments. These normalisation genes show consistent levels of expression over defined sample sets. The raw data obtained is firstly quality checked before further analysis using specific analysis software (for example, Refiner and Expressionist from GeneData and GeneSpring from Silicon Genetics). Different algorithms (such as MAS5 from Affymetrix and RMA), whereby treated and control groups could be compared, are used for normalisation of the data. Default parameters provided in the software are applied for all analysis. A threshold value of 2-fold change is usually applied to the data to aid in interpretation, although statistical analysis is essential. Dose-responses or time-effects can be evaluated by special statistical methods.

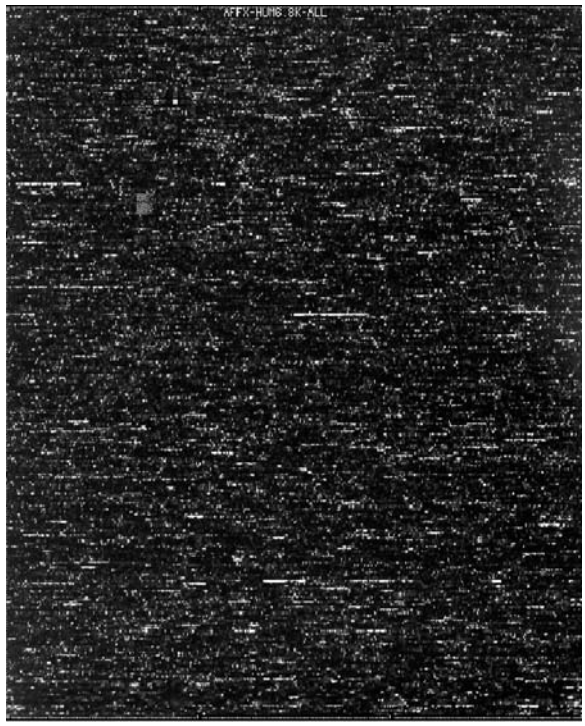

Fig. 2. Computer image of an Affymetrix GeneChip after sample hybridisation

CRITICAL ASSESSMENT OF THE METHOD

Affymetrix, as a tool, is well accepted in the scientific community and is highly reproducible and sensitive (down to 1.5pm mRNA). Chip-to-chip variation is kept to a minimum and correlation coefficients of over 0.99 have been published (Technical note, Affymetrix). The major advantage of this method is that you can simultaneously monitor the expression changes of tens of thousands of individual genes. Mechanisms of toxicological response can be teased out of the data and gene expression patterns (signatures) may lead to a more predictive approach to early toxicological assessment. However, the data is cumbersome and the amount of data generated is enormous, and therefore large databases are essential. Interpretation of such large datasets is difficult and care must be taken not to over-interpret such data. For all array technologies, a second method should be employed to confirm a small number of the gene expression changes, for example real-time PCR (see section Real-time PCR (Taqman)).

MODIFICATIONS OF THE METHOD

The actual protocol for running Affymetrix arrays is very standardised – as recommended by the manufacturer. However, it is clear that this method is only

a tool, and many researchers have reported different uses for this technology (and not only in toxicology).

There are also obviously many other companies offering similar global expression arrays. These include MWG, Agilent, Illumina, GE Healthcare, to name just a few. All are based on oligonucleotides, with spotted arrays being the most popular. This is in contrast to the Affymetrix array described above which is based on a photolithographic method of oligonucleotide synthesis (Pease et al. 1994). In addition to Affymetrix, spotted cDNA arrays have been widely used for mechanistic toxicity testing (for example, Kultima et al. 2004).

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EXAMPLES

Many researchers have used genome-wide expression profiling to elucidate toxic mechanisms and/or to find marker gene(s) for specific toxicity endpoints. Ellinger-Ziegelbauer et al. (2004) have used Affymetrix technology to characterise genotoxic carcinogens in rat liver. Ezendam et al. (2004) have used toxicogenomics for the elucidation of the mechanism of toxicity after sub-chromic hexachlorobenzene exposure in rats. Hewitt et al. (2005) have reported the use of the Affymetrix technology to elucidate the teratogenic mechanism of two drugs (retinoic acid and a novel pharmaceutical agent). In addition, many in vitro studies have been reported, for example in hepatocytes (Harris et al. 2004; Jessen et al. 2003; Kostrubsky et al. 2003).

III.H.2.3 Focused Arrays

PURPOSE AND RATIONALE

Focused arrays are usually knowledge driven, in that only a sub-set of important genes (usually chosen after performing global expression profiling) are characterised at a time. These subsets could be in the 10s or 100s of genes depending on the research question. This enables a much quicker and cheaper analysis and will aid in the higher-throughput of such toxicogenomic studies. Interpretation of data is usually easier, due to fewer genes and in most cases more biological background information for the chosen genes is available. Obviously, the fewer the genes the less information is available to enable toxicity mechanism elucidation.

PROCEDURE

Both spotted (cDNA and oligo) and on-chip synthesized (oligo) arrays are available. As an example this chapter will describe only the procedure for a two-colour system using an oligo spotted array (MWG Biotech AG).

Total RNA is used as starting material. The RNA is collected in DEPC water and Oligo d (T) 12–18 is added to the solution and incubated in a Thermomixer at 70°C. The solution is immediately transferred after incubation to ice. A mix comprised of dNTP (amino-allyl dUTP), DTT buffer and RNase Block is added to the RNA-Oligo d (T) solution. After adding reverse transcriptase, the mixture is incubated at 48 °C in a Thermomixer. Reverse transcriptase is added into the mixture and incubated. In order to hydrolyze the RNA strand, NaOH is added and the mixture incubated at 70 °C. After cooling to room temperature, the cDNA solution is neutralized with HCl. In order to precipitate the cDNA, NaOAc, glycogen and 95 % ethanol are added to the mixture and incubated over-night at –20 °C. The precipitated cDNA solution is then centrifuged for 15 min at high speed at 4 °C,

the supernatant removed and the pellet washed with ice cold 70 % ethanol by spinning for a further 15 min. After the careful removal of the ethanol, the pellet is left to dry at room temperature. These modified dUTP nucleotides then served in the second step as binding sites for the monofunctional forms of Cyanine 3 (Cy3) and Cyanine 5 (Cy5) fluorescence dyes.

Fluorescent dyes, especially the cyanine dyes Cy3 and Cy5, are the most popular choice for dual colour microarray analysis. The main benefit of using Cy dye fluorophors in particular is that the two dyes can be excited and detected from the same slide. Fluorescent dyes can be directly incorporated into nucleic acid by either enzymatic or chemical methods. Basically there are two fluorescent dye-labeling strategies:

1. Labeling the first strand: – direct labeling
2. Post-cDNA-labeling: – indirect labeling

In the **direct labeling method**, mRNA is converted into a labeled cDNA population. This is achieved by copying the transcripts into cDNA molecules with a reverse transcriptase while incorporating a modified Cy dye nucleotide.

In the **indirect labeling method**, amine modified cDNA is first synthesised by incorporating aminoallyl-modified nucleotides in first strand cDNA by a reverse transcriptase. After hydrolysis of the RNA template, and purification of the amine-modified cDNA, chemical labeling with N-hydroxyl succinimidyl-ester derivative of the Cy dye is performed. A high excess of Cy dye NHS-ester is needed for an efficient reaction. The Cy dye-cDNA is then purified to remove Cy dye that is not incorporated into labeled cDNA.

The labeled cDNAs are purified (using standard methods) and quantified using UV-spectrophotometry prior to hybridisation.

Equal amounts of the Cy3-labeled cDNA and Cy5-labeled cDNA are combined and co-hybridised on the microarrays. As in any hybridisation, the following are the goals when annealing labeled probes to targets spotted on oligonucleotide microarrays:

- To achieve the highest possible ratio of signal-to-background noise,
- To minimise cross-hybridisation between labeled probes and arrayed elements that share small regions of homology,
- To ensure that the strength of the signal is proportional to the concentration of labeled target.

These goals can only be achieved by optimising experimental parameters, the amount of labeled probes in the hybridisation mixture, the volume of the hybridisation mixture, the ionic and buffering conditions, temperature and time of pre-hybridisation and hybridisation. The hybridisation process can be partitioned into three basic steps:

- Pre-hybridisation treatment of array slides,
- Hybridisation of probes with targets, and
- Post hybridisation washing of slides.

The pre-hybridisation and hybridisation processes are run at the same temperature, i.e. 42 °C. As with global expression arrays, after hybridisation, the arrays are scanned using a fluorescent scanner (Figure 3).

EVALUATION

As with global expression analysis, normalisation of the spot intensities is essential. This can be more trou-

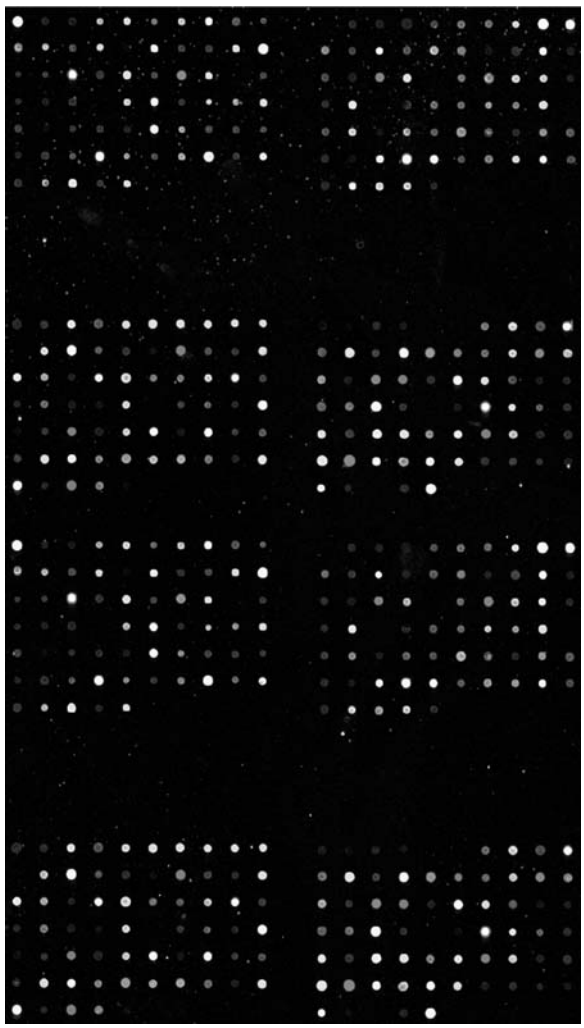

Fig. 3. Computer image of an oligonucleotide 2-colour custom array (MWG) after hybridisation with both treated and control samples (labeled with Cy3 and Cy5 dyes)

blesome as global normalisation requires a very large number of genes that are not differentially expressed. Therefore, other alternatives can be used. These include, housekeeping genes (genes whose expression is thought to be consistent in all conditions), reference RNA (an RNA mix of 20 known cell types/lines sold as a standard that can be labeled with one of the cy-dyes), spike controls (inclusion of non-mammalian RNA to hybridise to specific oligonucleotides spotted onto the array), or other more sophisticated bioinformatics algorithms. After normalisation, expression analysis is similar to that for genome-wide arrays (see section Affymetrix GeneChip).

CRITICAL ASSESSMENT OF THE METHOD

The major advantage of using focused arrays is that data interpretation is simplified by the selection of a small subset of highly relevant genes. For compound screening, prioritisation, etc, this approach has great benefit. Obviously, the limitations of these arrays are that unknown, unexpected gene expression changes will not be observed. It is unlikely that new mechanistic hypothesis can be drawn.

Compared to direct labeling, the indirect labeling method described takes longer and requires more steps. However, the added advantage of brightness, lack of enzymatic bias, and lower cost makes this method worthwhile.

For all array technologies, a second method should be employed to confirm a small number of the gene expression changes (for example real-time PCR).

MODIFICATIONS OF THE METHOD

The general labeling method has been widely used and is a standard technique. However, the types of arrays using these cy-labeled samples have been very varied and lab-specific. Depending on the provider the length of the oligonucleotide on the array can vary (for example; Septozens – 70mer; MWG Biotech – 50mer; GE Healthcare – 30mer; Affymetrix – 13mer). Affymetrix also offers custom array services – based on their one-colour technology, discussed in section 1.2.

There are many commercially available focused arrays, encompassing all areas of biological research, including toxicology. Many reports have been published using these cDNA microarrays (for example, Pennie et al. 2001; Landowski et al. 2004; Tomascik-Cheeseman et al. 2004).

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EXAMPLE

Many laboratories have carried out gene expression analysis using a customised array. For example, a hypothesis-driven array was used to study the effects of two teratogenic compounds in pregnant rats (mother and foetus) (Hewitt et al. 2005). The data suggested a specific maternal metabolic deregulation that caused an increase in specific circulating bile acids, which are known to cause teratogenic effects in the rat. This was later confirmed by more traditional biochemical blood tests. Gene expression associated with stress and DNA damage and repair has also been reported in mouse tissue (Tomascik-Cheeseman et al. 2004). Richburg et al. (2002) have reported on the use of a custom mouse testis cDNA array to study effects of chemicals on spermatogenesis.

III.H.2.4 Real-time PCR (Taqman)

PURPOSE AND RATIONALE

The quantitative analysis of gene expression changes is important if we are to trust data generated by larger gene arrays. The real-time PCR technique (for example, Taqman from ABI) allows fast and very sensitive detection of even rare RNA molecules and is routinely used for the validation of array data. This high specificity is due to a complementarity between the primer set, the internal probe and the target. It is widely believed to be the most sensitive and accurate method for mRNA quantitation (Wang and Brown 1999). PCR product accumulation is measured only during the exponential phase of the reaction. Very small amounts of RNA are required, ensuring economic use of precious samples, as well as the possibility of using micro-dissected tissue.

PROCEDURE

Taqman exploits the 5' nuclease activity of the DNA-Polymerase to cleave Taqman probes during PCR. This cleavage results in the liberation of a quencher dye and a subsequent fluorescence of a reporter dye. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

Purified RNA is subjected to reverse transcription using random hexamers and TaqMan reverse transcription reagents. Individual primers (both forward and reverse) and probe used for the real time PCR assay are designed using the PrimerExpress software (Applied Biosystems, Darmstadt, Germany) and oligonucleotides are synthesized. The PrimerExpress software produces sequences that comply with requirements regarding the melting point, G/C content, length and configuration. The amplicon should not exceed 150bp. Probes are labeled with the fluorescent dyes FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamin) at the 5'- and 3'-end, respectively. Real-time PCR is performed (ABI Prism 7000 Sequence Detection System). The primer concentration should be optimized prior to the reaction with a fixed probe concentration. All samples are run in triplicate and a standard curve is generated for each experiment. Each sample cDNA is used and normalized to 18S ribosomal RNA (rRNA) control. For amplification up to 50 cycles are run with the following parameters: 2 min at 50°C, 10 min at 95°C and for each cycle 15 s at 95°C for denaturation and 1 min at 60°C for transcription. Two different negative controls are performed, one omitting the reverse transcription step and one omitting target RNA.

EVALUATION

Two methods are usually used for the evaluation of real-time PCR data, namely the standard curve method and the comparative threshold (C_T) method. The standard curve method relies on the use of purified cDNA pasmid standards, which will result in only a relative quantification. Other more specific standards can be used, e.g., in vitro transcribed RNA, which gives an absolute qantitation, however, this method is very labour intensive and not commonly used (Martell et al. 1999). The standard curve is included in each PCR run, and therefore provides a correction control for the PCR efficiency, making inter-assay comparisons easier. The comparative C_T method uses algorithms to calculate relative expression levels, compared to a calibrator (e.g., a control sample). A detailed description of the mathematics is given by Livak and Schmittgen (2001). After calculation the normalized expression of the target gene in the unknown sample relative to the normalized expression of the control (calibrator) sample is produced. It is important when using this method that the PCR efficiency of the target gene and the housekeeping gene are equal, and when it is then more samples can be run in one PCR run (i.e., no wells lost to the standard curve).

In addition, it is common to normalize the expression data with a housekeeping gene, which will correct for minor variations in the amount of RNA used or for differences in reverse transcription (Figure 4). The housekeeping gene is one that is universally expressed, and does not change under the conditions of the assay employed. 18s RNA, β-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), cyclophilin and mitochondrial ATP synthase 6 have all been reported (Zhong and Simons 1999; Gerard et al. 2000). Assays are evaluated only when negative controls do not show any amplification products.

Statistical analyses (e.g., ANOVA followed by Dunnett's test) are performed and significant differences between treated samples compared to vehicle control are determined.

CRITICAL ASSESSMENT OF THE METHOD

Real-time PCR can be considered to be the most sensitive, specific and reproducible technology for quantitation of gene expression. Reliability is very high and the data generated is of the highest quality. This method would be the method of choice, when expression of a limited number of genes is required. The obvious drawback is that the number of gene expressions possible is limited. Therefore, higher-throughput gene arrays for the study of larger numbers of genes are needed.

Care must be taken when choosing housekeeping genes for normalisation, as there are many citations reporting the gene expression regulation of all of these commonly used housekeepers.

MODIFICATIONS OF THE METHOD

More recently double stranded DNA-binding dyes, (e.g., SYBR Green), have been introduced (Giulietti et al. 2001) which removed the need for an expensive, specific probe to be designed. Other sophisticated tools have been developed to work in conjunction with the Taqman method, for example molecular beacons, scorpions and hybridisation probes. These techniques rely on the FRET (Fluorescence Resonance Energy Transfer) principle but do not require the nuclease activity of the Taq polymerase. The different real-time

Table 1 Real-time PCR formats currently available

Company	PCR System
Applied Biosystems	ABI Prism 7700SDS ABI Prism 7900 HT SDS GeneAmp 5700 SDS
Bio-Rad	iCycler iQ
Cepheid	Smart Cycler
Corbett Research	Rotor Gene
Roche Molecular Biochemicals	LightCycler
Stratagene	Mx4000 Multiplex Quantitative PCR

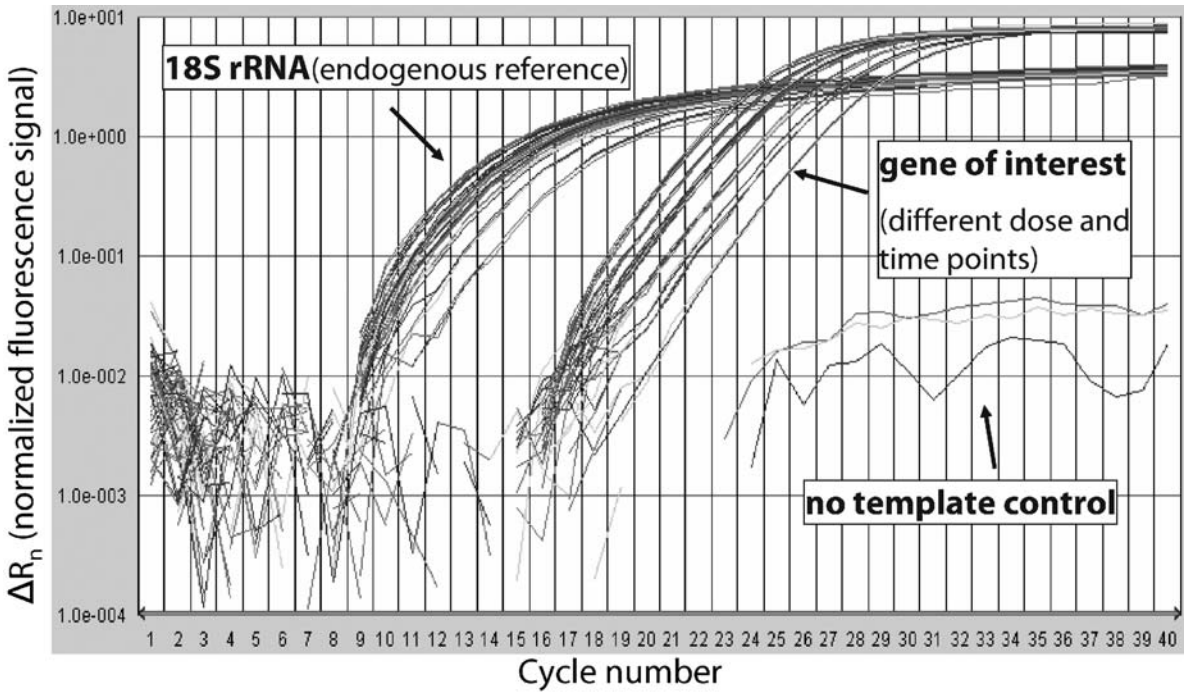

Fig. 4. Representative real-time PCR (Taqman) trace showing the housekeeping gene, 18S and the test gene of interest. Increased fluorescence intensity indicates increasing levels of RNA

PCR formats currently available on the market are given in Table 1.

Recently (2004), Applied Biosystems have introduced "microfluidics cards" or low-density gene expression arrays. These cards follow the main Taqman principles, but are based on a 384-well plate design. Therefore, multiple samples and genes can be monitored, quantitatively, at the same time. Maley et al. (2004) have also reported the use of a multiplexed Taqman model for high throughput screening applications.

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EXAMPLE

There are numerous publications where real-time PCR analysis has been used to follow the changes in expression of specific toxicity relevant genes. Giulietti and co-workers (2001) have used real-time PCR techniques to evaluate cytokine profiles in both mouse and human cells and tissues. Heregulin (a member of the neuregulin family) and its binding receptors (ErbB-2, ErbB-3 and ErbB-4) are induced by gentamicin treatment, and are therefore postulated to play an important role in hair cell regeneration following ototoxic shock (Zheng et al. 1999). Campbell et al. (2004) recently showed, using Taqman, the importance of matrix metalloproteinases in kainic acid induced excitotoxicity in the rat brain.

III.H.3 Toxicoproteomics

Daniel Liebler said: 'Proteomics techniques are not going to be done just as demonstrations of powerful technology, but will really be integrated into studies in basic laboratory science, animal and non-animal models of diseases and environmental exposures, and actually in human clinical studies as well' (Hood 2003). Toxicoproteomics can be described as a 'marriage' of classical toxicology assays and the new discipline of protein expression profiling. Applications have been described for mechanistic investigations (Cutler et al. 1999; Bandara et al. 2003) for the pathological classification of diseased tissue (Roberts et al. 2003; Kröger et al. 2004) and ultimately for the development of assays capable of accurately predicting cancer (Petricoin et al. 2002; Veenstra et al. 2004).

In general, the proteome can be analysed by a broad spectrum of methods. All of them follow similar principles. After treatment of laboratory animals/cells, parallel protein extraction and protein separation is performed. Changes in the proteome are then investigated by differential expression analysis with bioinformatics/biostatistics tools before the identification of proteins of interest is done (Anderson and Anderson 1998; Ryan and Patterson 2001; Schrattenholz 2004). The most powerful protein separation technologies are liquid chromatography (LC) and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). For protein identification there is a set of mass spectrometry methods (MS) with ever increasing accuracy and sensitivity. Global measurements of protein expression can be performed by various combinations of these key technologies (Wilkins et al. 1997; Liebler 2002). By stable isotope tagging methods, the protein quantitation can be coupled to identification by MS (Gygi et al. 1999). Other combinations include liquid chromatography (LC) as well as retentate chromatography (RC) protein separation technologies with online MS (Merchant and Weinberger 2000; Issaq 2001) or 2D-PAGE followed by MS (Rabilloud 2000).

Proteomic platforms do not yet deliver the same information as in the genomic field. Indeed, the relationship between gene number and proteome size is far from simple when taking into account splice variants and posttranslational modifications of proteins. Furthermore, the concentration range of the proteome exceeds 7 orders of magnitude (high dynamic range) (Wilkins et al. 1996; Harrison et al. 2002). Single proteomic platforms are not sufficient to display the enormous proteome in total. For this reason, only combinations of multiple proteomics technologies may be needed for complex problem areas like toxicology.

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III.H.3.1 Extracting Proteins from Biological Samples

PURPOSE AND RATIONALE

For the investigation of expression changes due to xenobiotic/toxin exposure, proteins have to be extracted from samples of body fluids, tissues or cells. For this purpose, many protein extraction protocols have been developed and adapted to different sample types (Link 1999). All protocols follow the main objective to recover as much of the proteome as possible with as little contamination by other biomaterials as possible. Sample preparation has to be performed prior to any proteomics technology used in investigative studies, or predictive toxicology. Depending on the method used for protein expression analysis, fractionation steps have to follow or have to be implemented in the extraction procedures (Wilkins et al. 1997; Rabilloud 2000; Liebler 2002).

PROCEDURE

Protein extraction varies greatly depending on the biological sample type. It must, however, meet some common rules:

- Efficient protein solubilisation
- Avoidance of proteolysis
- Avoidance of protein modifications
- Depletion of interfering substances.

Prior to protein extraction, lysis procedures have to be performed. Lysis conditions are very important for the success of the protein extraction and depend strongly upon the sample used. Due to sample diversity, the biological sample can either be pulverised, homogenised, sonicated, or otherwise disrupted to yield a mixture that contains cells, subcellular components, and other biological debris in an aqueous buffer or suspension. Afterwards, the proteins are extracted generally in the presence of:

- Detergents, which help to solubilise membrane proteins and aid their separations from lipids.
- Reductants, which reduce disulfide bonds or prevent protein oxidation.
- Denaturating agents, which disrupt protein-protein interactions, as well as secondary and tertiary structures within proteins.
- Enzymes, which digest contaminating nucleic acids, carbohydrates, and lipids.
- Protease inhibitors, which prevent proteolytic protein degradation.

In the case of very complex protein extracts or when high abundant proteins dominate the samples, fractionation steps have to be performed in addition to the extraction procedure. Regardless of the method used, the principle of separating proteins is to split them into fractions based on their diversity in chemical/physical properties. Analysis of these fractions increases the total number of proteins that can be resolved in the following proteomic procedures used. Some recent developments in sample preparation allow a biochemical fractionation into different cellular constituents, such as, special cell organelles or cell structures (Figure 5) (Cordwell et al. 2000; Abdolzade-Bavil et al. 2004). In addition, chromatography approaches are highly applicable (Issaq 2001). As an initial fractionation step are available: reverse phase, anion and cation exchange, size exclusion, and affinity chromatography columns. The latter is particularly attractive as a means of extracting a subset of proteins from a complex mixture or depleting highly abundant proteins from samples like body fluids (Burnouf 1995).

EVALUATION

After sample preparation, protein yield can be measured by optical density. Several methods are

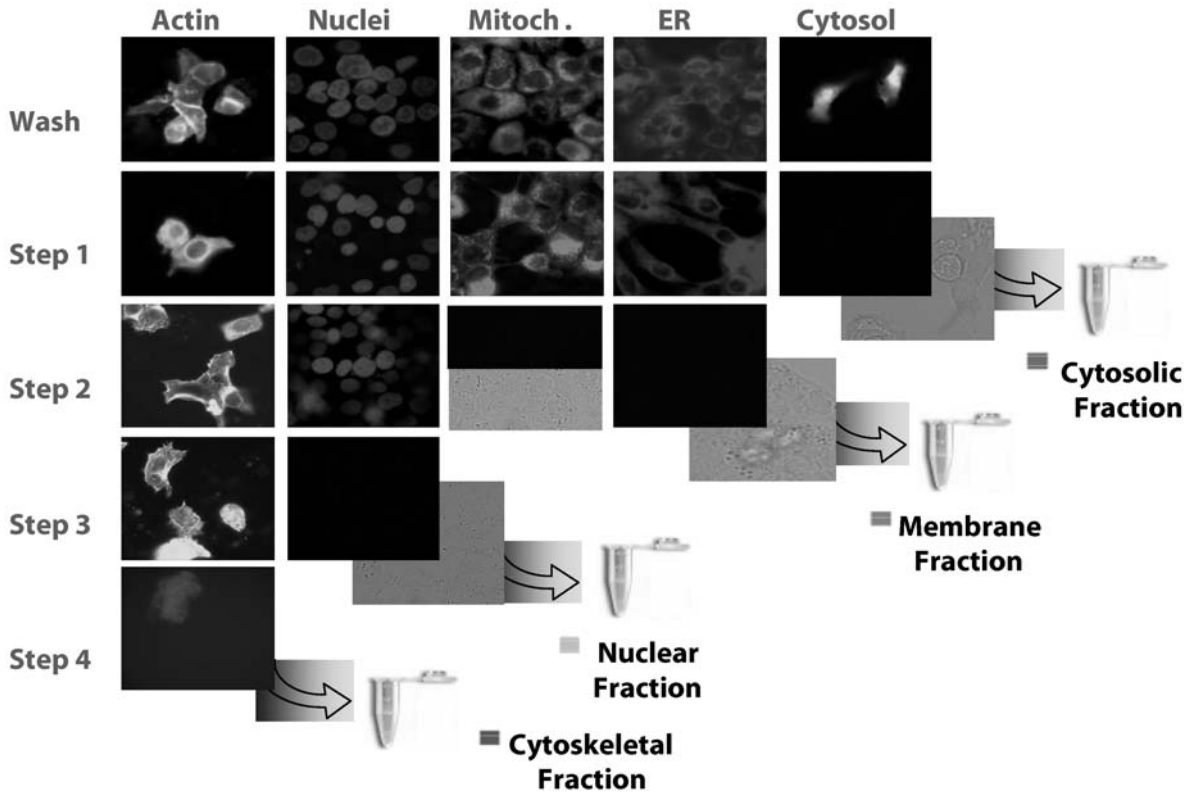

Fig. 5. Schematic view of a subcellular protein extraction kit developed by the Merck KGaA

available. In general, measurements after Bradford are used (Bradford 1976). Careful must be taken due to incompatibility of many detergents, reductants and denaturating agents with these methods.

CRITICAL ASSESSMENT OF THE METHOD

Since differential expression analysis means to compare the quantities of a set of proteins in two samples, every step during sample preparation has to be highly reproducible. In order to maximise reproducibility, complete protein extraction in a one step procedure is recommended. On the other hand, proteome fractionation increases the detection of low abundant proteins as well as the number of proteins for further analysis whenever reproducibility decreases and uncontrolled loss of proteins is unavoidable. Well-defined conditions are recommended for all procedures.

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III.H.3.2 Two Dimensional Gel Electrophoresis/ Mass Spectrometry (2DE/MS)

PURPOSE AND RATIONALE

Originally, the 2DE-method was developed independently by Klose, O'Farrell and Scheele in 1975 to

increase the separation power for protein identification and characterisation from complex mixtures. For more than 2 decades, proteins could not be easily identified from the 2DE-gels, even with the introduction of automated protein sequencers, until this technology has been linked with MS in the mid 1990s. Since then, the 2DE/MS method has undergone a tremendous development (Görg et al. 2004) and has been used by many researchers for differential protein expression analysis (Fountoukalis et al. 2000; Rabilloud 2000; Liebler 2002).

The proteome is constantly changing due to stimuli of growth, environmental factors, chemicals, drugs, as well as disease processes. Understanding molecular mechanisms underlying these changes is necessary to understand complicated pathologies. 2DE/MS approaches have been described mainly to detect specific protein expression patterns induced by particular groups of xenobiotics or toxins. These examples show usefulness both for investigative studies and with regard to discovery of new safety markers for predictive toxicology (Man et al. 2002; Kröger et al. 2004; Fella et al. 2005).

PROCEDURE

During 2DE, protein extracts undergo a combination of two different types of separation. First, proteins are resolved on the basis of their isoelectric points (pI) by isoelectric focussing (IEF). Second, focused proteins are resolved on the basis of their molecular weight (MW) by electrophoresis on a polyacrylamide gel (SDS-PAGE). Since any protein as well as its modifications are characterised by a particular pI/MW pair, they always migrate to the same location in the 2DE gels. After separation, proteins are visualised in the gels by standard staining techniques (Figure 6). In order to create a digital picture of the partial proteome, the gels are scanned before quantification. Differential analysis can be performed by using specialised software programs. Protein spots on the gels are detected, the values for the signal to noise ratios are calculated as relative intensities and gel matching is performed by overlaying each gel with every other gel. After normalization, group-wise comparisons can be performed by treatment-related changes in protein expression values. After specific proteins of interest have been detected, they are excised from the gel, fragmented into peptides and subjected to MS analysis (see section Mass Spectrometry (MS)).

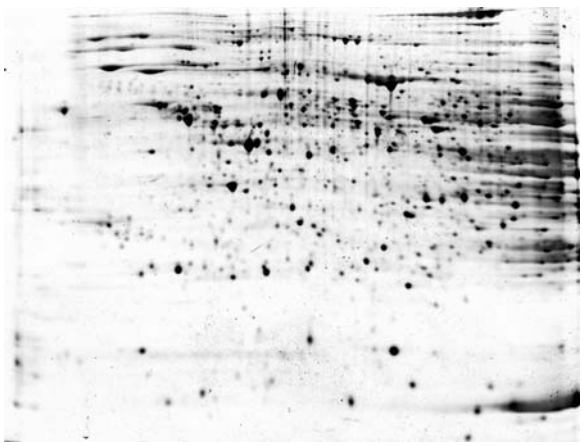

Fig. 6. Representative 2-DE gel of a rat liver proteome; in the first dimension proteins were separated in a pH gradient (4–7), in the second dimension based on their molecular weight; protein visualization was realized using fluorescent staining with ruthenium

EVALUATION

Differentially expressed proteins are identified by comparison of 2DE gels from treated and control samples. By combining group-wise statistics with N-fold regulations, proteins are selected with a low p-value and are up or down regulated. Dose-responses or time-effects can be evaluated by special statistical methods.

CRITICAL ASSESSMENT OF THE METHOD

The simultaneous identification, characterisation and quantification of numerous proteins and their post-translational modifications is mandatory to evaluate how multiple cellular mechanisms are influenced by xenobiotics/chemicals. This is of special interest for investigative studies as well as for the discovery of new safety markers in toxicology which can be used to screen novel compounds in the drug evaluation process. 2DE/MS proteomics approaches meet these expectations and have been used in many studies of toxicological interest (Kennedy et al. 2002; Merrick and Tomer 2003; Wetmore and Merrick 2004). However, without pre-fractionation of samples, the platform gives preference to higher abundant proteins. The 2DE gels have only limited coverage of proteins present in a sample because of the high dynamic range of the proteome. On the other hand, when including pre-fractionation steps to overcome this problem, the method is too time-consuming. This is also the reason why the 2DE method is not suited for a fast screening of higher sample numbers, therefore, it is inadequate for setting up predictive proteomic patterns.

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III.H.3.3 Mass Spectrometry (MS)

PURPOSE AND RATIONALE

In toxicoproteomics, the identification of proteins is central. Today, mass spectrometry (MS) is the method of choice for this purpose. The MS technique has roots going back to J.J. Thompson, but the current instantiations are largely due to the work of J. Fenn and M. Karas (Fenn et al. 1989; Karas and Hillenkamp 1988). In the past decade, MS technology has undergone tremendous change and progress in this technology has been covered in many reviews (Wilkins et al. 1997; Rabilloud 2000; Aebersold and Mann 2003). MS instrumentation can offer different types of analyses. First of all, MS provides accurate molecular mass measurements of intact proteins as well as of peptides from proteolytic digests. The latter one is called peptide mass fingerprinting (PMF). Second, sequence analysis of peptides from proteolytic digests can be obtained. Data from peptide mass measurements and sequencing can be searched directly against databases frequently to obtain definitive identification of the proteins of interest (Thiele 2003). In order to discover differentially expressed proteins related to toxic effect, MS has to be performed after any protein separation technology both for investigative studies and partly in predictive toxicology.

PROCEDURE

All MS methods use three essential components for the mass determination of proteins/peptides that have been converted to gas-phase prior to detection. The components are an ion source to produce the ions, a mass analyser which separates the ions by their mass (m) to charge (z) ratio and a detector (Liebler 2002). The resulting MS data are recorded as spectra, which display ion intensity versus the recognised m/z value.

Two techniques that have become preffered for ionisation of proteins/peptides is electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI). Although different combinations of ionisation techniques and mass analyser exist, MALDI usually is coupled with a time-of-flight (TOF) (Figure 7) tube as a mass analyser while ESI is tradionally combined with quadrupole mass analysers. Instruments capable of MS/MS have the ability to select ions of particular m/z ratio from a mixture, to fragment selected ions and to record the precise masses of the resulting fragment ions. If this process is applied to the analysis of peptide ions, in principle the amino acid sequence of the peptide can be deduced.

EVALUATION

The protein identification technique in which MS is used to measure the masses of proteolytic peptide fragments is the so-called peptide mass fingerprinting (PMF). Numerous computer programs based on different algorithms have been developed to allow protein identification by matching their amino acid compositions against databases of full-length protein (SWISS-PROT) or gene sequences (NCBI) (Mann et al. 1993; Yates et al. 1993). These programs search the databases by comparing what would be expected if the protein in the database were split into the pattern of fragmentation. Once a reliable match is found, the protein sequence stored in the database can be used to identify the protein and predict its full sequence.

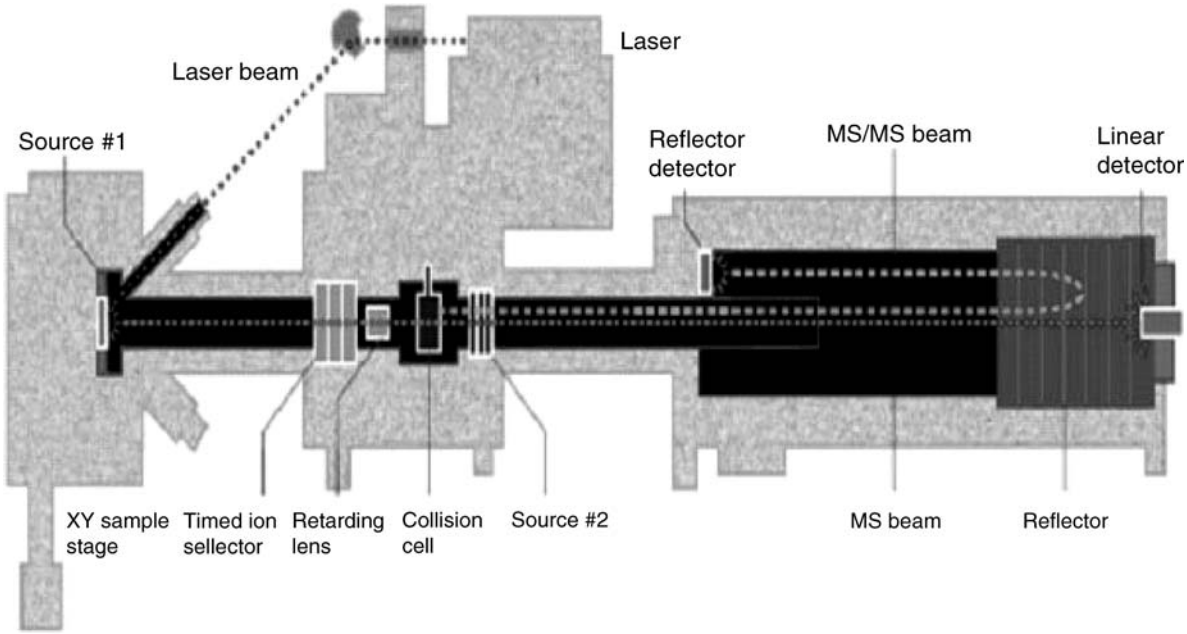

Fig. 7. Schematic view of the 4700 Proteomics Analyzer (Applied Biosystems), a MALDI-TOF/TOF mass spectrometer

Matched proteins are ranked according to their score from the lowest best match to the highest worst match. The more fragments obtained from a single protein, the higher the confidence in the protein match and identity. In the end, best ranked proteins are summarised in a list and shown as results (Clauser et al. 1999; Perkins et al. 1999).

When peptide ions undergo fragmentation in a MS/MS spectrometer, the most significant cleavages are along the peptide backbone. The spectrum derived from one precursor ion shows a descending series of fragment ion m/z values which can be used to identify the protein by querying these fragments with databases (Yates 1995). Computer use the precursor to select all peptides from the database with the same mass. Then, theoretical MS/MS spectra are generated from each of the selected peptides and compared to the spectrum being analysed. A correlation score is calculated for each match between the experimental and theoretical MS/MS before best matches are shown and reported.

CRITICAL ASSESSMENT OF THE METHOD

Since MS instrumentations today are robust and sensitive, protein identification by PMF is quite good. The rapid development of protein and gene sequence databases provides an ever more consistent platform for searches. Furthermore, improvements in search algorithms and the application of sophisticated statistical methods has improved the reliability (Clauser et al. 1999). However, there remain several limitations. The lack of complete and accurately annotated databases limits the quality of matches that can be achieved. The greater number of highly homologous proteins in higher organism complicates the distinguishing between closely related proteins. Last but not least, information of peptide sequences and sites of modifications cannot be deduced from PMF measurements.

For determination of peptide sequences as well as protein modifications, MS/MS has become the definitive approach. However, for database searches MS/MS spectra have to be perfect. This is not always given because there are differences in tendencies of peptide bonds to fragment and certain amino acids show unique fragmentation characteristics.

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III.H.3.4 Liquid Chromatography / Mass Spectrometry (LC/MS)

PURPOSE AND RATIONALE

In toxicoproteomics, mass spectrometry (MS) is the method of choice for protein identification. Since most applications are concerned with the relative abundances of proteins, the coupling of this method with separation technologies allows a more sophisticated proteome view. LC/MS is a versatile combination of liquid chromatography (LC) and mass spectrometry. This approach has exploited online LC based separation of proteins immediately prior to entry into tandem mass spectrometry (MS/MS) for protein identification or posttranslational characterisation (Bischoff and Luider 2003). The multidimensional protein identification technology (MudPIT), first described by Link in 1999, is represented by a 2 dimensional LC separation coupled online with MS/MS to analyse hundreds of proteins. The platform has also been called shotgun proteomics since entire protein lysates are digested by trypsin into peptide fragments prior to MudPIT separation and identification (Wolters et al. 2001; McDonald and Yates 2003). Although the LC/MS platform has a great potential for protein discovery in toxicoproteomics, its ability for quantitative sample comparison is still missing. Only in combination with labeling procedures (see section Quantitative Mass Spectrometry (QMS)) will LC/MS approaches have an impact on investigative studies as well as predictive toxicology.

PROCEDURE

The LC/MS approach starts with a crude protein mixture that is digested into peptides, which are then resolved by chromatography and analysed by MS/MS. The basic idea with this method is to obtain MS/MS spectra for as many peptides as possible. In order to get high quality spectra, the instruments typically used for this approach work with a data dependent control to select precursor ions for the recorded MS/MS data. To maximise the number of analysed peptides, protein mixtures can be resolved by different LC steps prior to MS. Primarily, classical LC/MS is performed in the reversed phase mode where the mobile phase is directly compatible with the requirements for peptide ionisation. Resolution in the mixture is achieved by a LC gradient, which leads to several peptide fractions. In the second approach, MudPIT, samples undergo a higher resolution by placing a strong cation exchange column in line immediately ahead of the reverse phase column, to which most of the peptides bind. This yields in a higher number of fractions/peptides that can be analysed by MS. Finally, the resulting MS/MS spectra obtained from each approach are mapped to protein/gene sequences from databases and peptides/proteins can be identified.

EVALUATION

During the MS/MS process, selected peptide ions undergo fragmentation. The spectrum derived from one precursor ion (peptide) shows a descending series of fragment ion m/z values which can be used to identify the protein by querying databases like SWISS-PROT (proteins) and/or NCBI (genes). The precursor ion is used to select all peptides from the database with the same mass. Then, theoretical MS/MS spectra are generated from each of the selected peptides and compared to the spectrum being analysed. A correlation score is calculated for each match before best matches are shown and reported (Yates 1995).

CRITICAL ASSESSMENT OF THE METHOD

The use of LC/MS analysis is the most reliable approach for protein identification. It is based on MS/MS spectra, which directly indicate peptide sequences. Both high- and low-abundant proteins can be analysed from complex protein mixtures. The LC/MS platform

has been used successfully for the identification of unknown proteins (Schirmer et al. 2003; Sam-Yellowe et al. 2004). However, this technology is not quantitative for peptides and proteins resolved from unlabeled samples. For this reason, toxicoproteomics related questions, where protein expression values from different samples have to be compared, can only be addressed by a combination with labeling procedures (see section Quantitative Mass Spectrometry (QMS)).

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III.H.3.5 Quantitative Mass Spectrometry (QMS)

PURPOSE AND RATIONALE

Quantitative comparison of diseased and normal cell lines/tissues is generally necessary to deduce the effects of xenobiotic/toxin exposures. Stable isotope tagging methods provide a useful means of determining the relative expression levels of individual proteins between samples in a mass spectrometer (Lill 2003; Schneider and Hall 2005). First approaches for quantitative mass spectrometry (QMS) go back to R. Aebersold and co-workers (Gygi et al. 1999). They used isotope-coded affinity tags (ICAT) to chemically label cysteine residues in proteins before quantitation by MS. Meanwhile, several stable isotope tagging reagents targeting different amino acids are available. The latest development for chemical labeling is the isotope tag for relative and absolute quantitation (iTRAQ) (Ross et al. 2004). These reagents target all N-termini as well as lysine residues of the peptides before MS based quantitation can be performed. In addition, metabolic isotopic labeling procedures have been described (Ong et al. 2002). However, these methods are limited to cell cultures where stable isotopic amino acids can easily be incorporated. QMS is useful both for investigative studies and with regard to discovery of new safety markers for predictive toxicology.

PROCEDURE

Two or more samples are treated with isotopic reagents to tag them before they are pooled with equal protein amounts. The tags are chemically identical but differ in their masses due to isotopes in the reagents. In order to reduce complexity, proteins can be separated by either LC- or 2DE-techniques before they are enzymatically digested. MS based detection can be performed either with MALDI or ESI. Analysis of the MS spectra allows identification of the proteins present. Examination of the following MS/MS scans allows relative quantitation of the differently tagged peptides. The ratio corresponds to the amount of protein in the different samples.

The ICAT reagents contain a biotin affinity tag to allow the enrichment of cysteine-containing peptides with avidin affinity chromatography. An acid-cleavable linker is incorporated to allow the removal of the biotin affinity tag before MS and MS/MS analysis is performed. The iTRAQ methodology utilizes isobaric tags containing both reporter and balancer groups. The reporter group allows quantitation and is balanced by a second group, which is depleted of the same stable isotopes. This balancer maintains each isotopic tag at exactly the same mass in a MS spectrum. During the MS/MS process, the reporter is quantitatively cleaved during collision-induced dissociation (CID). An isotope series represents the quantity of a single peptide of known mass from different samples in the MS/MS spectrum. Since the peptide remains attached to the isobaric tags until CID is conducted, the peptide is simultaneously fragmented for sequence identification.

Metabolic labeling generally exploits the incorporation of isotopic amino acids during the process of cellular metabolism, which includes protein synthesis. Cellular groups are grown in media containing different isotopic amino acids. After cell harvesting, the protein concentrations are equalised and pooled before MS as well as MS/MS analysis takes place.

EVALUATION

The QMS platform combines the identification of proteins with their quantitative detection in one procedure. While protein identification can be deduced from peptide mass fingerprinting (PMF) or MS/MS spectra (see section Mass Spectrometry (MS)), protein quantitation is based on analysing either peak areas or signal intensities, or a combination of both. Several computer programs, in most cases reagent specific ones, are available. For each peak, quantitation values are calculated before differentially expressed proteins are identified by the comparison of control and treated samples.

CRITICAL ASSESSMENT OF THE METHOD

In toxicoproteomics, the quantity of a protein is an important parameter that has to be measured in relation to other proteins in a defined space (cells, tissues, body fluids). However, the wide concentration range of the proteome (large dynamic range) can exceed 6 or 7 orders of magnitude. Therefore, differential expression analysis must consider these diverse properties to achieve an optimal separation among proteins in a quantitative manner that is compatible with protein identification. QMS based proteomic methods could meet these expectations. Especially for global protein profiling strategies primarily focused on safety marker discovery and investigative purposes they have a great potential. Particularly in combination with multidimensional LC, these methods offer the possibility to detect low-abundance proteins (Hansen et al. 2003; Oda et al. 2003). However, sample simplification steps by LC are too time consuming for screening purposes, which would be necessary for pattern based predictive toxicology. Last but not least, depending on the reagent target as well as the quantity of labeling reaction, only selected peptides of proteins are accessible for QMS.

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III.H.3.6 Retentate Chromatography / Mass Spectrometry (RC/MS)

PURPOSE AND RATIONALE

The process was described first by Yip and Hutchens in 1993 and patented in 1998 by Ciphergen Inc. who advanced it to a commercial product (Wright 2002; Bischoff and Luider 2003; Wetmore and Merrick 2004). The so-called Surface Enhanced Laser Detection/Ionisation (SELDI) process combines two well-established methods, namely retentate chromatography (RC) and time-of-flight mass spectrometry (TOF-MS) in a data analysis integrating system. In predictive toxicology, the technology can be applied to the problem of identifying patterns within the complex proteome that discriminate between normal and pathological states (Petricoin et al. 2002; Li et al. 2002; Liotta et al. 2003). In toxicology, the aim of this approach is to set up databases with particular protein patterns resulting from group specific xenobiotic/toxin exposures.

PROCEDURE

Protein extracts can be directly applied to the SELDI chips (metal bars with spots coated by specific surfaces). These spots represent either chromatographic surfaces (hydrophobic, cationic, anionic, normal phase, etc.) or are pre-activated for the coupling of capture molecules (oligonucleotides, proteins or even enzymes) prior to sample loading. Only a subset of the proteins from the complex extract binds to the surfaces. This interaction is specific as the chromatographic binding is based on the inherent amino acid sequence of any given protein, as well as on the pH, detergent and salt concentration in the lysate buffer. After a short incubation period, the SELDI chips are rinsed to remove unbound proteins, and the bound

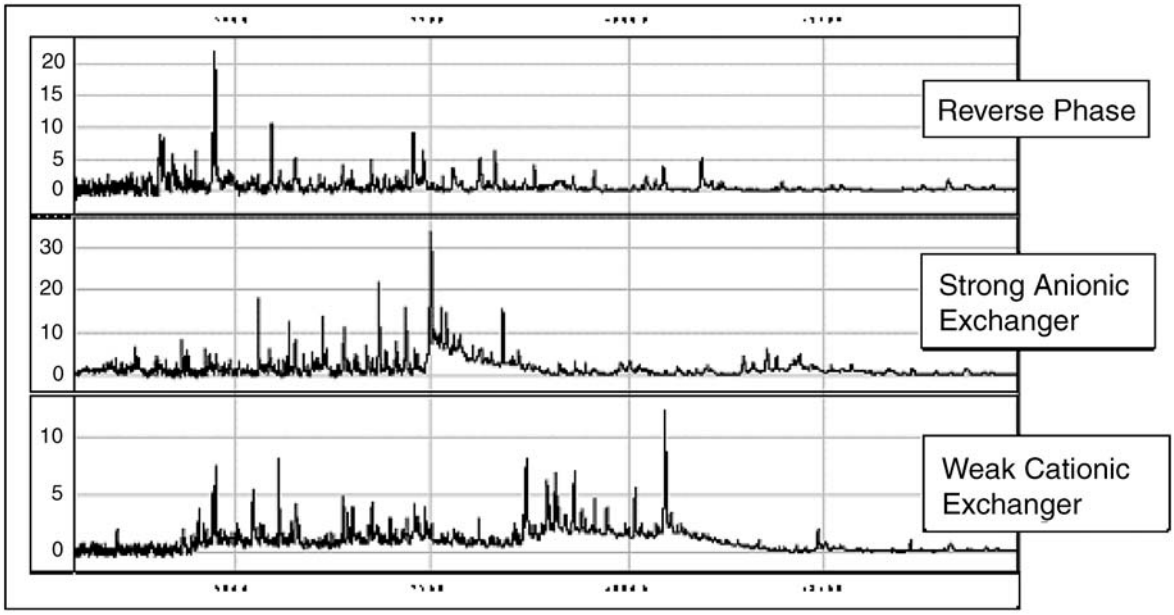

Fig. 8. SELDI-spectra of rat liver proteins derived from different chromatographic surfaces

proteins are co-crystallised with energy absorbing molecules. The chips are analysed in a TOF-MS. The results are initially visualised in a graph with the m/z of the sample components on the x-axis and the corresponding relative signal intensities on the y-axis. In this way, a specific protein pattern can be created from any sample, either belonging to control or treated groups. For differential expression analysis, the protein patterns are subjected to specialised computer programs. Group wise statistical comparisons are applied to processed spectra for single biomarker discovery. In order to classify subsets of the spectra by their characteristic patterns of relative expression intensities, bioinformatics tools have to be applied to the spectra.

EVALUATION

After xenobiotic/toxin exposure, differentially expressed proteins are identified by the comparison of SELDI spectra from control and treated samples. By combining groupwise statistics with N-fold regulations, single biomarkers (m/z) can be selected. As to be expected from the complexity of the proteome, in many cases no single marker will be able to discriminate between the groups. Rather, a complex pattern of multiple markers will be acquired (Figure 8). Discovery of such markers/patterns can be successful by application of multivariate statistics methods on the data set. However, for the identification of specific protein expression patterns bioinformatics tools are recommended. Many have been developed and most fall into two main types of approaches: supervised and unsupervised learning methods (Banks and Petricoin 2003; Yasui et al. 2003). Supervised methods require knowledge on certain group characteristics on which classification can be performed. The result is a system that can be trained to recognise and distinguish between groups. Unsupervised methods cluster groups due to similarities/differences in expression levels without previous knowledge of group characteristics. The result is specific clusters, which hopefully represent different sample groups. For the creation of databases useful in predictive toxicology, it is essential to implement biostatistics/bioinformatics tools that can identify validated patterns correlating strongly to specific toxicity end-points.

CRITICAL ASSESSMENT OF THE METHOD

The concept of comparing expression data on the proteome level for toxicity prediction screens has been gathering interest. The SELDI technology is able to generate these prediction screens of protein expression patterns in a semi-high throughput approach with respect to sample processing and data acquisition (Bandara and Kennedy 2002; Veenstra et al. 2004). For this reason, the method is useful to set up databases of xenobiotic/toxin specific expression patterns, which could be screened by novel compounds during the drug discovery and evaluation process. However SELDI has several drawbacks. Firstly, the molecular weight range

of SELDI is limited to 30 000 Da, which means that only a part of the proteome is presented in the SELDI spectra. If this subproteome can be used to establish predictive toxicology screens still needs to be evaluated. Secondly, the identification of proteins/pattern related masses (m/z values) is not routine meaning that prediction screens are based on protein mass data of differentially expressed peaks without further knowledge of protein identification. This is also the reason why this method is inadequate for investigative toxicology approaches.

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Chapter III.I
Application of Toxicoproteomics in Profiling Drug Effects

Mostafa Kabiri

PURPOSE AND RATIONALE

Traditionally, toxicologists define the preliminary risk of a new compound to human safety using animal models supported by histopathological and biochemical approaches. However, despite decades of experience, the extrapolation dilemma and the relevance of animal data to real-life, long-term exposure in humans remained unclear. The genomics revolution of the recent years led to development of many new and innovative technologies that can change this paradigm and address uncertainty issues in the current toxicological practice and safety assessment through the identification of novel key genes, marker proteins, or protein profiles. Thus, these technologies provide a superior alternative to traditional rodent and canine bioassays to identify and accurately assess the safety of chemicals and drug candidates for human safety.

Toxicogenomics, the use of DNA microarray for comprehensive RNA expression analysis, has recently caused a great deal of interest (Pennie et al. 2000; Nuwaysir et al. 1999). This technology has been used to monitor changes in gene expression in response to drug treatment. However, analysis of the information produced by toxicogenomics shows some limitation (Anderson and Seilhamer 1997; Mann 1999; Srinivas et al. 2001). Fundamental studies have illustrated the usefulness and potential of the Toxicoproteomics, the proteomic approach, to complement RNA microarray data. *Proteomic technology helps identify corresponding changes in the level of protein,* which is critical because the protein is the basic component of a cell. Additionally, toxicoproteomics helps resolve issues involving differential protein modifications. These are critical for the function of many proteins, in that they may lead to changes in the activity of gene products. Primarily, the manifestation of protein modifications is the reason for undesired, compound-related effects. Toxicoproteomics helps to determine such changes, and to gain insight into the mode of action of drugs.

PROCEDURE

Available Technology Platforms

The most common implementation of proteomic analysis involves protein separation two-dimensional gel electrophoresis (2DGE), quantification of proteins with analytical methods for their identification in mass spectrometer (MS), and at the very least data integration and analysis using bioinformatic tools.

2DGE

Initially, proteins in a sample are separated according to their isoelectric point in a pH-gradient. Next, the proteins are separated according to size on a SDS-polyacrylamide gel. A dye marker such as coomassie blue, silver or fluorescent dyes then detect the resolved proteins. In order to analyze differentially expressed protein spots in an experimental set of gels a computerized detection and matching system is required. Finally, MS identifies selected protein spots.

MS

Mass fingerprinting of excised and trypsin-digested gel spots is the method of choice to identify proteins. The masses of the tryptic fragments in a sample are accurately and quickly measured using a matrix assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) instrument. In this technique, purified or partially purified proteins are mixed with a crystal-forming matrix, placed on an inert metal target, and subjected to a pulsed laser beam to produce the phase ions that traverse a field-free flight tube. They are then separated according to their mass-dependent velocities. The mass peak list obtained is searched by means of in silico digest of sequence databases for comparison and identification of proteins.

EVALUATION

Performed Studies to Figure out the Mechanism of Organ Toxicity by Proteome Analysis

The proteomic investigation has been applied to a series of compounds to examine the response of in

vitro and in vivo models after exposure to toxicants. The main focus of these studies has been to understand the mechanism of their toxicity. In an attempt to develop a rodent liver proteomic toxicity database, Anderson and colleagues characterized the effect of a range of xenobiotics on protein expression in the liver (Anderson et al. 1996a). Using this database, it was possible to detect, classify and characterize a broad range of hepatotoxins. Toxicoproteomics was the key tool used to gain new insights into the molecular mechanism involved in cyclosporine A (CsA) nephrotoxicity. The initial study reported by Steiner et al (1996) investigated changes in the kidney protein pattern of CsA treated rats in order to determine the nephrotoxic mechanism of this drug. Using this proteomic approach, the investigators discovered an association between decreased calcium binding protein, calbindin-D 28 kDa, and CsA-mediated medullar nephrotoxicity (Aicher et al. 1998). Data on H1 receptor antagonist, pyrilamine, and the non-genotoxic carcinogenic analogue, methapyrilene, showed differing proteomic profiles despite a similar chemical structure. Widespread changes in hepatic protein composition were observed for methapyrilene but not for pyrilamine (Cunningham 1995). The proteomic approach further assisted numerous mechanistic investigations followed by explanation of regulatory changes implemented at the protein level. This included testing of pharmaceuticals for carcinogenic potential, hepatocellular hypertrophy, and peroxisome proliferation (Arce et al. 1998; Anderson et al. 1996b). The proteome profiling in these studies were partially used for lead prioritization, emphasizing the potential role for toxicoproteomics in lead candidate selection.

To examine the significance of the 2-DGE technology in determining changes in protein expression Seshire et al. (2004) investigated the response of rat primary hepatocytes to increasing concentration of the peroxisome proliferator fenofibrate. When primary cells were treated with 0.5 mM fenofibrate over 24 hours, a total of 30 protein spots were strongly affected. Fig. 1 shows the 2-D pattern obtained after separation of 350 μg protein sample from treated cells as an example. Many of the identified proteins are involved in cell-proliferation, protein metabolism, and energy. In addition, proteins associated with defense reactions to cellular stress are expressed at high levels in response to fenofibrate exposure. These results are consistent with mRNA abundance as indicated in microarray experiments (data not shown). However, some differentially expressed proteins were detected, for which no changes at RNA level were measured. Hence, the results show the potential of toxicoproteomics to serve as a complementary technology to microarray-based approach.

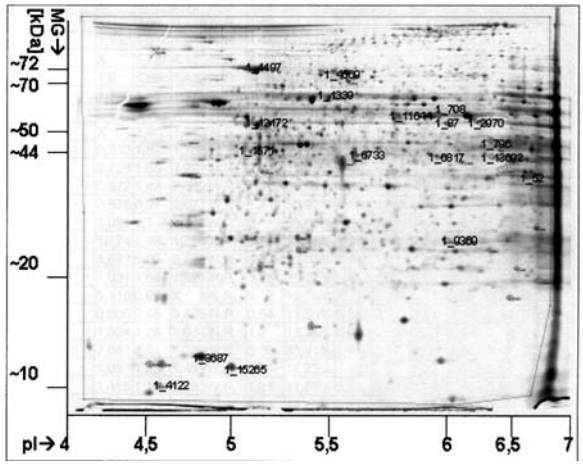

Fig. 1. Protein pattern of treated cells compared to control obtained after separation of 350 μg protein sample. Primary rat hepatocytes were exposed to 0.5 mM fenofibrate for 24 h. After the first dimensional run, proteins were separated on a 12.5 % SD-polyacrylamid gel and stained with SYPRO Ruby. Spot Ids marks differentially regulated proteins

CRITICAL ASSESSMENT OF THE METHOD

Alternative Proteomic Technologies and Options

Although 2DGE is unchallenged in its ability to resolve thousand of proteins, it has several limiting factors. Firstly, it is labor-intensive, requires large quantities of proteins, and may not be suitable to serve as an effected diagnostic tool. Secondly, the application of 2DGE is limited because it fails to detect proteins at the extremes of separation either by size or by isoelectric point, and because it is insufficiently sensitive for low-abundant proteins (Moseley MA 2001). Therefore, additional innovative methods are needed to measure broad protein abundances and activity.

SELDI-TOF

SELDI-TOF is beginning to offer an alternative to 2DGE. Surface-enhanced laser desorption/ionizitation (SELDI) is an affinity-based mass spectrometric method in which proteins of interest are selectively adsorbed to a chemically modified surface on a biochip (Ciphergen Protein Chip® Arrays).

This system has already been used to identify markers of prostate cancer and changes in renal cell carcinoma. It also has been applied to the discovery of new toxicity markers (Grizzle et al. 2004; Jr et al. 1999; Paweletz et al. 2001; Vlahou et al. 2001). Taking advantage of the recent development in SELDI and the

Protein Chip technology, it will be possible to simultaneously analyze protein profiles of body fluids such as serum and urine samples very rapidly. The SELDI mass spectrometry in conjunction with bioinformatics tools could greatly facilitate the discovery of new and improved toxicologic biomarkers.

ICAT

A recent and exciting development by Aebersold and colleagues is the isotope-codes affinity tag (ICAT) method, which can be used to label proteins before separation (Gygi et al. 1999). By using two different isotopes for labeling, it is possible to perform a binary comparison in a single step. After labeling, test and control sample are pooled and digested with proteases to produce peptide fragments. ICAT-labeled peptides are separated and analyzed by tandem MS. Due to the fact that the ICAT method is designed to combine labeling, separation, and the analysis of peptides into a single automated procedure, it is possible to scan several thousand peptide pairs a day.

Phosphoproteomics and Detection of Post-translational Modifications

Proteomics is complicated by the fact that the absolute quantification does not always reflect the molecular function of proteins, because protein activities are highly regulated post-translationally. Post-translational modifications modulate the function of proteins and thus directly impact their capacity to participate in cellular regulatory events (Cravatt and Sorensen 2000). Due to post-translational modifications, the numbers of proteins in human are estimated to be at least three times the amount of genes. Therefore, the elucidation of protein post-translational modifications is the most important justification for biochemical and structural relationships. Hence, these modifications need to be evaluated.

However, establishing a proteomics platform initially requires implementation and combination of a series of systems to allow a flexible and reliable approach for analysis and identification of differences observed on 2-D gels. Proteomics in this sense is more interdisciplinary, combining aspects of biology, chemistry, toxicology and pharmacology, bioinformatic and information sciences. Use of bioinformatics is essential for analyzing the massive amount of data generated by proteomics.

The throughput of proteomics is currently much lower than that of RNA microarrays, largely due to the requirement of MS analysis, or similar technologies. However, the microarray-based approaches of protein detection may overcome this limitation.

While the combination of 2DGE with protein analytic techniques has been established for toxicoproteomics, *the integration of bioinformatic and appropriate software has yet to be implemented.*

For toxicoproteomics, the ideal proteomics platform would be one that is:

- Sensitive enough to detect high- and low-abundance proteins
- Easily implemented and performed quickly
- Able to detect modifications and alternative splice forms in addition to abundance
- Able to deliver sophisticated data for protein-protein-networking

There are many obstacles to overcome in regard to current limitations of toxicoproteomic technologies. Thus, in the near future proteomics will play an important role in the research toxicology.

MODIFICATIONS OF THE METHOD

Subcellular Proteomics

One-step characterization of a eukaryotic cell proteome is difficult if not impossible to achieve. There is a growing trend in eukaryotes proteomics toward characterization of subcellular structures. The reason for this shift from global proteomics to subcellular proteomics is the complexity of eukaryotic cells and subcellular organelles. Therefore the proteomic analysis of subcellular organelles will be an important aspect of toxicoproteomics.

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Biographies of the Editors

H. Gerhard Vogel studied Pharmacy and Medicine at the Universities Erlangen and Tübingen (Germany). He received his Licensure as a Pharmacist in 1952 and as a Physician in 1956. His Doctorate in Medicine was gained in 1955. In 1963 he was designated a Fachpharmakologe DPhG. In 1967 he became Associate Professor in Pharmacology and Toxicology of the Department of Medicine, University of Marburg. In 1970 he was acknowledged as a Specialist for Pharmacology (Arzt für Pharmakologie). From 1972 onwards he has taught students of Medicine, Pharmacy and Biology as an Honorary Professor in Pharmacology and Toxicology of the Faculty of Medicine, University of Marburg and from 1979 as an Honorary Professor in Pharmacology and Toxicology of the Faculty of Medicine, University of Frankfurt.

After working as a resident in medicine in a city hospital, he joined the Department of Pharmacology of Farbwerke Hoechst AG, Frankfurt as a pharmacologist and endocrinologist in 1958. In 1969 he was nominated as Director of the Department of Pharmacology at Hoechst, in 1977 Head of Pharma Research Experimental Medicine, in 1980 Head of Pharma Research, Preclinical Evaluation and Development, and in 1989 Head of the Decision Board on Pharmaceutical Development. At present, he is working as a Consultant for Pharmaceutical and Medical Research and Development.

His scientific work was devoted to elaboration of new pharmacological methods, pharmacology of plant extracts from various countries, pharmacology of steroid hormones, pharmacology of peptide hormones, pharmacology of cardiac glycosides, biomechanics and biochemistry of connective tissue, experimental gerontology, experimental dermatology, development procedures for pharmaceutical products.

He has published more than 160 papers, mainly on new pharmacological methods, endocrinology, especially peptide endocrinology, biomechanics and biochemistry of connective tissue, such as skin and bone, and on other topics.

He is a member of many international and national scientific societies, such as The New York Academy of Science, American Association for the Advancement of Science, German Society of Experimental and Clinical Pharmacology and Toxicology, International Society for Bioengineering and the Skin, European Association for the Study of Diabetes, German Society of Endocrinology, German Society of Diabetology, German Society Connective Tissue Research, German–Chinese Society of Medicine.

He is a coeditor and contributor to several scientific textbooks.

In 1997 he published together with Wolfgang H. Vogel the book Drug Discovery and Evaluation. Pharmacological Assays (Springer-Verlag Berlin Heidelberg New

York), which appeared in a second completely revised, updated and enlarged edition with many contributions by his colleagues in 2002. A third edition is currently in preparation.

Franz Jakob Hock received his Ph.D. degree (Sc.D.) in Zoology at the Institute of Neuroethology and Biocybernetics at the University of Kassel, Germany. He joined the Department of Pharmacology of the pharmaceutical company Hoechst AG in 1976. He initially worked on methods in general pharmacology and nephrology, before becoming head of a laboratory devoted to pharmacological methods for drugs influencing memory and learning. He was then appointed Head of the Laboratory of General and Safety Pharmacology at the Frankfurt site of Aventis Pharma Deutschland GmbH.

He received the degree of Fachpharmakologe DGPT (*"certified expert pharmacology"*) in 1981. In 1983 he spent a sabbatical year at the University of California, Irvine, at the Center for the Neurobiology of Learning and Memory (Director Prof. Dr. James L. McGaugh).

He lectured for several years to students in Biology at the University of Kassel and the Technical University Darmstadt. He has published over 100 original papers on methods in Pharmacology and on new compounds.

He is currently a member of the Task Force General/Safety Pharmacology German/Swiss Pharmaceutical Companies, Safety ad hoc Group EFPIA. He served several times as a member of the program committee of the Safety Pharmacology Society. He is member of several domestic and international scientific societies.

He now works as a consultant in pharmaceutical business development.

Jochen Maas is head of the Drug Metabolism and Pharmacokinetics departments of Aventis in Frankfurt and in Paris. Educated at the universities of Heidelberg, Munich and Zurich, his background includes degrees in Biology and Veterinarian Medicine. During his studies and researching for his thesis at the "Gesellschaft für Strahlen- und Umweltforschung" in Munich-Neuherberg he collected his first experiences in Pharmacokinetics and in working with radiolabeled compounds.

After a short period as assistant veterinarian in an animal hospital he joined the former Hoechst AG as Laboratory Head in 1992, performing various kinds of animal studies with radiolabeled isotopes in the life-science area of pharmaceutics, veterinarian medicine and crop science. During this time he completed his education as a specialist for Radiology. Following the break-up of the different parts of the company he became Section Head, responsible not only for carrying out animal studies but also for the analysis, evaluation and assessment of all kinds of pharmacokinetic and toxicokinetic animal studies.

He took over the responsibility for the complete Drug Metabolism and Pharmacokinetics Department in Frankfurt in 2000. His activities ranged from in-silico approaches at very early stages of the value chain, in-vitro studies, and in vivo animal studies up to Phase I, II and III clinical studies. The whole scope of these activities

from early research to late development is included in the pharmacokinetic part of this book.

In 2003 he was appointed Head of Drug Metabolism and Pharmacokinetics in Paris working simulataneously in France and Germany. The main objective of his work is an intensive knowledge transfer from early parts of the value chain ("Research") to the late stages ("Development") and vice versa across the different disciplines.

Dieter Mayer studied veterinary medicine at the University of Munich, Germany. Thereafter, he worked on a thesis on biochemical mechanisms of heavy metal intoxication at the Institute of Pharmacology and Toxicology at the Ludwigs-Maximilian-University of Munich.

In 1975 he joined Hoechst AG Frankfurt, Germany. He worked there at the Institute of Industrial Toxicology and was in charge of the safety assessment of Acesulfam, an artificial sweetener. Further projects consisted of fluoro-carbons as replacements for chlorofluoro-carbons. He contributed to the development of several pesticides.

Dieter Mayer was a member of the German MAK (TLV) committee for about 12 years. He worked at the University of Davis, California and at the Centre International de Toxicologie (affiliation of Hoechst AG), Evreux, France, where he held the position of Scientific Director.

In 1986 he became Head of the central toxicology department at Hoechst AG. This entailed responsibility for the entire Hoechst portfolio, and hence also for pharmaceuticals. He was involved in the successful development of antiinfectives, cardiovascular drugs, several insulins, CNS drugs and anti-rheumatics. In 1998 he was promoted to Vice President of Lead Optimization (Toxicology, DMPK and Clin. Pharmacology).

Prof. Mayer has been teaching toxicology at the University of Frankfurt since 1991 and has authored more than 90 scientific articles, abstracts and oral presentations.

Today Dieter Mayer is a Consultant for the global pharmaceutical industry and is a member of the Advisary Board of National German Research Associations.

Index

c

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X