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Drug Discovery and Evaluation: Methods in Clinical Pharmacology

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Drug Discovery and Evaluation: Methods in Clinical Pharmacology

Hans Gerhard Vogel, Jochen Maas, Alexander Gebauer (Eds.)

Drug Discovery and Evaluation: Methods in Clinical Pharmacology

With 153 Figures and 95 Tables

Springer

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Introduction

A.1 General Introduction

Hans Gerhard Vogel

Drug discovery and evaluation is a multidisciplinary process. Discovery starts with experiments in isolated organs or in biochemical pharmacology, for example in vitro receptor binding studies. New chemical compounds have to be compared with known drugs used in therapy. Positive results have to be confirmed in various animal tests. The therapeutic advances may be higher potency, fewer side effects, or a new mode of action. Many methods are described in the literature and are reviewed in *Drug Discovery and Evaluation. Pharmacological Assays*, the first book of this series.

The strategy of drug development has changed in recent years. Instead of sequential studies in toxicology and pharmacokinetics, the parallel involvement of the various disciplines has been preferred. Exposure of drugs to the body is investigated by pharmacokinetic studies on absorption, distribution, and metabolism at an early stage of development, and contributes to the selection of drugs. Safety pharmacology has been coined as a special issue in addition to traditional toxicity tests.

These studies are reviewed in the second book of the series as *Drug Discovery and Evaluation. Safety and Pharmacokinetic Assays*.

Clinical Pharmacology and Clinical Pharmacokinetics belong together. There are no pharmacodynamics without pharmacokinetics and vice versa. We therefore combined both disciplines in the third volume of *Drug Discovery and Evaluation. Methods in Clinical Pharmacology*, with the aim of demonstrating the mutual dependency to the reader.

An important objective of clinical pharmacology is the early and ongoing assessment of the **safety and tolerability** of a new drug. This is done by assessing the type, frequency and severity of side effects, assessing in which patient population these side effects may occur at which dose or exposure, for what duration, and whether these side effects are reversible. The importance of an adequate selection of animal models, assessing the significance of the preclinical data obtained for humans and adequately planning the study conduct in the first in human study, has recently been shown quite dramatically.

The first dose step in the first in man study with a humanized monoclonal antibody induced a cytokine release syndrome in all actively treated healthy volunteers, all of whom suffered life threatening, acute shock and subsequent multi organ failure. Obviously these severe and serious adverse events were not predicted by the animal studies conducted prior to human studies. As a consequence of this event, the regulators worldwide have changed several processes so that this should not happen again.

Pharmacogenomics have an increasing input to drug development. Genomic information should enable the pharmaceutical industry to target specific patient populations that are more likely to respond to the drug therapy, or to avoid individuals who are likely to develop specific adverse events in clinical studies. In this volume, the possibilities of pharmacogenomic guided drug development are discussed.

Finally, also in the name of my colleagues Jochen Maas, Alexander Gebauer, I would like to express our gratitude and our sincere thanks to all authors who contributed their knowledge to this book

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Clinical Pharmacokinetics

B.1 Introduction

Jochen Maas

In the editorial to *Drug Discovery and Evaluation: Safety and Pharmacokinetic Assays* I emphasized that the different disciplines should overcome their occasional 'silo thinking', which results in a separated point of view due to the scientific home base of the author. We tried to overcome these silos between the different preclinical disciplines in that volume and we did so in this 3rd book in the clinical situation as well.

Clinical Pharmacology and Clinical Pharmacokinetics belong together. Some scientific organizations and pharmaceutical companies have these disciplines combined in one department; others have both functions separated from each other in different departments. Nevertheless, there is no pharmacodynamics (normally covered by pharmacology) without pharmacokinetics (normally covered by pharmacokinetics) and vice versa. We therefore combined both disciplines in the clinical volume of *Drug Discovery and Evaluation*, hoping to demonstrate the mutual dependency to the reader.

A further aspect which had to be taken into consideration was the fact that no clinical evaluation can be performed without having data in hand. Often, the data *production* is regarded as a "second class" science, but which is promoted to "first class" by the data *evaluation*. This may be true for individuals who are not involved in the daily life of Drug Discovery and Development. What would we do without bioanalytical, radioanalytical or biomarker data? What would we evaluate? I guess it is time to address these aspects in a clinical pharmacology and pharmacokinetic textbook, too. We therefore added some chapters which are closely related to technologies and methods of generating data without them even the best scientists would not be able to draw conclusions.

And one final aspect also had to be taken into consideration: no clinical studies are possible if specific preclinical requirements (and predictions) are not satisfied. Most of these aspects are covered in the second volume of *Drug Discovery and Evaluation*, but the most important preclinical studies are mentioned in this volume again. Here again we have to take into account a cross functional thinking: the preclinical data are evaluated by clinical pharmacologists and clinical pharmacokineticists simultaneously to design the optimal first clinical studies. The better and more accurate the planning of the first clinical studies based on preclinical pharmacokinetic and pharmacological in vivo and in vitro studies, the higher their outcome and predictability for the expensive further clinical testing.

And last but not least, I want to thank all the authors for their contributions despite very busy times in the pharmaceutical industry. Special thanks to Roland Wesch for his daily support and help to complete this volume of *Drug Discovery and Evaluation*. Without him the book would never have been completed.

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B.2 Dose Finding in Single Dose Studies by Allometric Scaling

Tobias Pähler · Jochen Maas

PURPOSE AND RATIONALE

The entry of a new chemical entity (NCE) into the clinical phase marks an important milestone in its development phase. Part of the planning of the first in man (FIM) study is the prediction and calculation of the first dose mainly based on the analysis of the available preclinical dataset. Various procedures and methods are available and described in the literature. All have in common that they are based on the assumption that new chemical entities exhibit the same or at least similar characteristics that influence the NCE exposure in plasma or tissues, which were observed in animals or in vitro systems. This chapter wants to highlight some of the methods and techniques that are currently in use by the pharmaceutical industry and try to illustrate their advantages and disadvantages.

An increasing importance was put in the last years on safety considerations in the conduct of all human studies. Therefore, increased safety considerations influence also the calculations of the FIM dose in a single dose study. Although the aim of an FIM dose prediction is not to cause any adverse events in the human probands, the 2006 incident after a single dose of Tegenero TGN1412, a monoclonal antibody, resulted in a serious health damage of the study probands (Nada and Somberg 2007). A direct consequence of this serious incidence was a currently draft guideline by the European Medicines Agency (EMEA 2007) entitled "Guideline on requirements for FIM clinical trials for potential high risk medicinal products." Apparently, the safety classification of biologicals, such as monoclonal antibodies, is different from those of small chemical molecules, although not every monoclonal is a high risk compound. On account of this in most cases, the dose prediction of biologicals is based on a case by case review and therefore not covered by this chapter. In comparison to small chemical molecules, the dose prediction of biologicals can result in an increased need of studies in the preclinical package depending, for example, on a novel mode of action like agonist to human specific cell receptors.

That is why in the preclinical work on small chemical entities an increased effort is taken to predict, not only the human plasma exposure after a single oral dose administration, but also to predict minimal biological and/or desired pharmacodynamic effects.

Besides small chemical molecules and biologicals, a third group of drugs in dose prediction is represented by cytotoxic agents in the development of oncology compounds. Because of their usually desired cytotoxic pharmacodynamic effect, the dose prediction of these compounds is different, since a pharmacodynamic treatment effect is aspired in the FIM study that recruits patients. The use of patients in oncology studies represents a major difference to the execution of small chemical molecules FIM studies and therefore has different ethical and safety fundamentals.

A good prediction of human pharmacokinetics is mandatory for a safe FIM dose calculation. The total plasma clearance in humans is of overriding importance, when describing the amount of plasma volume from which the applied NCE is removed after an intravenous administration (McNamara 1991). Various means are available to describe and predict the human *CL* already in the preclinical development phase.

PROCEDURE

B.2.1 Human Clearance Prediction by Allometric Scaling

The underlying principles of allometry are described in the literature for more than 100 years, but it was in the 1930s when Huxley and Teissier defined the still valid algebraic power function of $[y = bx^{\alpha}]$. It was also realized by several authors that by using a power law function and logarithmic coordinates, it was possible to describe the relation between brain and body size in mammals (Gayon 2000). The importance of the allometric power function in the interpretation of animal pharmacokinetic data was then pushed again by the publications from Dedrick et al. (1970) and Boxenbaum (1982), respectively, based on their empirical investigations. During the analysis of plasma clearance values from methotrexate in five

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mammalian species (including man), both described the measured values against body weight in a double log diagram by the function $CL = a^*(W)^b$ for that mainly renally cleared compound. This formula is still valid in the use of dose scaling and is usually referred to as "simple allometric scaling." To improve the clearance prediction quality from in vivo animal data, Mahmood and Balian (1996a, b, c) investigated a brain weight correction as proposed by Boxenbaum (1984) and a maximum life span correction by McNamara (1991). Based on their empirical analysis, Mahmood and Balian were able to define a guidance based on the calculated value of exponent of the allometric power function. The limits of this rule of exponents can be found in Table B.2 1.

EVALUATION

As an example of allometric scaling with the rule of exponents, the following case is used. From preclinical intravenous in vivo animal studies, the following plasma clearance values are available: rat 0.91 L/h (BW 0.25 kg), rabbit 4.44 L/h (BW 3.0 kg), and dog 31.0 L/h (BW 15.8 kg). These values plotted in a double log diagram and calculation of the slope linear power function results in an allometric exponent value of $b = 0.837$ based on the simple allometric scaling approach (Fig. B.2 1).

According to the rule of exponents, the calculated exponent of $b = 0.837$ indicates that allometric scaling corrected by the maximum life span potential should be used ($0.71 \leq b \leq 1.0$). The utilized factors for maximum life span correction and brain weight correction can be found in Table B.2 2. Figure B.2 2 gives an example of a maximum life span correction based on the previous case mentioned above.

All *CL* values from rat, rabbit, and dog are multiplied with the MLP values from Table B.2 2. The results are again plotted into a double log diagram (Fig. B.2 2).

This human *CL* prediction obtained from a recommended minimum of three different species can now be used in the process of an FIM dose calculation.

According to the following formula, a dose can be calculated.

$$FIM\ Dose = \frac{(AUC \times CL_{\text{human predicted}})}{(F_{\text{estimated}} \times \text{Safety factor})}$$

As an input for the expected exposure, it is recommended to use an $AUC_{0-24\ h}$ value obtained from the most sensitive species at the no observed adverse effect level (NOAEL) at the last day of a toxicokinetic study. Assuming similar toxicology in animals and humans, this calculation should end up in a safe FIM dose. To further increase safety, a safety factor of usually 10 is recommended

Table B.2-1

Allometric model recommendation based on the rule of exponents

Calculated exponent [$CL = a^*(W)^b$]	Model
$0.55 \leq b \leq 0.70$	Simple allometric scaling
$0.71 \leq b \leq 1.0$	Allometric scaling corrected by the maximum life-span potential
$b > 1.0$	Allometric scaling corrected by the brain weight
CL might be underestimated using simple allometric scaling when $b < 0.55$ and overestimated using brain weight correction when $b > 1.3$.	

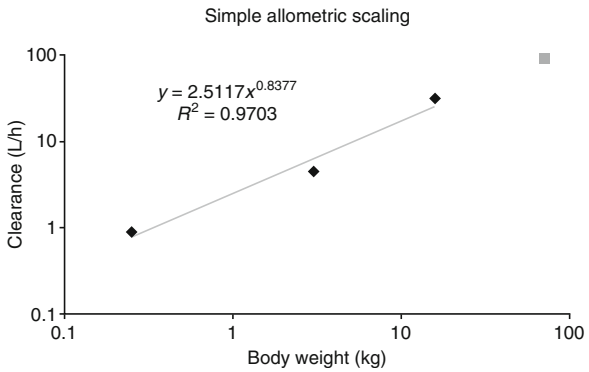

Figure B.2-1

Simple allometric scaling. The black diamond represent plotted values for rat, rabbit, and dog. The gray square represents a predicted human *CL* value of 88 L/h for a BW of 70 kg

Table B.2-2

Maximum life period and brain weight correction factors

Species	Brain weight (Percentage of body weight)	MLP (Years)
Mouse	1.45	2.67
Rat	0.751	4.68
Guinea pig	1.27	6.72
Rabbit	0.391	8.01
Dog	0.531	19.7
Monkey	1.32	22.3
Man	2.19 (1,530 g for 70 kg)	93.4

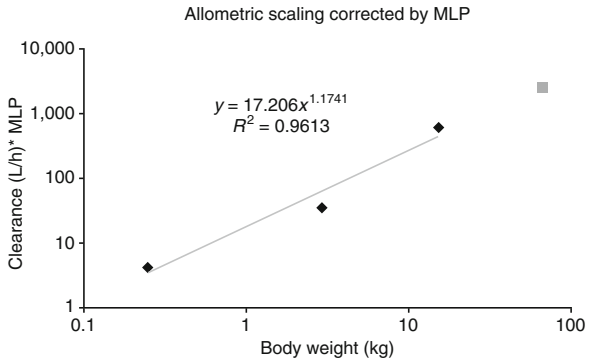

Figure B.2-2

Allometric scaling corrected by maximum life span. The obtained power function can now be used to predict the human plasma clearance *CL* = (17.206*(*BW*$^{1.1741}$))/93.4. Using a BW of 70 kg would predict a *CL* of 27 L/h in humans

(US FDA 2005). This factor can be adjusted according to known effects in toxicology studies, for example, increased to 50 in case irreversible tissue damage. A good estimation of the human oral bioavailability after the administration of the NCE helps selecting a safe dose. As a starting point, an oral bioavailability of 100% can be used as a conservative approach, but animal pharmacokinetics and in vitro solution simulations in gastric fluids can complete the estimations. For a prediction of the hepatic first pass effect in mainly hepatically cleared compound, intrinsic *CL* values from human hepatocytes studies can be used according to the following scheme.

The determination of the in vitro metabolic *CL* in human hepatocytes during the preclinical development phase offers the possibility to upscale a total human liver clearance from the cells. After the calculation of the elim ination rate constant in hepatocytes, this value corre sponds to intrinsic *CL* ($CL_{\text{int, in-vitro}}$), usually given in the dimension mL/h/10^6 cells. This $CL_{\text{int, in-vitro}}$ can be up scaled to a $CL_{\text{int, in-vivo}}$ calculating with 99 × 10^6 cells/g liver and a liver weight of 25.7 g/kg body weight (Houston 1994; Davies and Morris 1993).

An integration of $CL_{\text{int, in-vivo}}$ in the well stirred liver model or parallel tube model (Pang and Rowland 1977) can be done according to the following equations:

$$CLH = \frac{QH * \text{fu} * CL\text{int}}{QH + \text{fu} * CL\text{int}} \text{well} - \text{stirred liver model}$$

$$CLH = QH * \left(1 - e^{\frac{CL\text{int} * fu}{QH}}\right) \text{parallel} - \text{tube model}$$

Using also a liver blood flow (Q_H) of 87 L/h and a correction by the fraction unbound (fu), obtained from protein binding studies, transfers to a human *CL* prediction.

A clearance up scaling from available in vitro date provides elimination characteristics of a new chemical entity generated in human tissue. A calculation of the *CL* also allows predictions with respect toward the bioavail ability, especially for metabolically cleared compound: for example, high *CL* values close to the overall plasma flow in the liver would indicate intensive metabolism and a high first pass loss of absorbed compound in the liver indicat ing to a low bioavailability.

It should be noted here that the scaling and modeling from in vitro parameters may underpredict the human *CL* and that additional correction factors may be needed (Houston and Galetin 2003). Summarized in a 2008 pub lication from Houston et al., the authors propose a correction factor of 4.5 when using human hepatocyte data for the up scaling of the total human *CL*.

CRITICAL ASSESSMENT OF THE METHOD

The main advantage of a dose prediction with the use of allometric scaling and the rule of exponents is that usually all the needed data are generated during the preclinical development phase of a NCE. Its underlying empiric prin ciples (similar metabolic pathways in all species) and availability for small molecules that are mainly cleared intact hepatically and renally are critical. It means that also in cases in which all observed preclinical results seem to lead to a reasonable dose prediction, discrepancies can be observed in the following FIM study. Frequently observed is an underestimation of the human plasma *CL*. With respect to safety this is not of concern, it leads to initial dose steps in an FIM study with no or only a few detectable plasma concentrations. As an improvement of the allometric prediction quality it is proposed to intro duce further in vitro data of the NCE in the prediction. The normalization of *CL* values with the help of the intrinsic metabolic activity or a correction by fraction unbound data is mentioned (Lave et al. 1995, 1996; Obach et al. 1997). In the case of a single CYP P450 metabolism, the use of the Simcyp clearance and Interac tion Simulator® can be utilized in the prediction with described good results (Shiran et al. 2006). An in vitro in vivo extrapolation with Simcyp® was slightly superior to allometry methods and resulted in a reduced error range.

Nevertheless, allometric scaling utilizing the rule of exponents offers a rather easy approach for the estimation of an FIM dose in humans with a sufficient prediction rate, but mandatory preconditions are similar pharmacokinetics and metabolic pathways in all species. Allometry works best

with NCE elimination from the body occurring unchanged or only contributed by a limited number of enzymes in metabolizing the NCE, as a difficulty also remains the problem to predict outliers in the human up scaling process. It must be kept in mind that all results must be interpreted carefully and all available preclinical data should be used in the process of dose scaling. To increase the safety for the human volunteers in FIM studies always, the most conser vative approach obtained should be used.

MODIFICATIONS OF THE METHOD

B.2.2 FDA Approach

A further approach to calculate an FIM dose is described in the US FDA (2005) Industry Guidance "Estimating the maximum safe starting dose in the initial clinical trials for therapeutics in adult healthy volunteers." This guidance emphasizes also on small molecules with an oral admin istration route and offers the possibility to calculate a "maximum recommended starting dose" (MRSD) with only limited preclinical animal data. This approach is based on empirical data using a species specific "body surface area conversions factor" (BSA CF). An overview on the BSA CF for different species is given in *Table B.2 3*. As a major element, the NOAEL dose of the most sensitive species is used with the BSA CF and a human body weight of 60 kg to calculate a "human equivalent dose" (HED).

$$HED = NOAEL_{\text{most sensitive species}}(\text{mg/kg}) \times BSA - CF \times 60\ (\text{kg})$$

Table B.2-3

Conversion of animal dose to human equivalent dose (HED)

Species	BSA-CF
Mouse	0.08
Hamster	0.13
Rat	0.16
Guinea pig	0.22
Rabbit	0.32
Dog	0.54
Monkeys (cynomolgus, rhesus)	0.32
Marmoset	0.16
Baboon	0.54
Micro-pig	0.73
Mini-pig	0.95

This HED expressed as milligram per subject is then divided by a safety factor of usually 10 to obtain the MRSD in mg per subject. The above mentioned consid erations in adjusting the safety factor within the scaling process are also valid for the US FDA approach.

An evaluation of the US FDA approach and allometric scaling with the rule of thumb with sanofi aventis com pounds revealed that allometric scaling ended up in only in 1 case out of 22 with a higher dose recommendation compared to the US FDA approach.

B.2.3 "Rule of Thumb" – Early Human *CL* Scaling from Rat Data Only

As mentioned in the introduction, an early dose predic tion becomes more and more relevant with respect to increased safety demands and an intended shortening of preclinical development times in the pharmaceutical industry. Caldwell describes in his publication from 2004 a reasonable prediction method for the human plasma clearance (*CL*) based solely on the plasma clearance in rats. This empirical evaluation from Caldwell and his coworkers propose a factor of 40 to multiply with the observed *CL* in rats. The use of this single species approach is routinely possible, since a plasma *CL* value in rats is generated in the development phase of a NCE. With human plasma *CL* prediction, it is possible to make predictions on the human plasma exposure and, for exam ple, by the use of discovery pharmacodynamic data support the selection of tablet strengths for the upcoming FIM study. This approach does not reach the prediction quality of a three species or more allometric scaling calculation, but it can give a valuable rough assumption in the early phase.

B.2.4 Human *CL* Scaling by "Liver blood Flow" – Scaling with Monkey Data

The use of the liver blood flow (LBF) scaling model also represents a single species approach to predict the human *CL*. Assuming that *CL* is mainly hepatic and that the blood to plasma ratio is constant across species, *CL* can be expressed in each of the preclinical species as a fraction of the LBF (Ward and Smith 2004). According to the formula $CL_{\text{human}} = CL_{\text{animal}} \times (LBF_{\text{human}} / LBF_{\text{animal}})$ and using LBF rates from the literature 85 (rat), 30 (dog), 45 (monkey), or 21 (human) mL/min/kg, respectively, a human *CL* can be calculated (Davies and Morris 1993; Brown et al. 1997). An evaluation on *CL*

prediction models resulted in the best prediction quality using the LBF model with monkey data (Ward et al. 2005).

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B.3 Multiple Dose Studies

Steven G. Woolfrey · James Gilmour Morrison

PURPOSE AND RATIONALE

The development of a New Chemical Entity (NCE) in man is highly regulated (ICH E8 1997), and normally starts with a single ascending dose study in healthy young male volunteers. As virtually all drugs are administered repeat edly, it is important that the safety and pharmacokinetics are also investigated following multiple dosing. A single ascending dose study is therefore normally followed by a multiple ascending dose study in healthy volunteers. In this, the investigator aims to establish the safety, maxi mum tolerated dose at steady state, and pharmacokinetics of the NCE before proceeding into patients. This may be one of the few opportunities for assessing the pharmaco kinetics of the compound in detail following multiple dosing, albeit not usually in patients. The route and, ideally, the dose regime should be those proposed for the final product at registration. The choice of subjects is often young healthy male volunteers, but regulatory authorities encourage the use of females at an early stage in develop ment. Healthy volunteers may also include the elderly and individuals of different races if appropriate for the development program.

The aim of this chapter is to describe the process for setting up and assessing the pharmacokinetic components of multiple ascending dose phase I studies in man. It is assumed that a single ascending dose study will have already been carried out and data are available from that study. For the purpose of this chapter, it is assumed that young healthy male volunteers are being used for the assessment and that one is measuring plasma levels of drug substance given by the oral route, once daily. The need to measure metabolites, in addition to parent drug, is covered in Chap. 6 Regulatory Guidelines Regarding Metabolite Identification and Testing and the hADME Study (Incl. Dosimetry) and Its Results.

A commonly used approach for the design of multiple ascending oral dose studies in healthy volunteers is to administer multiple doses of the NCE to steady state, with detailed pharmacokinetic assessment on Day 1 and at steady state: In order to characterize the approach and time to steady state, pre dose samples are also taken throughout the dosing period. Typically, this type of study is carried out in small groups of separate subjects per dose group, without consideration of the statistical power.

PROCEDURE

The design of a typical multiple ascending dose study in healthy male volunteers is shown below, involving four ascending dose groups. Additional groups could be used if necessary.

B.3.1 Protocol Outline

Randomized, double blind, placebo controlled study to assess the tolerability and pharmacokinetics of 14 days repeated oral doses of r, x, y, and z mg of ABC1234 in healthy male volunteers.

B.3.1.1 Primary Objective

To assess the safety and tolerability after repeated oral doses of ABC1234 to steady state.

B.3.1.2 Secondary Objective

To assess the pharmacokinetic parameters after repeated oral doses of ABC123 to steady state.

B.3.1.3 Study Design

A double blind design of three or four treatment groups of 12 subjects, nine treated with ABC1234, and three with placebo. Subjects were randomly assigned to a group and each group was treated with an increasing dose of drug in the fasted state, once daily for 14 days. The safety and pharmacokinetic data were assessed at each dose level before progressing to the next higher dose.

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.3,

B.3.1.4 Inclusion Criteria

Caucasian men between 18 and 45 years in good health. Permitted concomitant medications were as used in the single ascending dose study. Any concomitant drugs that could affect the safety and pharmacokinetics of the NCE were considered for exclusion.

B.3.1.5 Treatments and Doses

Each daily treatment was given as a combination of capsules with 200 mL of water in the morning, after an overnight fast. A light breakfast was allowed 2 h after dosing.

B.3.1.6 Pharamacokinetic Data

In order to characterize the pharmacokinetics of ABC1234, frequent blood samples for the measurement of plasma concentrations of total drug were taken over a dosing interval on Day 1 (12 blood samples) and at the end of the study over the last dosing interval on Day 14 (12 blood samples). Beyond Day 14, additional samples were taken in order to characterize the terminal half life at steady state, in this case an extra two samples. Blood samples were taken pre dose (C_{trough}) on selected days during the dosing period to measure the approach and time to steady state. In all cases, duplicate plasma samples were collected, one transported to the site of bioanalysis and the other retained at the clinical site, in case of problems in transport.

A pre dose blood sample was collected to investigate allelic variants of drug metabolism enzymes and drug transporters as part of the development process. It was planned only to use these data as part of a larger dataset at the end of Phase I to examine the pharmacokinetics with respect to allelic variants.

No urine was collected in this study as it had been determined previously that renal excretion was not a major elimination route for parent drug.

The protocol indicated that the pharmacokinetic analysis would be carried out using a model independent approach and included a description of the software, pharmacokinetic parameters, statistical techniques, and quality assurance procedures.

EVALUATION

The plasma concentration time data were assessed for each individual profile using a model independent pharmacokinetic analysis approach. Actual blood sampling times were used in the analysis. Data were only excluded if there was a fully documented reason indicating a problem with the bioanalysis, dosing or subject compliance.

The following parameters were determined for each individual plasma concentration time profiles of ABC123:

- Day 1 C_{max}, t_{max}, $AUC_{last,}$ and AUC_{τ}
- Day 14 C_{max}, t_{max}, AUC_{τ}, Cl_{ss}/F, V_z/F, MRT, and $t_{1/2}$
- C_{trough} plasma concentrations on Days 2 14

The Day 1 and steady state data for C_{max} and AUC_{τ} were compared in order to determine the accumulation ratio of the compound (R_{Cmax} and R_{ac}).

Statistical analysis was carried out to assess dose proportionality for C_{max} and AUC_{τ} on Day 1 and at steady state separately, using the empirical power model (Parameter = $\alpha \times dose^{\beta}$), along with an "estimation" interpretation, according to the recommendations of Gough et al. (1995). Within subject and total standard deviations for $\log(C_{max})$ and $\log(AUC_{\tau})$ were also estimated.

Accumulation was examined statistically for log (Ratio of Day 14/Day 1) for C_{max} and AUC_{τ}, with a linear fixed effects model. Accumulation ratios were assessed for each dose level separately as well as pooled across dose levels within the fixed effects model framework.

The occurrence of steady state was assessed by fitting C_{trough} values to a nonlinear mixed effects model in order to predict the time to achieve 90% of the steady state trough concentration, taking into account any dose differences. Cl_{ss}/F and V_z/F were only calculated on Day 14 if steady state had been achieved, but were not subject to any statistical analysis.

Half life determined at steady state was compared across the doses using a linear mixed effect model.

Summary statistics were calculated for all parameters and the data then listed on an individual basis and with the summary statistics. The data were plotted to show both the individual and mean values so that trends could be easily identified and investigated. Reporting was carried out according to ICH guidelines (ICH E3 1995).

CRITICAL ASSESSMENT OF THE METHOD

B.3.2 Use of Previous Data to Design Study

In order to optimize the sampling regime and choose the doses for the multiple ascending dose study, it is recommended that data in humans from a single ascending

dose study are used to predict the pharmacokinetics on multiple dosing, including accumulation, time to steady state, and exposure at steady state. Such modeling and simulation can help provide confidence that the likely steady state exposures will be within safe limits, but are adequate to allow a pharmacological effect in Phase II.

B.3.3 Approach to a Double-Blinded Study

Multiple dose Phase I studies are normally carried out in a double blinded manner in order to aid the interpretation of the safety data. The Bioanalyst and Pharmacokineticist, however, effectively "unblind" the study in their analyses. Pragmatic procedures, therefore, need to be written into the protocol which protect the safety assessment from accidental "unblinding." It should be remembered that often the bioanalysis cannot wait until the completion and "lock" of the safety data, particularly if the pharma cokinetic data has to be released prior to the next dose increase.

B.3.4 Dosing in Fed or Fasted State

The effect of the prandial state on the pharmacokinetics of the NCE may be unknown at the point of carrying out a multiple ascending oral dose study. If it has been examined prior to the multiple dose study, then the prandial state that significantly maximizes the exposure (typically greater than two fold) is recommended. If data is unavailable or previous data has shown no major influ ence of food, then dosing in the multiple ascending oral dose study should be carried out after an overnight fast.

B.3.5 Blood Sampling

The blood sampling regime is fundamental to the successful outcome of any pharmacokinetic assessment. Care should, therefore, be taken to ensure that an adequate number of samples, appropriately placed during the dosing regime, are built into the design. The data from the single dose escalation study and predictions of the pharmacokinetics on multiple doing should be extensively used to help decide on the blood sampling regime. Practical consid erations also need to be considered in the actual timing of blood samples: Sampling during the night when the subjects are normally asleep is possible, but should obviously be kept to a minimum and avoided if possible.

B.3.6 Genotyping Data

The pharmacokinetics of some drugs can be affected by genetic polymorphisms of drug metabolism enzymes, which may lead to an apparent high level of pharmacoki netic variability and, in extreme cases, safety concerns. By the time an NCE enters clinical development some knowledge is available on the likely impact of the major genetic polymorphisms from in vitro data. It is recommended that subjects are screened for the major or compound specific metabolic genetic polymorphisms, in order to assess the effect of these on the pharmacokinetics of the NCE at the end of the phase I program. At this point data will be available from a limited number of subjects showing a number of allelic variants of metabolism enzymes.

MODIFICATION OF THE METHOD

B.3.7 Additional Pharmacokinetic Parameters After Oral Dosing

There are many pharmacokinetic parameters that can be calculated to characterize the pharmacokinetics of a compound after multiple oral dosing and the most common used for summarizing the data are those given in the proposed protocol.

Three optional parameters that are often calculated, include the apparent volume of distribution, clearance, and mean residence time, all estimated at steady state in multiple ascending dose studies. It must be remembered that the apparent volume of distribution and clearance are influenced by the bioavailability of the compound, which may well be unknown early in clinical development.

There are a variety of apparent volumes of distribution terms that may be calculated. It is generally regarded, however, that the volume of distribution at steady state (V_{ss}/F) is the most robust (ICH E3 1995), although the apparent volume term for the terminal phase (V_z/F) is the one usually calculated. Where possible, V_{ss}/F is recommended.

Other parameters that are sometimes stated include C_{min}, $C_{average}$ (at steady state), t_{last} and $t_{1/2\ effective}$, the latter being the half life estimated from the time to achieve steady state.

In some cases, there may be a lag in the absorption of a compound, possibly due to the dispersion of an oral dose form. Such a delay is usually observed in the single ascending dose study, and it may be important to include t_{lag} in the multiple dose study. If a lag time is not observed

after a single dose, it is probably unnecessary to measure this parameter in the multiple dose study, unless there is a significant change in dosage formulation.

B.3.8 Impact of Dosing More Frequently Than Once Daily

In some cases a NCE is administered more frequently than once daily, in order to achieve adequate exposure during the dosing interval. The principles that have been devel oped for multiple dosing assuming once a day dosing, also applies to the situation when dosing is more frequent. In essence, the pharmacokinetic analysis is carried per dosing interval.

With once daily dosing the impact of diurnal variation on exposure is usually not apparent. With more frequent dosing, however, diurnal variation can have a significant effect on exposure and, ultimately, the therapeutic effect. This can be assessed by characterizing the pharmacokinet ics at steady state under two dose periods, these being carefully chosen to maximise the likely differences (e.g., one during the day and one at night).

B.3.9 Need to Measure Drug in Urine

The need to sample urine for the assessment of the NCE will depend whether the compound is eliminated via the kidneys to any great extent and this is normally assessed in the single ascending dose study. It is recommended that if >5% of the dose is renally excreted as parent, then the quantification of drug in urine via a validated assay needs to be carefully considered, as part of the multiple ascending dose study. The need to measure urinary metabolites is covered in Chap. 6 Regulatory Guide lines Regarding Metabolite Identification and Testing and the hADME Study (Incl. Dosimetry) and Its Results.

To quantify urinary excretion of parent in a multiple ascending oral dose study, it is recommended that a urine collection is made over a dosing interval at steady state from which the following parameters can be calculated:

- Amount excreted under a dosing interval ($Ae_{0\text{-}T}$)
- Fraction of the dose excreted per dosing interval ($Fe_{0\text{-}T}$)
- Urinary Clearance ($Cl_{R0\text{-}T}$)

Care needs to be taken to ensure a complete collection of urine (as the volume of urine/dosing period is critical to the assessment) and that the urinary collection and storage process maintain the integrity of the compound.

EXAMPLE

To illustrate the type of data that can be obtained using the study design discussed above a typical example is described below.

In this example, the pharmacokinetics of ABC1234 had already been studied in a single ascending dose study in healthy male volunteers. Based on predictions of steady state, the available safety data and likely pharma cological levels, it had been decided to carry out a multiple ascending dose study at four dose levels up to a maximum daily dose of 60 mg. Predictions indicated that steady state would be achieved before Day 14: The assay for the mea surement of drug was adequately sensitive with a limit of quantification (LOQ) of 0.2 ng/mL. The study was carried out in a double blinded manner in young healthy male volunteers, with the objective of characterizing the safety and pharmacokinetics of the compound after once daily dosing for 14 days.

The compound was administered in capsules once daily for 14 days to groups of 12 subjects, 3 of which were matched placebos. A blood sampling regime, based on that from the single ascending dose was used on days 1 and 14: Pre dose samples (C_{trough}) were taken from days 2 14. After the last dose on Day 14, blood sampling was continued up to 72 h post last dose in order to characterize the terminal half life at steady state. Previous human studies had indicated urinary excretion was <5% of the dose, thus, urine was not collected for the assessment of urinary pharmacokinetics. A pre dose blood sample was collected from all subjects in order to genotype for meta bolic polymorphisms, although the results were not con sidered in the current analysis.

The pharmacokinetic profiles of ABC1234 on days 1 and 14 are shown in *Figs. B.3 1* and *B.3 2*, with the approach to steady state given in *Fig. B.3 3*. The derived pharmacokinetic parameters are shown in *Tables B.3 1* and *B.3 2*, which included V_z/F, Cl_{ss}/F, and MRT. No evidence of a lag time in absorption (t_{lag}) had been observed in the single ascending dose study and the dosage form was identical, hence it was not measured here. All blood samples were collected within ±15% of the theo retical sampling time specified in the protocol.

ABC1234 appeared rapidly in plasma following oral administration of 10, 20, 40, and 60 mg of ABC1234 to healthy young male subjects. On Day 1, following a single oral administration, plasma concentrations were quanti fiable up to the last sampling time (24 h post dose) at all dose levels. On Day 14 following repeated daily adminis tration, plasma concentrations were quantifiable up to the last sampling time (72 h post dose) at the 20, 40, and

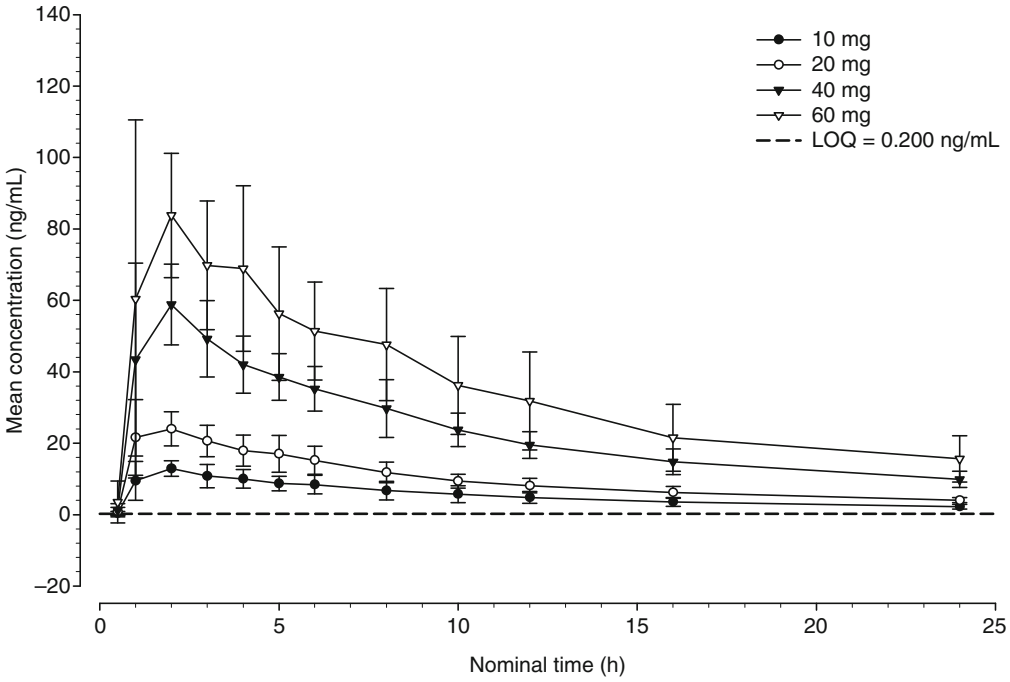

Figure B.3-1

Plasma concentrations (Mean ± SD) of ABC1234 after a single oral administration on Day 1

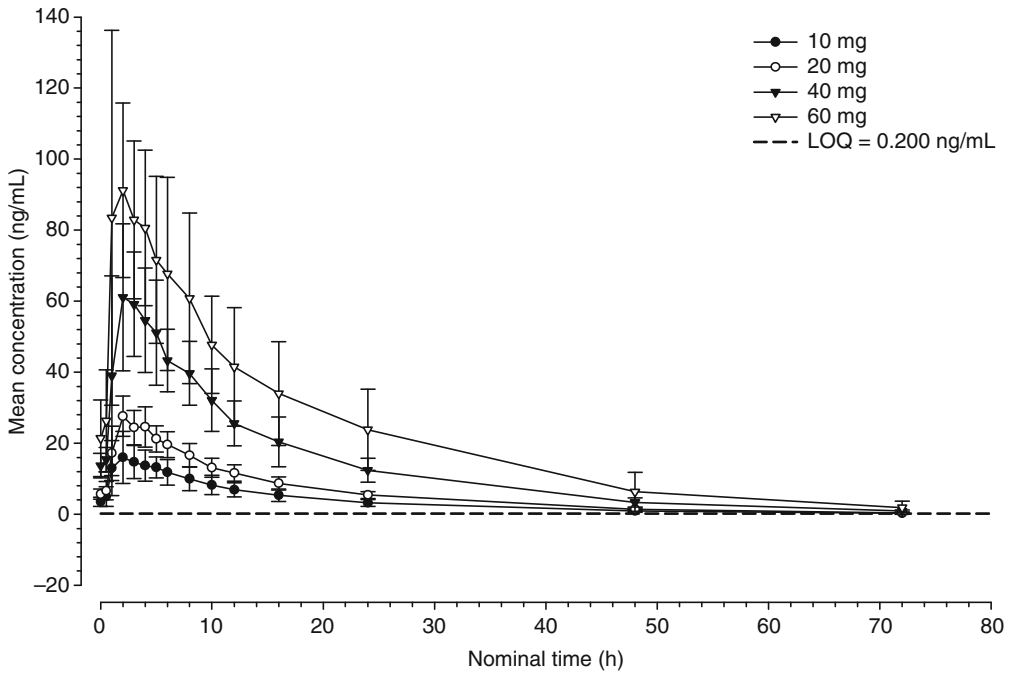

Figure B.3-2

Plasma concentrations (Mean ± SD) of ABC1234 at steady state on Day 14 after repeated daily oral administration for 14 days

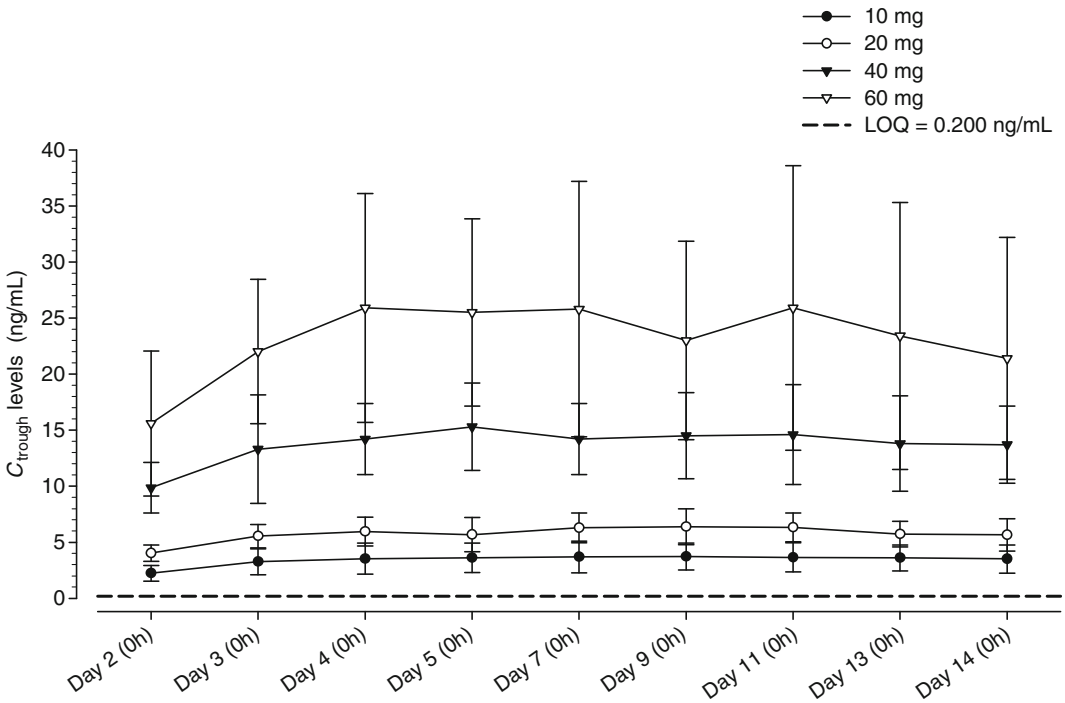

Figure B.3-3

C_{trough} plasma concentrations (Mean ± SD) of ABC1234 following daily administration for 14 days

Table B.3-1

Pharmacokinetic parameters of ABC1234 after a single oral administration

PK parameter	10 mg	20 mg	40 mg	60 mg
C_{max} (ng/mL)	13.7 ± 2.76 (20) [13.5]	28.6 ± 5.09 (18) [28.1]	61.6 ± 13.6 (22) [60.2]	101 ± 26.3 (26) [97.7]
t_{max} (h)	2.00 (1.00, 3.00)	2.00 (1.00, 2.00)	2.00 (1.00, 2.00)	2.00 (1.00, 4.00)
AUC_{last} (ng h/mL)	129 ± 38.0 (29) [125]	234 ± 47.3 (20) [230]	558 ± 99.7 (18) [551]	840 ± 229 (27) [816]
$AUC_{0\text{-}24}$ (ng h/mL)	131 ± 38.3 (29) [126]	237 ± 47.7 (20) [232]	563 ± 100 (18) [556]	849 ± 233 (27) [824]

Tabulated values are Mean ± SD (CV%) [Geometric Mean] except for t_{max} where values are Median (Min, Max): Minor differences between AUC_{last} and $AUC_{0\text{-}24}$ reflect small variation in the actual sampling times around the 24 h point, but all were within ±15% of nominal

60 mg dose levels. ABC1234 was not quantifiable in any of the pre dose Day 1 samples for subjects who received ABC1234, or in any of the samples analyzed from subjects who received placebo.

Mean C_{max} ranged from 13.7 to 101 ng/mL on Day 1 and 18.1 to 106 ng/mL on Day 14, over the dose range studied. The increase in C_{max} showed no major deviation from dose proportionality, although it should be noted that the lower 90% confidence interval for the β estimate of the log transformed power model was slightly greater than 1 on Day 1 (90% CI: 1.01,1.19). For a six fold increase in dose, C_{max} increased by 7.20 fold on Day 1 (90% CI: 6.14, 8.45) and 6.23 fold on Day 14 (90%CI: 4.99, 7.79).

There was no accumulation of C_{max} over the dosing period (accumulation ratio pooled across doses was 1.08; 90% CI: 0.998, 1.16). Within subject and total subject variability was low (19% and 26% respectively).

Table B.3-2

Pharmacokinetic parameters of ABC1234 at steady state on Day 14 after daily oral administration for 14 days

PK parameter	10 mg	20 mg	40 mg	60 mg
C_{max} (ng/mL)	18.1 ± 7.41 (41) [16.9]	27.8 ± 5.45 (20) [27.3]	66.3 ± 18.8 (28) [64.1]	106 ± 35.6 (34) [102]
t_{max} (h)	2.00 (1.02, 5.02)	2.00 (2.00, 3.00)	3.00 (1.00, 4.00)	2.00 (1.00, 6.00)
$t_{1/2}$ (h)	14.1 ± 2.33 (17) [13.9]	12.7 ± 1.09 (9) [12.6]	12.6 ± 0.978 (8) [12.6]	12.6 ± 2.13 (17) [12.4]
$AUC_{0\text{-}24}$ (ng h/mL)	190 ± 58.9 (31) [183]	313 ± 60.5 (19) [308]	723 ± 177 (25) [705]	1,150 ± 402 (35) [1,100]
V_z/F (L)	824 ± 211 (26) [802]	882 ± 151 (17) [871]	783 ± 186 (24) [763]	726 ± 213 (29) [698]
Cl_{ss}/F (L/h)	41.5 ± 11.7 (28) [39.9]	48.6 ± 9.28 (19) [47.8]	43.2 ± 10.3 (24) [42.1]	41.2 ± 13.6 (33) [39.0]
MRT (h)	17.4 ± 2.10 (12) [17.3]	16.7 ± 1.05 (6) [16.6]	16.4 ± 1.40 (9) [16.4]	17.3 ± 2.78 (16) [17.1]

Tabulated values are Mean ± SD (CV%) [Geometric Mean] except for t_{max} where values are Median (Min, Max).

Mean $AUC_{0\text{-}24}$ increased in a manner that was dose proportional on both Day 1 and 14: For a six fold increase in dose, $AUC_{0\text{-}24}$ increased by 6.80 fold on Day 1 (90% CI: 5.77, 8.02) and 6.19 fold on Day 14 (90% CI: 5.09, 7.52).

Consistent with the dose proportional increase in C_{max} and $AUC_{0\text{-}24}$, V_z/F, Cl_{ss}/F and MRT were independent of dose.

Over the dosing period there was minimal accumula tion of $AUC_{0\text{-}24}$ (accumulation ratio pooled across doses was 1.33; 90% CI: 1.29, 1.38). Within subject and total subject variability was low, with values of 7.5% and 24%, respectively.

On repeated once daily dosing, steady state was reached after the second or third dose at all dose levels (*Fig. B.3 3*). Median time to steady state pooled across doses was 2.1 days (90th percentile 2.4 days).

Arithmetic mean $t_{1/2z}$ of ABC1234 on Day 14 follow ing repeated daily administration was 12.6 14.1 h over the dose range studied. The difference in mean $t_{1/2z}$ between doses was not statistically significant (p = 0.2333), consistent with the dose proportional nature of the pharmacokinetics.

In conclusion, ABC1234 appeared rapidly in plasma with a median t_{max} of 2 3 h and then declined with a terminal half life of approximately 13 h. C_{max} and $AUC_{0\text{-}24}$ increased in a dose proportional manner, reaching steady state by about Day 2. After repeated daily administration, there was minimal accumulation of $AUC_{0\text{-}24}$ (accumula tion ratio: 1.33) over the dosing period. Within subject and total subject variability for $AUC_{0\text{-}24}$ was low (7.5% and 24%, respectively).

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B.4 Dose Linearity and Proportionality

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In drug development, one preferable aim is to develop a new drug candidate with linear pharmacokinetic properties to facilitate dose and dose regimen adjustment in patients. "Linear pharmacokinetics" implies that any concentration time profiles normalized for dose and time are superimposable (Ludden 1991). Thus, one of the necessary conditions for linear pharmacokinetics is dose proportionality, and its assessment is a fundamental pharmacokinetic analysis conducted during the clinical development of a new drug candidate.

B.4.1 Dose Proportionality

If the concentration of the drug (usually in plasma) at any given time is proportional to the dose of the drug administered, then that drug is said to be dose proportional (Smith 2004). Meaning, if the dose is for instance doubled (or tripled or halved), so is the concentration. Mathematically, dose proportionality at a given time point implies that for any dose equal or above zero

$$C \propto \text{dose} \qquad (B.4.1)$$

or replacing the proportionality with an equality

$$C = \alpha\,\text{dose} \qquad (B.4.2)$$

where C is the concentration at a given time point after dosing and α is some regression constant. A relationship between dose and C in case of dose proportionality is illustrated in ❯ *Fig. B.4 1*.

When the concentration is normalized for dose, Eq. B.4.2 passes into Eq. B.4.3 for any dose above zero, illustrating that dose normalized concentrations being constant are conditions equivalent to dose proportionality of these concentrations.

$$C/\text{dose} = \alpha \text{ (for dose} > 0) \qquad (B.4.3)$$

Instead of raw concentrations, the two most common surrogates, area under the curve (AUC) and maximum concentration (C_{max}), are generally used. However, there is no reason why other dose dependent concentration measures (e.g., trough concentrations) cannot be used. A dose proportional compound should exhibit dose proportionality for any dose dependent concentration measure (e.g., minimum concentration, steady state concentration, amount excreted via kidneys in a given time period).

B.4.2 Dose Linearity

Dose linearity is not to be mixed up with linear pharmacokinetics. Dose linearity is a weaker condition, even weaker than dose proportionality. It can be described by simple linear regression of the exposure measure C against dose

$$C = \alpha_0 + \alpha\text{dose} \qquad (B.4.4)$$

where α_0 is an intercept term and α is a regression constant. If the intercept term α_0 is zero, then Eq. B.4.4 simplifies to Eq. B.4.2 which is the definition of dose proportionality. Dose proportionality implies: no drug exposure if the dose is zero. If the intercept term α_0 takes a nonzero value, the result is dose linearity, but without dose proportionality (Cawello et al. 1999). Although no dose is administered, the exposure is larger than zero, which is typically observed for endogenous compounds. This is why a formal assessment of dose linearity is of minor practical importance of most drug candidates. The relationship between dose and C in case of dose proportionality and general dose linearity is illustrated in ❯ *Fig. B.4 1*.

B.4.3 Mechanisms Leading to Lack of Dose Proportionality

Lack of dose proportionality (implying nonlinear pharmacokinetics) may be due to many mechanisms but is typically due to the saturation of some component in the system, such as metabolizing enzymes or transporters. Ludden (1991) classified nonlinearity into causes due to nonlinear absorption, nonlinear distribution, or nonlinear elimination.

Common causes of dose nonproportionality due to nonlinear absorption include saturation of carrier mediated

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.4,

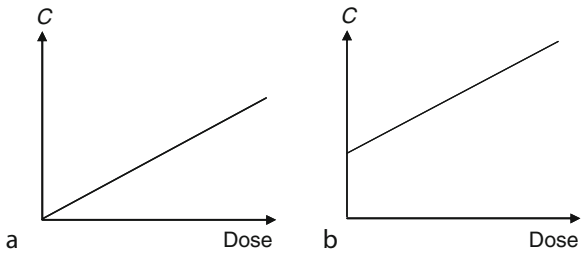

Figure B.4-1

Relationship between dose and concentration (*C*) in case of dose proportionality (a) and general dose linearity (b), both with $\alpha > 0$

uptake, poor aqueous solubility or slow release from the formulation, and saturation of presystemic metabolism. Common mechanisms of nonlinear distribution include saturable protein binding, red blood cell binding, and tissue distribution. Lack of dose proportionality due to saturable elimination includes saturable elimination at metabolic enzymes, saturable renal elimination at transporters, and autoinduction.

Lack of dose proportionality may have implications with regard to safety and efficacy. For a drug that shows dose dependent absorption, typically higher doses lead to less absorption and sub proportional drug concentrations. In this case, efficacy becomes a concern. For a drug that shows saturable elimination, higher doses lead to higher than proportional concentrations and increased risk of adverse events. This becomes more of a concern when a drug has a narrow therapeutic window. Related to this is the issue of insufficient predictability.

B.4.4 Clinical Assessment of Dose Linearity/Proportionality

The assessment of dose linearity/proportionality typically starts with early exploratory single dose clinical studies (Sect. B.4.1) providing PK data over a considerable dose range (Frick et al. 2006). Already in this early phase of the clinical development, these data are going to support exposure response relationships and dose selection in patients, and thus a potential submission (US FDA 2003). The assessment of dose linearity/proportionality may be deepened during drug development by more complex study designs (Sect. B.4.2) to further support PK/PD relationships and ends with comprehensive confirmatory studies (Sect. B.4.3) to support drug labeling, dosage form modifications (EU CPMP 1999) or the use of several dosage strengths (EU CPMP 2001).

B.4.5 Statistical Assessment of Dose Linearity/Proportionality

Dose proportionality is a mathematically ideal concept, which physiologically will never be met in a strict sense. For instance, there is no biological setting where equation (2) can hold for an unlimited range of doses. In addition, even if the true expected concentrations for a drug would behave along with ideal dose proportionality within a certain dose range, due to biological variability within and between subjects this could never be fully proven.

This leads to the assessment of dose proportionality being a statistical question, that is, to testing and estimation problems. Statistical analyses will have to analyze to what extent the data are compatible with the model of dose proportionality, to quantify deviations from the ideal and to support the derivation of clinical implications.

The clinical question which has to be answered is whether the deviations of expected exposure from dose proportionality are of clinical relevance or not. Deviations are relevant if they are large enough to go along with a risk from modified clinical features of the drug within the meaningful window of doses.

B.4.5.1 Descriptive Analyses

Descriptive analyses would normally include the presentations of descriptive statistics for concentration related parameters (typical statistics: number of non missing observations, mean, standard deviation, minimum, median, maximum, geometric mean, coefficient of variation). This can be supplemented by the presentation of the corresponding descriptive statistics for dose normalized parameters.

Graphical display could include scatterplots of PK parameters over dose for the raw or as well for the dose normalized parameters. For the latter, dose proportionality is represented by a dose independent (horizontal) level of the values (e.g., *Fig. B.4 8*). In addition, this presentation accounts for the fact that dose normalization typically standardizes the variability.

B.4.5.2 Discrete Model

For any study design with a set of fixed doses, one approach to test for deviations from dose proportionality can be based on classical linear models with a fixed effect for dose for log transformed dose normalized parameters. If a significant dose effect is found, strict dose proportionality

can be considered refuted. However, this does not yet imply that the deviations are of any clinical relevance.

Pairwise comparison of exposure allows estimating the ratio of PK parameters for two given doses. Given dose proportionality, the ratio is expected to be equal to the ratio of doses. Alternatively, ratios for dose normalized parameters could be assessed and compared with the value of 1 which is to be expected under dose proportionality (e.g., *Table B.4 6*).

B.4.5.3 Power Model

Quantifying deviations from dose proportionality for any dose demands the use of statistical models where parameters can be estimated together with measures of imprecision (usually confidence intervals) and where dose proportionality is characterized by certain values of the model parameters. Assuming log normal distributions for exposure related parameters like AUC and C_{max} suggests to model deviations from dose proportionality in a multiplicative rather than in an additive manner. A statistically beneficial way to model this is known as the power model

$$C = \alpha \ \text{dose}^{\beta} \qquad (B.4.5)$$

Ideal dose proportionality is met when $\beta = 1$ that is, when $C = \alpha$ dose.

In the above, deviations from dose proportionality ($C = \alpha$ dose) are indeed modeled as factors depending on β, which can be seen in

$$C = \alpha \ \text{dose}^{\beta} = \alpha \ \text{dose} \times \text{dose}^{\beta \ 1} \qquad (B.4.6)$$

For the interpretation of results from the power model it may help keep in mind that the following applies: for the r fold of a given dose, one can expect the $r^{\beta-1}$ fold of the exposure that can be expected in case of dose proportionality.

$$C/\text{dose} = \alpha \ \text{dose}^{\beta \ 1} \qquad (B.4.7)$$

Values of β above 1 represent higher exposure (super proportional), values of β below 1 represent lower exposure (sub proportional). Differently viewed at, the factor $r^{\beta-1}$ can be considered as the dose normalized ratio between exposure of the r fold of a dose versus the initial dose. Different scenarios are given as examples in the *Table B.4 1* below.

For instance, for a value of 1.25 for β, in case of doubling the dose ($r = 2$), the exposure would be more than doubled, in particular, it would be 19% higher than expected under dose proportionality. *Figure B.4 2* shows the factor for deviation from dose proportional exposure in dependence of the dose factors for different values of β.

Table B.4-1

Factor for deviation from dose proportional exposure for different values of β and *r*

Dose factor *r*	β				
	0.5	**0.75**	**1**	**1.25**	**1.5**
0.1	3.16	1.78	1.00	0.56	0.32
0.5	1.41	1.19	1.00	0.84	0.71
1.0	1.00	1.00	1.00	1.00	1.00
2.0	0.71	0.84	1.00	1.19	1.41
10	0.32	0.56	1.00	1.78	3.16

Obviously, and this is inherent to the model used, the estimate for $r^{\beta-1}$ can respect any given limits only for a certain range of r (or if β is equal to 1). Thus, it will be of clinical importance to assess whether the range for "r" is large enough to cover clinically relevant dose ranges.

One important beneficial statistical feature of the power model is that log transformation leads to a very simple linear model with a normal distribution for $\log(C)$:

$$\log(C) = \log(\alpha) + \beta \times \log(\text{dose}) + \text{Error}(\text{for dose} > 0) \qquad (B.4.8)$$

Based on standard statistical methods for linear models, estimates for β together with confidence limits can be derived (Smith et al. 2000). Based on that, for a given dose ratio, a confidence interval for the "deviation factor" $r^{\beta-1}$ can be calculated. This leads to the analysis being linked to questions of bioequivalence. If for instance, a classical range of 0.8 1.25 can be considered for a drug as a range for bioequivalence, the deviation from dose proportionality may be considered as irrelevant as long as the confidence interval for $r^{\beta-1}$ does not violate this interval of 0.8 1.25.

B.4.6 Exploratory Assessment of Dose Linearity/Proportionality: Single Dose Study Design

PURPOSE AND RATIONALE

The primary objective of first in human studies is gener ally to assess the safety and tolerability of a drug in healthy volunteers. These early clinical studies always provide

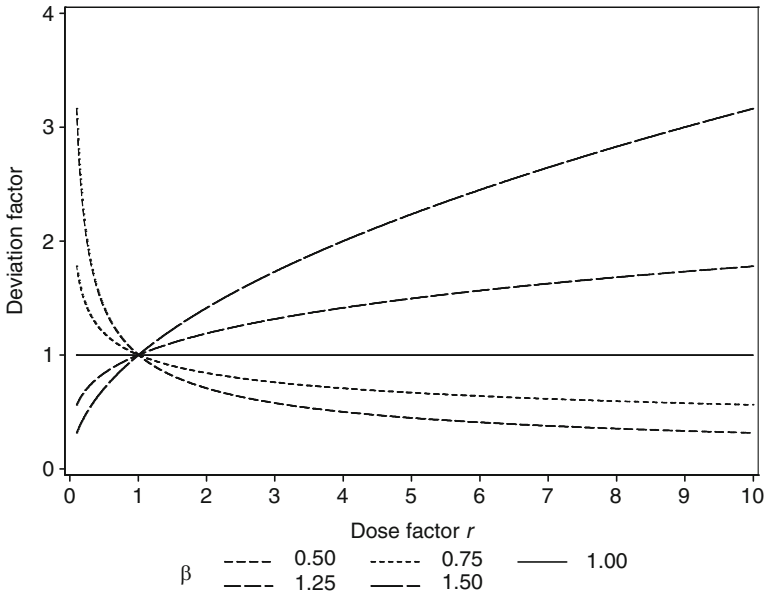

Figure B.4-2
Factor for deviation from dose proportional exposure for different values of beta (β)

pharmacokinetic (PK) and sometimes pharmacodynamic (PD) data over a range of doses, mostly within a single dose approach. The secondary objective is thus the evaluation of pharmacokinetic parameters to describe the dose effect on drug absorption (t_{max}, t_{lag}), elimination ($t_{1/2z}$), and exposure (C_{max}, AUC) overall to assess the drug linearity/proportionality. The selection of the starting dose is generally based on preclinical data from the most sensitive species in toxicology studies as described in more detail in Chap. B.2. The tested dose range should cover the potential therapeutic dose and should allow the determination of a maximum tolerated dose in humans and the safety margin.

PROCEDURE
The design of an exploratory assessment of dose linearity/proportionality during conduct of a first in human study for candidate drug X001 is presented below. The description is limited to pharmacokinetic data although safety/tolerability and pharmacodynamic data were also obtained.

B.4.6.1 Title

A double blind, randomized, placebo controlled safety/tolerability and pharmacokinetic study of escalating oral single doses of X001 under fasted conditions in healthy young male subjects.

B.4.6.2 Objectives

Primary objectives were to assess the clinical and laboratory safety/tolerability and secondary objectives were to assess the pharmacokinetics (PK) and pharmaco dynamics (PD) following ascending single oral doses of X001 under fasted conditions.

B.4.6.3 Study Design

It was a single center, double blind, placebo controlled, randomized, escalating single oral dose study. Eight healthy young male subjects per dose step were to be randomized and treated with 1, 2, 5, 10, 20, 40, 80, 160, 300 or 500 mg X001, or placebo. Six subjects in each dose step were randomized to X001 and two subjects to placebo. In total 80 plus 8 additional subjects for optional dose levels were planned. The placebo controlled study design was chosen for safety and PD assessment not man datory for the PK objective.

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 ($n + 1$) was based on the full range of safety parameters of the last dose (n) and pharmacokinetic data of the previous dose ($n - 1$).

Subjects entered the study unit the evening before the study of drug administration, and were to be assessed for

their baseline characteristics on the morning of the day of drug administration. After oral dose, subjects remained in the study unit for 48 h.

B.4.6.4 Inclusion Criteria

Healthy young male subjects aged between 18 and 45 years with a body weight between 50 and 90 kg and a body mass index between 18 and 28 kg/m^2 were included.

B.4.6.5 Treatments

In this study, capsules containing 1, 10, 50, or 200 mg X001 were used to provide flexible doses between 1 and 500 mg. The capsules were administered with 240 mL of non carbonated water after an overnight fast and with a 4 h post administration fasting period.

EVALUATION
Standard safety/tolerability criteria (e.g., adverse events, biochemistry, hematology, urinalysis, ECG, and vital signs) and additional pharmacodynamic parameters (e.g., postprandial blood glucose) were assessed in this study but are out of the scope of this chapter.

B.4.6.6 Criteria for PK Evaluation

For pharmacokinetic evaluation, concentrations of X001 in plasma were determined by LC MS/MS and following pharmacokinetic parameters were calculated using standard non compartmental techniques: at least maximum concentration (C_{max}), time to maximum concentration (t_{max}), area under the concentration time curve from time of drug administration to last quantifiable concentration time point (AUC_{last}), area under the concentration time curve from time of drug administration extrapolated to infinity (AUC), terminal elimination half life ($t_{1/2z}$), and time to the first quantifiable concentration (t_{lag}).

B.4.6.7 PK Sampling and Bioanalytical Methods

Blood samples to determine X001 were collected before dosing, and at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h post dose. X001 concentrations in plasma were assayed using liquid chromatography tandem mass spectrometry (LC MS/MS) with a validated lower limit of quantification (LLOQ) for X001 of 1 ng/mL.

B.4.6.8 Statistical Methods

Plasma concentrations and pharmacokinetic parameters were listed by standard descriptive statistics (N, Mean, SD, SE, Min, Median, Max, CV%, and Geometric Mean).

B.4.6.8.1 Dose Proportionality

For C_{max} and AUC, dose proportionality was evaluated using the log transformed power model with dose as the fixed effect:

$$\text{Log(parameter)} = \text{Log}(\alpha) + \beta \times \text{Log(dose)} + \text{Error} \qquad (B.4.9)$$

This model was tested for lack of fit using the plot of residuals. Since there was no evidence of lack of fit (residuals randomly distributed around the origin), estimates for β with 90% confidence intervals were obtained by ordinary least squares. Estimates with 90% CI for PK parameter increases associated with an r fold ($r = 2$ and r = highest dose/lowest dose) increase in dose were obtained by exponentiating r to the powers of the β estimate ($\hat{\beta}$) and confidence limits, that is.

$$r^{\hat{\beta} \pm t_{95,df} SE(\hat{\beta})} \qquad (B.4.10)$$

B.4.6.8.2 Dose Effect

For $t_{1/2z}$, dose effect was assessed using a linear fixed effects model on log transformed values:

$$\text{Log}(t_{1/2z}) = \text{dose} + \text{Error} \qquad (B.4.11)$$

Point estimate and 90% CI for the geometric means of $t_{1/2z}$ were provided pooled across dose levels and separately for each dose group.

B.4.6.9 Results

B.4.6.9.1 Plasma Concentrations

Mean X001 plasma concentration time profiles following a single oral dose of 1, 2, 5, 10, 20, 40, 80, 160, 300, and 500 mg X001 are presented in *Fig. B.4 3*. X001 was rapidly absorbed showing peak plasma concentrations approximately 1 h post dose, irrespective of dose.

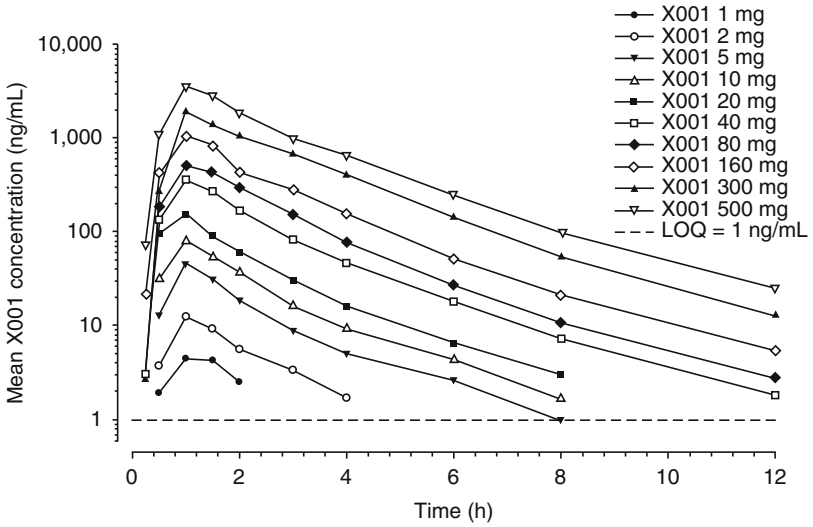

Figure B.4-3

Mean X001 plasma concentrations (semi-logarithmic scale)

Table B.4-2

Key PK parameters and descriptive statistics of X001 by dose

	AUC (ng*h/mL)				C_{max} (ng/mL)				$t_{1/2z}$ (h)			
Dose (mg)	Mean	CV%	SD	Geo. Mean	Mean	CV%	SD	Geo. Mean	Mean	CV%	SD	Geo. Mean
1	10.5	20	2.1	10.4	6.99	36	2.5	6.61	0.968	63	0.609	0.852
2	25.7	37	9.4	24.4	13.1	5	0.7	13.1	1.36	27	0.36	1.31
5	84.4	35	29.1	80.5	50.5	42	21.0	47.3	1.93	18	0.36	1.90
10	160	18	29	158	93.0	28	26.4	90.2	1.62	18	0.29	1.60
20	283	33	94	269	164	40	66	152	1.58	4	0.07	1.58
40	714	33	237	683	390	30	117	375	1.79	19	0.33	1.77
80	1,170	26	301	1,140	554	21	117	543	1.76	14	0.25	1.74
160	2,150	7	155	2,140	1,210	28	334	1,170	1.78	12	0.22	1.77
300	4,350	32	1,370	4,150	2,140	16	346	2,110	2.07	31	0.65	2.00
500	7,910	18	1,440	7,800	3,880	14	540	3,850	2.04	28	0.57	1.98

Depending on the dose, the subjects were exposed to X001 up to maximum 12 h post dose.

B.4.6.9.2 Pharmacokinetic Parameters

A summary of the descriptive statistics of main X001 PK parameters is given in *Table B.4 2*.

The relationship of individual AUC values versus dose, with linear regression and the 95% confidence range is illustrated in *Fig. B.4 4*. X001 exposure increased with increasing doses. The results of dose proportionality analysis are summarized in *Table B.4 3*. 90% CI of β estimates for C_{max} and AUC were 0.97 1.03 and 1.00 1.06, respectively, thus including the unity and dem onstrating dose proportionality. The mean terminal elim ination half life ($t_{1/2z}$) increased from 1 to 2 h with increase in dose. Statistical analysis revealed that the dose had a significant effect ($p<0.001$) on the terminal elimination half life ($t_{1/2z}$), the pooled average was 1.61 h (90% CI: 1.52 1.70 h) indicating an overall short half life of X001.

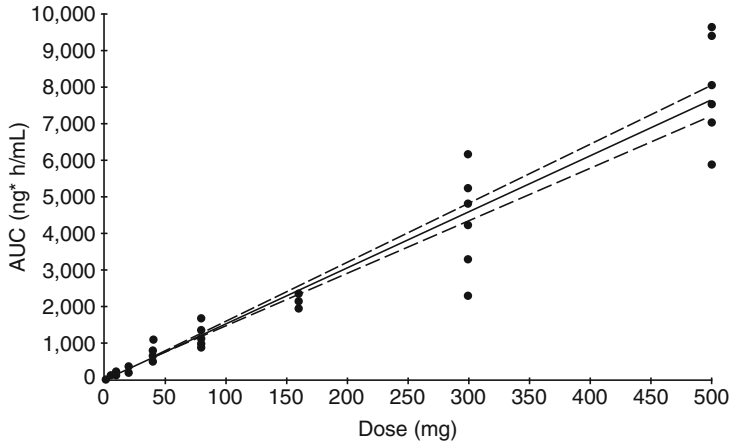

Figure B.4-4

Relationship of individual AUC values for X001 versus dose with linear regression (*bold line*) and the 95% confidence range (*dashed line*)

Table B.4-3

Estimates with 90% CI for *r*-fold increase in dose

Parameter	Dose ratio	Ratio	
		Estimate	90% CI
Cmax (ng/mL)	(*r*) = 2	2.00	(1.96 to 2.05)
	(*r*) = 500	506	(414 to 619)
	β-Estimate	1.00	(0.97 to 1.03)
AUC (ng.h/mL)	(*r*) = 2	2.04	(1.99 to 2.08)
	(*r*) = 500	592	(487 to 720)
	β-Estimate	1.03	(1.00 to 1.06)

(*r*) = 500 = highest/lowest dose

CRITICAL ASSESSMENT OF THE METHOD

The described evaluation provides a tool, also called "online PK," which allows adjusting the dose in this first in human study on a very flexible basis. Consequently, this flexible dose scheme is described already in the study protocol. The main prerequisite is, beside an adjustable dosing form, an immediate shipping and evaluation of the bioanalytical samples. Generally, the PK results of dose step n are available before starting the dose step $n + 2$.

As shown in *Fig. B.4 3* for the two lower doses of 1 and 2 mg X001 could only be detected up to 2 and 4 h, respectively, resulting in very limited data points in the elimination phase of the drug. Therefore, the quantification limit of the analytical method might have impeded somewhat the reliability of the PK parameters obtained for these doses (1 and 2 mg). For example, the low value of approximately 1 h for the calculated $t_{1/2z}$ for the 1 and 2 mg doses (*Table B.4 2*) differs from the value of approximately 2 h for the doses from 5 to 500 mg. If the low doses are of further interest (e.g., as pharmacologically active dose), the lower limit of quantification of the analytical method should be improved in order to guarantee a reasonable PK assessment.

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. This kind of explorative data related to dose linearity/proportionality can be used to predict the exposure for further planned dose steps, inside and outside the dose range investigated so far. At least if a notable nonlinear effect is seen by this exploratory evaluation, then a more elaborate study will need to be performed as described in Sect. B.4.3.

MODIFICATION OF THE METHOD

Typically, this kind of early first in human study covers a very broad dose range often leading to dose disproportionality over the entire dose range. At very low doses, PK parameters are often not reliable due to the limits of analytical quantification. At high doses, saturation of absorption or elimination processes or limitations in drug release from dosage form due to insolubility influence the exposure and prevent a dose proportional increase. In these cases a pivotal investigation of dose linearity/proportionality becomes necessary. It typically

includes at least three different doses covering the anticipated to be marketed therapeutic dose range. More details are provided in Sect. B.4.3.

In some cases dose linearity/proportionality assessment using the described explorative method is not possible due to high interindividual variability in PK characteristics. In these cases an intra individual crossover design is preferred as described in Sect. B.4.2.

B.4.7 Assessment of Dose Linearity/Proportionality: Crossover Study Design

PURPOSE AND RATIONALE

Drug candidate X002 had already been carefully explored before this study for pharmacodynamics, pharmacokinetics, and safety in a large number of subjects. However, no formal evaluation of the dose exposure response relationship had been conducted so far. Thus, this study was conducted in order to more precisely define this relationship. The three doses of 0.075, 0.15, and 0.3 units/kg body weight (U/kg) chosen for this study embrace a common range of doses used in clinical practice, as could be concluded from phase 3 studies.

The number of doses was restricted to three to allow for intra subject comparisons in a crossover design.

PROCEDURE

The design of an exploratory assessment of dose linearity/proportionality in a crossover setting for candidate drug X002 is presented below. The description is limited to pharmacokinetic data although safety, tolerability, and pharmacodynamic data were also obtained.

B.4.7.1 Title

Dose exposure response relationship of X002 in patients.

B.4.7.2 Objectives

Primary objective was to investigate the dose exposure response relationship of X002 after single subcutaneous injections of 0.075, 0.15, and 0.3 units/kg body weight (U/kg).

Secondary objective was to assess the safety and tolerance of X002.

B.4.7.3 Study Design

It was a single center, single blind, randomized, single dose, 3 way crossover study comparing three single doses of X002 (0.075, 0.15, and 0.3 U/kg) injected subcutaneously. The design was a full crossover, that is, including all six possible treatment sequences. Subjects were to be randomized to one of the six sequences for the three doses of X002. The study consisted of five trial periods trial period 0 (screening visit), trial periods 1, 2, 3 (X002 treatment visits), and trial period 4 (follow up visit). The subjects were blinded with regard to which of the three single doses they were to receive at trial periods 1–3.

B.4.7.4 Inclusion Criteria

All subjects were to be male patients between 18 and 55 years, body mass index between 18 and 30 kg/m^2, and with in the context of the underlying disease normal findings in the following assessments: medical history, physical examination, laboratory values, electrocardiogram (ECG), blood pressure, pulse rate, and core body temperature, unless the investigator considered any abnormality to be clinically irrelevant and not interfering with the safety of the subject and the scientific integrity of the study.

B.4.7.5 Treatments

Subjects received X002 in single doses of 0.075, 0.15, or 0.3 U/kg body weight, according to the randomization schedule. X002 was injected subcutaneously into the predefined body region on three different study days.

EVALUATION

Standard safety/tolerability criteria (e.g., adverse events, biochemistry, hematology, urinalysis, ECG, vital signs, and local tolerability at injection site) and additionally pharmacodynamic parameters were assessed in this study but are out of the scope of this chapter. Pharmacodynamic (PD) was assessed up to 10 h after dosing or up to the end of PD activity based on prespecified criteria, whatever came earlier.

B.4.7.6 Criteria for PK Evaluation

PK parameters were derived from the serum concentrations and actual sampling times relative to dosing.

The following parameters were derived using standard non compartmental methods: area under the concentration time curve for the time between 0 and 2 h after dosing ($AUC_{0–2h}$, μU.min/mL) and between dosing and end of the period of pharmacodynamic assess ment ($AUC_{0–end}$, μU.min/mL), and mean residence time (MRT, h).

The following parameters were derived using a compartmental method: maximum concentration (C_{max}, μU/mL) and time to C_{max} (t_{max}, hours).

$AUC_{0–2h}$ was considered the primary PK parameter.

Further supportive parameters were derived and reported, but are out of the scope of this chapter.

B.4.7.7 PK sampling and Bioanalytical Methods

Serum concentrations were measured at time point 0 (prior to dosing) and after 10, 20, 30, 40, 50, 60, 70, 80, and 90 min as well as after 2, 2.5, 3, 4, 5, 6, 8, and 10 h after the injection of study medication. If the end of the period for pharmacodynamic assessment occurred earlier than the maximum of 10 h post study medication injection, no further samples were taken. Serum concentrations of X002 were analyzed using a radioimmunoassay. The lower limit of quantification (LLOQ) was 5.0 μU/mL.

B.4.7.8 Statistical Methods

All PK analyses were based on the PK population (subjects with evaluable PK profiles and treated without major protocol deviations). No adjustments of the alpha levels were made for multiple analyses.

B.4.7.8.1 Sample Size

No formal sample size calculation was performed for this study. The sample size of the total 18 subjects, 3 subjects per sequence, was considered a standard approach for evaluation of dose exposure response relations.

B.4.7.8.2 General Descriptive Methods

Serum concentrations and pharmacokinetic parameters were listed individually and summarized per dose by standard descriptive statistics (number of non missing observations, geometric mean, mean, standard deviation, standard error of the mean, minimum, median, 25% and 75% quantiles, maximum, coefficient of variation (%)). Individual profiles per subject and median profiles were plotted by dose.

Box and whisker plots of primary pharmacokinetic variables were generated per dose.

B.4.7.8.3 Descriptive Methods for Assessment of the Dose–Exposure Relationship

The number of subjects with an observed strictly monotonically increasing dose exposure relationship was given.

The not normalized AUC values ($AUC_{0–2h}$ and $AUC_{0–end}$) and C_{max} were plotted over the dose per kg body weight (U/kg) as well as over the total dose (U), for each subject. Corresponding plots were generated for the exposure parameters normalized to a dose of 0.15 U/kg or to 10 U, respectively.

Geometric means for AUC values and C_{max} were plotted over dose together with a regression line forced through the origin point. Geometric means for AUC values and C_{max}, normalized to a dose of 0.15 U/kg were also plotted over dose together with a regression line.

B.4.7.8.4 Assessment of the Dose–Exposure Relationship Based on a Discrete Model for Doses per Kilogram Body Weight

AUC values (2 h and end), C_{max} and MRT were natural log transformed and analyzed using a linear ANOVA model with adjustment for dose, period, sequence and subject within sequence (discrete model). 95% confidence intervals for pairwise dose differences were calculated and re transformed to derive the respective confidence limits for mean ratios of the pair wise treat ment comparisons, that is, for 0.15 U/kg versus 0.075 U/kg and for 0.3 U/kg versus 0.15 U/kg. Dose propor tionality within the commonly accepted bioequivalence criteria (0.80 1.25) is confirmed for a doubling of the dose, when the confidence interval for a treatment ratio is within 1.60 2.50.

T_{max} was analyzed by nonparametric analysis for pair wise comparisons with 95% nonparametric confidence intervals for the respective median difference in dose.

B.4.7.8.5 Assessment of Dose Proportionality Relationship Based on a Power Model for Total Individual Doses

In addition, a power model was applied to assess dose proportionality. This analysis was based on the individual total actual doses (U).

B.4.7.9 Results

All randomized subjects were treated, completed the study, and were evaluable for the PK population (N = 18). Median X002 serum concentration time profiles following single subcutaneous doses of 0.075, 0.15, and 0.3 U/kg are presented in *Fig. B.4 5*. X002 was rapidly absorbed showing peak serum concentrations approximately 1 h post dose. Depending on the dose the median exposure to X002 lasted up to a maximum of 6 h post dose.

All subjects showed a strictly monotonically increasing dose exposure relationship in $AUC_{(02h)}$, $AUC_{(0end)}$, and C_{max} for X002. T_{max} generally increased slightly with dose. Descriptive statistics for key PK parameters of X002 are given in *Table B.4 4*.

Box plots for $AUC_{(0–2h)}$ of X002 are given in *Fig. B.4 6*, showing the monotonic relationship over the dose together with the expected increase in variability with increase in dose.

Geometric means for $AUC_{(0–2h)}$ of X002 are given in *Fig. B.4 7*, showing a relationship visually close to what is expected under dose proportionality.

Geometric means for $AUC_{(0–2h)}$ of X002, normalized on a dose of 0.15 U/kg are given in *Fig. B.4 8*. The

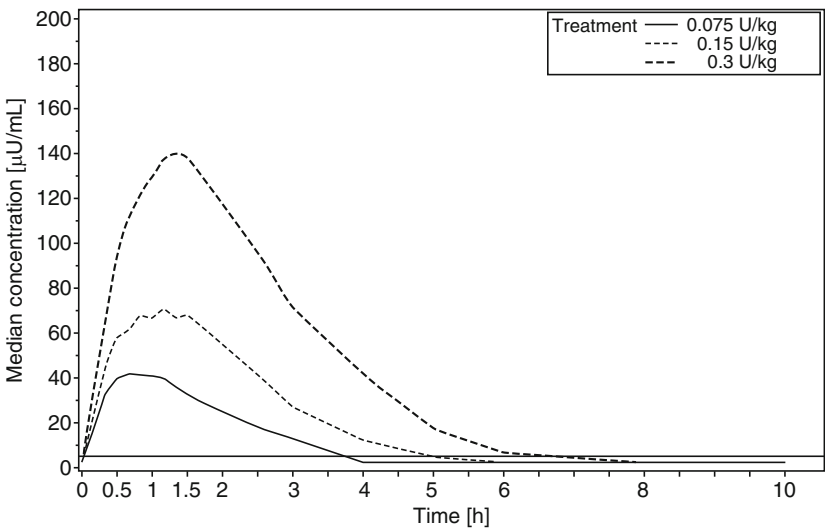

Figure B.4-5
Median X002 concentration (µU/mL) profiles over time after dosing

Table B.4-4
Key PK parameters of X002 and descriptive statistics by dose

Variable [Unit]	Geometric mean (arithmetic mean ± SD)		
	0.075 U/kg N = 18	0.15 U/kg N = 18	0.3 U/kg N = 18
$AUC_{(0–2h)}$ [µU·min/mL]	3,792 (3,855 ± 677)	6,676 (6,832 ± 1,461)	12,992 (13,237 ± 2,559)
$AUC_{(0–end)}$ [µU·min/mL]	5,341 (5,372 ± 589)	11,196 (11,284 ± 1,456)	24,891 (25,076 ± 3,209)
C_{max} [µU/mL]	42 (43 ± 9)	72 (73 ± 16)	140 (142 ± 25)
MRT [min]	115 (122 ± 50)	121 (125 ± 34)	134 (136 ± 28)
T_{max} [min][a]	47 [34 99]	57 [44 93]	72 [50 112]

[a] Median [minimum maximum] reported

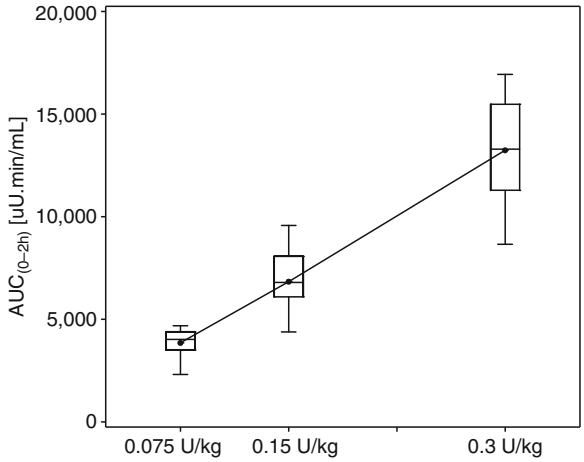

Figure B.4-6
Boxplots of $AUC_{(0-2h)}$ (uU.h/mL) for X002 per dose

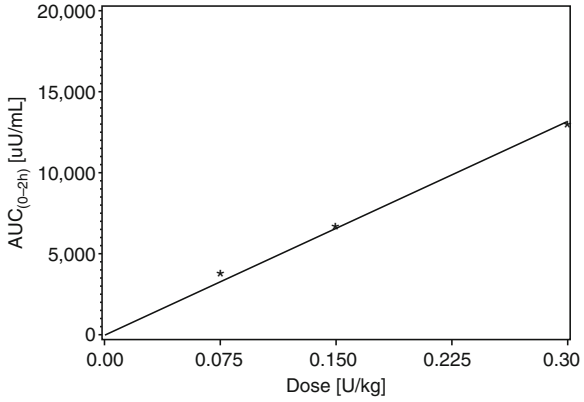

Figure B.4-7
Plots of geometric mean $AUC_{(0-2h)}$ for X002

estimated regression line shows a very slight decrease, indicating weakly sub proportional results for $AUC_{(0-2h)}$.

Point estimates for treatment ratios together with 95% confidence intervals are presented in *Table B.4 5*. All 95% confidence intervals are fully contained within the range of 1.60 2.50, and as a consequence the 90% confi dence intervals as well. Thus, exposure can be assumed to behave according to dose proportionality for a doubling of doses within the dose range investigated.

Due to the variation in body weight, the applied doses varied between 4 and 31 U: Individual plots of $AUC_{(0-2h)}$ over the total dose (U) are given in *Fig. B.4 9*. The figure also represents the finding of dose monotony in this exposure parameter for each subject.

Individual plots of $AUC_{(0-2h)}$ dose normalized for 10 U, over the total dose (U) are given in *Fig. B.4 10*.

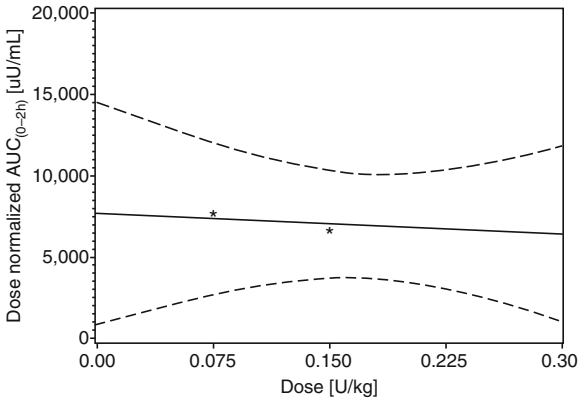

Figure B.4-8
Plots of geometric mean $AUC_{(0-2h)}$ for X002 dose normalized on 0.15 U/kg

Table B.4-5
Pairwise comparisons of key PK parameters for X002

Variable	Point estimate (95% confidence interval)	
	Ratio of 0.15 U/kg dose to 0.075 U/kg dose	Ratio of 0.3 U/kg dose to 0.15 U/kg dose
$AUC_{(0-2h)}$	1.8 (1.6 1.9)	1.9 (1.8 2.1)
$AUC_{(0-end)}$	2.1 (2.0 2.2)	2.2 (2.1 2.3)
C_{max}	1.7 (1.6 1.9)	2.0 (1.8 2.1)

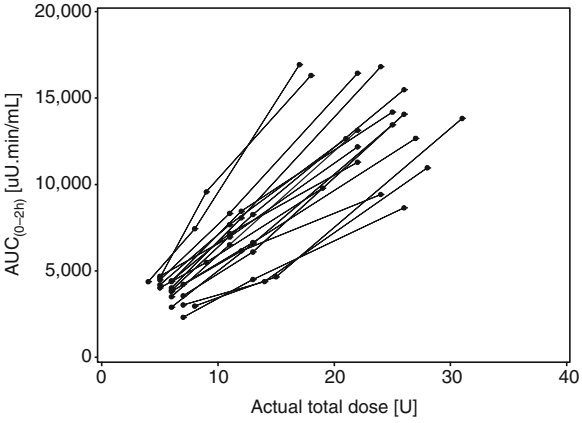

Figure B.4-9
Individual $AUC_{(0-2h)}$ (uU.h/mL) over actual total dose (U) per subject

Each deviation from a horizontal line indicates a deviation from dose proportional results for an individual. However, deviations are generally small.

The results of the analysis on basis of the power model for the actual total doses (U) are presented in Table B.4 6. The 90% confidence intervals for the dose normalized ratios for a value of $r = 2$ are fully contained within the classical bioequivalence range, confirming the dose proportional behavior of exposure for a doubling of dose. According to the results from the power model, dose proportionality could not be shown for the full dose range, corresponding to a factor of 7.75 between the highest and the lowest total dose. However, it was shown up to a 3.7 fold (r^*) for $AUC_{(0–2h)}$, up to 5.0 for $AUC_{(0–end)}$, and up to 3.0 for C_{max}. These dose ranges, for which dose proportionality can be assumed, are considered to cover clinical needs, because for X002 this covers the range of dose adjustments which may be applicable for a subject.

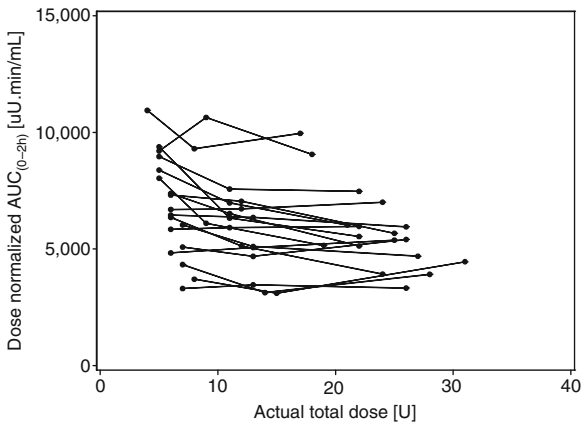

Figure B.4-10

Dose normalized individual $AUC_{(0–2h)}$ (uU.h/mL) over actual total dose (U) per subject

CRITICAL ASSESSMENT OF THE METHOD

Based on the crossover design, the study design allowed to assess dose exposure relationship and to investigate dose proportionality based on intra subject comparisons, which is not possible in any setting with parallel groups. Due to the crossover design with complete blocks, the number of doses investigated had to be limited to a small number.

Different statistical approaches were used and delivered consistent findings. The study allowed to draw conclusions for a clinical relevant range of doses. In addition, due to the dosing per kg of body weight and the variability of body weight between subjects, a broader range of doses could be observed.

MODIFICATION OF THE METHOD

The study was designed as an explorative study, because a strictly confirmatory study was not deemed necessary at this stage of the development of X002. In other setting this might be required. For this case, a single primary analysis used to decide about dose proportionality will have to be specified upfront.

Table B.4-6

Estimates with 90% CI for r-fold increase in dose

Parameter	Dose ratio	Ratio		Dose-normalized ratio	
		Estimate	90% CI	Estimate	90% CI
$AUC_{0–2h}$	(*r*) = 2	1.8440	(1.7784, 1.9119)	0.9220	(0.8892, 0.9559)
	(*r*) = 7.75	6.0964	(5.4781, 6.7838)	0.7866	(0.7069, 0.8753)
	(*r**) = 3.7324	3.1985	(2.9859, 3.4260)	0.8570	(0.8000, 0.9179)
	Beta Estimate	0.8828	(0.8306, 0.9350)		
$AUC_{0–end}$	(*r*) = 2	2.1576	(2.1145, 2.2016)	1.0788	(1.0572, 1.1008)
	(*r*) = 7.75	9.6960	(9.1353, 10.2917)	1.2511	(1.1788, 1.3280)
	(*r**) = 5.0073	5.9723	(5.6989, 6.2591)	1.1927	(1.1381, 1.2500)
	Beta Estimate	1.1094	(1.0803, 1.1385)		
C_{max}	(*r*) = 2	1.8164	(1.7425, 1.8936)	0.9082	(0.8713, 0.9468)
	(*r*) = 7.75	5.8315	(5.1580, 6.5940)	0.7524	(0.6655, 0.8508)
	(*r**) = 3.0718	2.6284	(2.4574, 2.8115)	0.8557	(0.8000, 0.9153)
	Beta Estimate	0.8611	(0.8012, 0.9211)		

(*r**) = highest dose ratio compatible with dose proportionality by the equivalence approach

Analysis to investigate the quality of fit for the models in use could be prespecified. In case of insufficient fit alternative models could be used.

Sample size considerations could also be deemed desirable. This could be power calculations for prespecified criteria or imprecision considerations for explorative studies.

For situations where a broader dose range has to be investigated, crossover designs with incomplete blocks could be set up.

B.4.8 Confirmatory Assessment of Dose Linearity/Proportionality: Single and Repeated Dose Crossover Design

PURPOSE AND RATIONALE

The aim of a confirmatory dose proportionality study is to assess the PK of a drug at doses bracketing the anticipated therapeutic dose (i.e., usually at one dose below therapeutic dose and one dose above), using the most appropriate design (i.e., crossover, within subject comparison) and an adequate number of subjects, in order to assess what are the variations in exposure when the dose needs to be adjusted, for example, in special populations or in case of concomitant medications.

In the case of candidate drug X003 the exploratory assessment of dose linearity/proportionality from first in human study did not allow accurate characterization of the deviation from proportionality of X003 pharmacokinetics because of the parallel group design, low number of subjects and relatively high variability of X003 PK. For a twofold increase in dose, there was a threefold increase in exposure with large 95% confidence interval of [1.9; 4.1] including 2. In addition, no multiple dose PK data was available at doses below the anticipated therapeutic dose of 400 mg bid.

PROCEDURE

The design of the confirmatory dose linearity/proportionality study during advanced clinical development for candidate drug X003 is presented below. The study description is limited to the primary objective of pharmacokinetic data although safety/tolerability data were also obtained as secondary objective.

B.4.8.1 Title

An open label, randomized, non placebo controlled, three treatment, three period crossover dose proportionality study after oral single and bid repeated (10 days) administrations of 200, 400, 800 mg X003 in healthy young male subjects.

B.4.8.2 Objectives

Primary and secondary objectives were to assess the deviation from dose proportionality and safety/tolerability, respectively, of X003 after 200, 400, and 800 mg single and twice daily repeated oral doses of X003 for 10 days.

B.4.8.3 Study Design

It was a single centers, randomized, non placebo controlled, open label, single and repeated BID oral dose, three treatment, three period, crossover study with a washout of 14 days between period. Healthy young male subjects were to be randomized and treated with 200, 400, and 800 mg X003. All doses were given as a single dose on Day 1, BID from Day 5 to Day 13 (evening and morning), and only on morning on Day 14.

Eighteen subjects were planned in order to have at least 12 subjects complete the study. Subjects were hospitalized the evening before first drug administration (Day 1) to Day 2 (morning) and from Day 13 (evening) to Day 15 (morning) of each period. The subjects visited the study unit every morning and every evening from Day 2 to Day 13 for blood sampling and/or study drug administration and on Day 15 evening, Day 16 to Day 18 morning for blood sampling. The duration of study participation for each subject was in total 12 15 weeks: 3 21 days for subject selection, 14 days for period 1, 14 days for washout period 1, 14 days for period 2, 14 days for wash out period 2, 14 days for period 3 and 10 12 days for follow up period.

B.4.8.4 Inclusion Criteria

Healthy young male Caucasian aged between 18 and 35 years with a body weight between 50 and 90 kg and a body mass index between 18 and 28 kg/m^2 were included.

B.4.8.5 Treatments

In this study, tablets containing 100 mg and 400 mg X003 were used to provide 200 mg (2 × 100 mg), 400 mg (1 × 400 mg), and 800 mg (2 × 400 mg) treatments. The tablets were administered with 200 mL of non carbonated water

on Day 1 at 8:00 am after the end of a standardized breakfast (single dose), on Day 5 to Day 13 at 8:00 and 8:00 pm, and on Day 14 at 8:00 am after the end of a standardized meal (repeated doses bid).

EVALUATION
Standard safety/tolerability criteria (e.g., adverse events, biochemistry, hematology, urinalysis, ECG, and vital signs) were assessed in this study but are not the subject of this chapter.

B.4.8.6 Criteria for PK Evaluation

For pharmacokinetic evaluation concentrations of X003 in plasma were determined by LC MS/MS and at least the following pharmacokinetic parameters were assessed using non compartmental analysis:

Day 1: Maximum concentration (C_{max}), time to max imum concentration (t_{max}), area under the concentration time curve from time of drug administration to time 12 h (AUC_{0-12h}) and to last quantifiable concentration time point (AUC_{last}), area under the concentration time curve from time of drug administration extrapolated to infinity (AUC), terminal elimination half life ($t_{1/2z}$).

Day 14: C_{max}, t_{max}, AUC_{0-12h}, $t_{1/2z}$.

Days 6, 7, 8, 9, 10, and 12: Trough concentration (C_{trough}).

B.4.8.7 PK Sampling and Bioanalytical Methods

Blood samples to determine X003 were collected before dosing and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 72, and 96 h after dosing on Day 1 and Day 14, and before morning dosing for trough determinations on Days 6, 7, 8, 9, 10, and 12. X003 concentrations in plasma were deter mined using a validated liquid chromatography tandem mass spectrometry (LC MS/MS) method with a limit of quantification (LOQ) for X003 of 0.5 ng/mL.

B.4.8.8 Statistical Methods

Plasma concentrations and pharmacokinetic parameters were listed by standard descriptive statistics (N, Mean, SD, SE, Min, Median, Max, CV%, Geometric Mean) for each dose level on Day 1 and Day 14.

Prior to analyses described below, X003 C_{max} (Day 1 and 14), AUC (Day 1), AUC_{0-12h} (Day 14), and $t_{1/2z}$ were log transformed, t_{max} were rank transformed.

B.4.8.8.1 Dose Proportionality

C_{max} and AUC at Day 1, and C_{max} and AUC_{0-12h} at Day 14 were analyzed with a "random intercepts and random slopes" mixed model in SAS PROC MIXED separately for Day 1 and Day 14. The parameters were assumed to follow a multiplicative power model, which is equivalent to the log transformed power model, and has the form:

$$\begin{aligned}\text{Log(parameter)} = {} & [\text{Log}(\alpha)_i - \text{Log}(\alpha)] + [\beta_i - \beta] \\ & \times \text{Log(dose)} + \text{period} + \text{Error}\end{aligned} \qquad (B.4.12)$$

In the model, Log(α) and β were the estimates of intercept and slope, respectively, estimated by generalized least squares (GLS) with restricted maximum likelihood (REML) estimates of random effects.

Lack of fit was assessed by visual inspection of resid uals plots. If there was no evidence of lack of fit (residuals randomly distributed around the origin), estimates for β with 90% confidence intervals were computed within the mixed model framework. Estimates with 90% CI for PK parameter increases associated with an r fold ($r = 2$ and $r = 4$ = highest dose/lowest dose) increase in dose were obtained by exponentiating r to the powers of the ($\hat{\beta}$) and confidence limits, that is,

$$r^{\hat{\beta} \pm t_{95,df} SE(\hat{\beta})} \qquad (B.4.13)$$

and also, subsequently, converting these to a dose normalized scale by dividing by r. Three cases were considered: $r = 2$ (doubling of dose), $r = 4$ (high/low dose), and the maximum dose ratio "r" compatible with dose proportionality by an equivalence approach (i.e., 90% confidence limits for the dose normalized increase in the PK parameter is within 0.80 1.25).

B.4.8.8.2 Dose Effect

For $t_{1/2z}$ and t_{max}, differences between doses were tested for significance with p values from the linear fixed effects model with fixed terms for sequence, period, day, dose, and the dose by day interaction, and a random term for subjects within sequence.

If the dose by day interaction was not significant ($p \geq 0.05$), the interaction term was dropped from the model and the model was refit. The p values for the dose and day effects were reported in the context of the reduced model.

If the dose by day interaction was significant ($p < 0.05$), the p value for the dose effect was computed in a mixed effects model, fit separately for Day 1 and Day 14, with fixed terms for sequence, period and dose, and a random

term for subjects within sequence. The p value for the day effect was computed in a mixed effect model, fit separately for each dose, with fixed terms for sequence and day, and a random term for subjects within sequence.

Each model described above was fit by GLS with REML estimates of random effects, using SAS PROC MIXED.

B.4.8.8.3 Accumulation Effects

To assess accumulation effects from Day 1 to Day 14, the PK parameters were analyzed with a mixed effects model with fixed terms for sequence, period, day, dose and the dose by day interaction, and a random term for subjects within sequence. The model was fit by GLS with REML estimates of random effects, using SAS PROC MIXED.

If the dose by day interaction was significant ($p < 0.05$), accumulation was assessed for each dose group separately within the mixed model framework. Otherwise, the term was dropped from the model and accumulation effects were assessed for all dose groups. For C_{max} and AUC_{0-12h}, the difference in means between Day 14 and Day 1, with 95% CI, was computed within the mixed model framework, and converted to an accumulation ratio of adjusted geometric means by the antilog transformation.

B.4.8.8.4 Steady State

The occurrence of steady state for X003 was assessed separately at each dose level by fitting the trough values with a nonlinear mixed effects model using the SAS NLMIXED procedure. The BID trough values corresponding to days 6, 7, 8, 9, 10, and 12 were utilized for the steady state assessment. Model lack of fit was evaluated by graphical inspection of the fitted curves and the within subject residuals, and by graphical inspection of histograms of the estimated subject specific random parameters (e.g., evidence of outliers, or subpopulations by gender, age, metabolizer status). For each subject and each dose level, the day at which 90% of the estimated subject specific steady state trough concentration is reached was predicted from the model. The overall time to steady state was determined as the 50th percentile (for average steady state) and the 90th percentile (for individual steady state) of these individual predicted values at each dose level. The 95% CIs for the 50th and 90th percentiles were calcu lated by nonparametric methods.

B.4.8.9 Results

B.4.8.9.1 Plasma Concentrations

Mean (SD) X003 plasma concentration versus time curves observed after single (Day 1) and repeated BID (Day 14) oral administrations of X003 are presented in *Fig. B.4 11*. X003 plasma concentrations were higher following a 10 day repeated BID oral administration (Day 14) com pared to the single dose at Day 1 and reached peak levels approximately 5 h post dose. Twelve hours post dose plasma concentrations were not below LOQ.

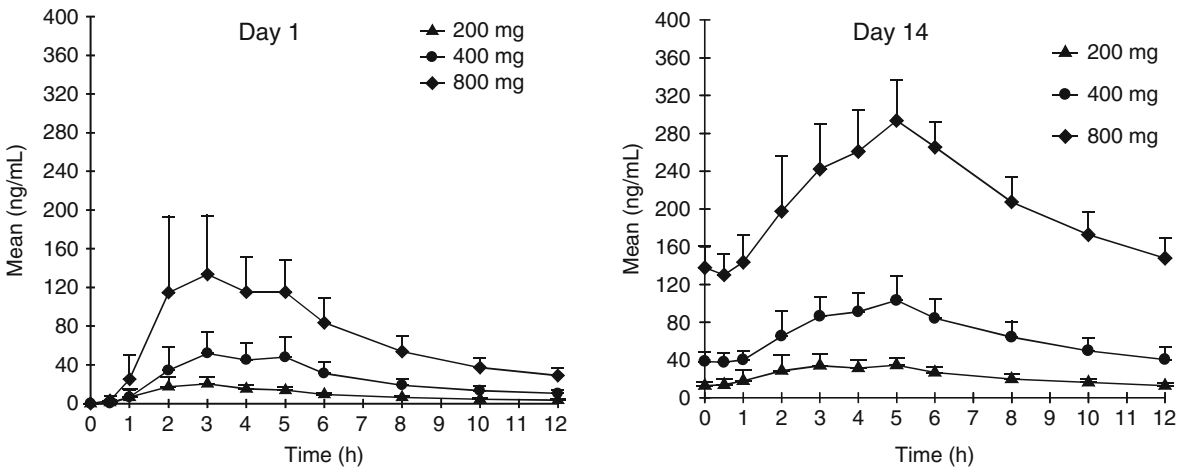

Figure B.4-11

Mean (SD) X003 plasma concentrations on Day 1 after a single and on Day 14 after repeated BID oral administration of X003 (linear scale)

B.4.8.9.2 Pharmacokinetic Parameters

A summary of X003 main pharmacokinetic parameters observed on Day 1 after a single oral administration and on Day 14 after a 10 day repeated BID oral administration of X003 is presented in *Table B.4 7*.

Steady State

Mean (SD) X003 C_{trough} observed from Day 6 to Day 14 during repeated BID X003 administrations are graphically summarized in *Fig. B.4 12*. Individual steady state, as expressed by 90th percentile, was reached after 3 to 5 treatment days. Average steady state, as expressed by 50th percentile, was reached after 3 to 4 treatment days, whatever the doses.

Accumulation

In the accumulation assessment, the dose by dose interaction was not significant for C_{max} and AUC_{0-12h}, allowing the assessment of a single accumulation ratio across dose for each PK parameter of X003. After a 10 day repeated BID oral administration, an accumulation ratio (95% CI) of 1.84 [1.65 2.04] in C_{max} and 2.72 [2.52 2.94] in AUC_{0-12h} was observed, whatever the administered dose (*Table B.4 8*).

Dose Proportionality

Results of the dose proportionality assessment, at Day 1 and at Day 14, are summarized in *Table B.4 9*.

C_{max} and t_{max}: After a 10 day repeated BID oral administrations of X003 doses ranging from 200 to 800 mg, X003 C_{max} values were reached 5 h after drug intake; no significant dose and day effects and no significant dose by day interaction was observed on t_{max}. As measured by ratio estimate and associated 90% CI, X003 C_{max} increased more than expected by dose proportionality: a twofold increase in dose led to a 2.62 [2.39 2.88] and 2.77 [2.62 293] increase in X003 C_{max} on Day 1 and Day 14, respectively.

AUC_{0-12h}: Individual and mean (SD) values of X003 AUC_{0-12h} on Day 14 are exemplarily graphically presented in *Fig. B.4 13*. As measured by ratio estimate and associated 90% CI, X003 AUC increased more than expected by dose proportionality (*Table B.4 9*): a twofold increase in

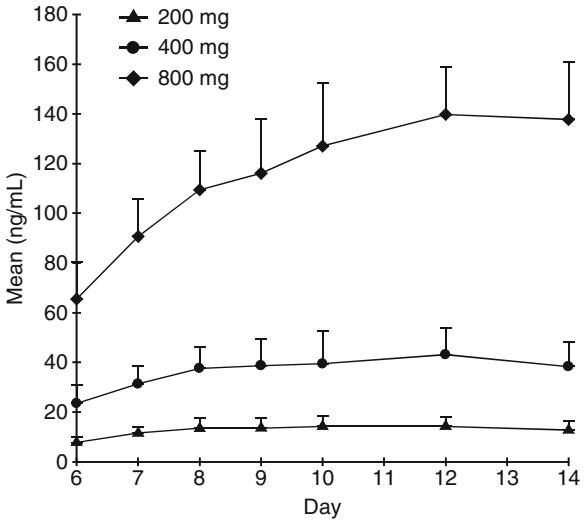

Figure B.4-12

Mean (SD) X003 trough concentrations from Day 6 to Day 14 during repeated BID oral administration of X003

Table B.4-7

Mean (CV%) X003 pharmacokinetic parameters observed after a single and repeated oral administration of X003 BID

PK Parameters	Day	200 mg N = 17	400 mg N = 16	800 mg N = 17
C_{max} (ng/mL)	1	23.1 (38)	67.2 (36)	162 (40)
	14	40.3 (30)	111 (17)	298 (13)
t_{max}[a] (h)	1	3 [2; 3]	3 [2; 5]	3 [2; 6]
	14	5 [2; 5]	5 [3; 6]	5 [2; 6]
AUC_{0-12h} (ng*h/mL)	1	111 (24)	310 (28)	846 (27)
	14	276 (23)	798 (19)	2,510 (12)
$t_{1/2z}$ (h)	1	9.81 (33)	17.6 (56)	19.6 (33)
	14	26.9 (32)	30.0 (29)	31.2 (32)
AUC (ng*h/mL)	1	160 (27)	474 (33)	1,310 (26)

[a] median value [Min; Max]

dose led to a 2.86 [2.67 3.06] and 3.06 [2.92 3.20] increase in X003 AUC on Day 1 and Day 14, respectively.

$t_{1/2z}$: The p value for the dose by day interaction was significant in the analysis of $\log(t_{1/2z})$ for X003 ($p \leq 0.001$). There was a dose effect at Day 1 and Day 14, and a Day effect for X003 at 200, 400, and 800 mg. From 200 to 800 mg, the mean $t_{1/2z}$ increased significantly from 9.8 to 19.6 h at Day 1 and from 26.9 to 31.2 h at Day 14 (*Table B.4 7*).

Table B.4-8

Accumulation ratio (Rac) with 95% CI for X003 C_{max} and AUC_{0-12h}

PK parameters	R_{ac} estimate	95% CI
C_{max} (ng/mL)	1.84	[1.65; 2.04]
AUC_{0-12h} (ng*h/mL)	2.72	[2.52; 2.94]

Table B.4-9

Ratio estimates and associated 90% CIs for dose proportionality assessed for a twofold increase in dose

PK Parameters	Day	Estimate	95% CI
C_{max} (ng/mL)	1	2.62	[2.39; 2.88]
C_{max} (ng/mL)	14	2.77	[2.62; 2.93]
AUC (ng/mL)	1	2.86	[2.67; 3.06]
AUC_{0-12h} (ng*h/mL)	14	3.06	[2.92; 3.20]

CRITICAL ASSESSMENT OF THE METHOD

The described evaluation provides confirmatory data on dose linearity/proportionality, which allows dose adjustment recommendations in the submission package of a drug. This type of study also supports bracketing approaches in bioequivalence studies in which different formulations and dose strengths are to be tested. In several cases it was accepted by authorities that bioequivalence for only the lowest and highest dose strength had to be demonstrated (US FDA 2008). Because of the three period crossover design the investigator should recruit approximately 50% additional subjects in order to have enough subjects completing all three periods and to guarantee appropriate PK evaluation. Overall, the crossover design is preferable for the assessment of dose linearity/proportionality because it minimizes the variability in PK parameters.

MODIFICATION OF THE METHOD

In case, steady state conditions are known, it might be sufficient to evaluate dose proportionality at steady state or alternatively single dose conditions only.

In some cases, the investigator might consider to evaluate dose proportionality also for major metabolites.

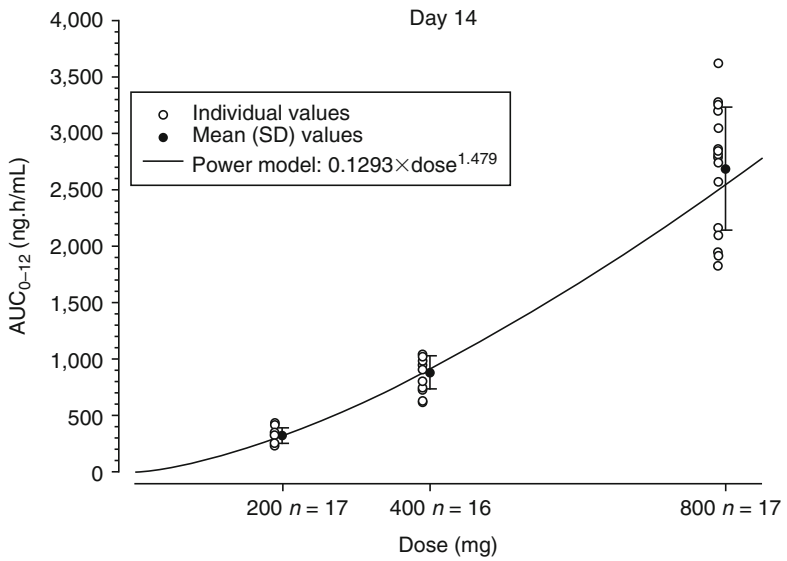

Figure B.4-13

Individual and Mean (SD) X003 AUC_{0-12h} values observed after repeated BID oral administration of X003

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B.5 Effects of Food Intake

Roland Wesch

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 and 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).

The effects of food intake include the physiological effects of food itself, as well as physicochemical interactions between food and the drug under investigation. For the latter, the categories of the biopharmaceutical classification system (BCS) become important (Amidon et al. 1995). For drugs belonging to BCS Class I (highly soluble, highly permeable) that rapidly dissolve from immediate release solid oral drug products, bioequivalence under fed conditions has been postulated (Yu et al. 2004). A comprehensive excellent review of the determinants of food effects on clinical pharmacokinetics can be found in Singh (1999).

PROCEDURE

The design of an exploratory food interaction bioavailability study with drug HMR123 is presented below (Part A). In this given project, the food interaction study was initiated in parallel to a human ADME 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. As the PK properties of the drug were not sufficient to support a BID dosing, modified release (MR) formulations were developed. The food effect of these MR formulations will be discussed in Part B.

B.5.1 Part A

B.5.1.1 Protocol Outline

Study of the effect of food on the pharmacokinetics of film coated tablets (3×200 mg) of HMR123 in healthy men.

B.5.1.1.1 Primary Objective

To assess the effect of food on the pharmacokinetics of 600 mg HMR123 in healthy men.

B.5.1.1.2 Study Design

An open, randomized, four period crossover study. There were four sequence groups of five 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.

B.5.1.1.3 Inclusion Criteria

Healthy men aged between 40 and 65 years. Body weights between −15% and +10% of the normal weight according to Broca.

B.5.1.1.4 Treatments

Treatment A: Single dose of 600 mg HMR123 on an empty stomach (overnight fasting) and start of high fat food intake 4 h later (reference).

Treatment B: Single dose of 600 mg HMR123 together with high fat food (drug administration 15 min after start of high fat food intake).

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.5,

Treatment C: Single dose of 600 mg HMR123 2 h after start of high fat food intake.

Treatment D: Single dose of 600 mg HMR123 on an empty stomach (overnight fasting) 1 h before start of high fat food intake.

B.5.1.1.5 Pharmacokinetic Data

Concentration of HMR123 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-inf}. The 90% confidence intervals of the point estimates of the ratio of C_{max} and AUC_{0-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. In this example it was 4 h. Single dose linearity/proportionality and unit strength will help to define the dose. In this example, dose proportionality has been demonstrated up to 700 mg, the unit strength of the tablets was 100 or 200 mg. Safety and tolerance data (maximum tolerated dose) will justify the dose. Taking all bits of information together, single doses of 600 mg were selected for this study.

The PK comparison in a food screen from a First in Man (FIM) study will influence the sample size.

It depends on the target indication, for example, acute or chronic use, and on the intended dosing regimen, for example, 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."

As the study participants serve as their own controls because of the crossover design, limitations might not be as strict as in the presented example. The BROCA index is only rarely applied now, and it has been replaced by the body mass index (BMI, body weight [kg] over height^2 [cm]). Moreover, if the drug under investigation does not exert any embryotoxic, teratogenic, or genotoxic effects, women of child bearing potential might be included.

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 h before the drug administration versus fasting overnight (at least 10 h) + at least 4 h after drug administration. At that time, a moderate fat/moderate calorie meal would be served.

MODIFICATIONS OF THE METHOD

There is a tendency to include a so called food screen already in the FIM study. The limitation of such an approach is the nonavailability 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 (see second part of this chapter for an example).

Only if the conditions for a waiver apply, a food interaction study is not needed for a submission package (FDA 2002). 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 AND FURTHER READING

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B.5.2 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.

B.5.2.1 Results – Pharmacokinetics

The HMR123 concentration time profiles show that food caused a delay in drug absorption and a lower peak concentration C_{max}. 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 B.5 1*.

A summary of the point estimates of the treatment ratios or of the difference between treatments for HMR123 is presented in *Table B.5 2*. The AUC_{0-inf} was similar for all treatments. When compared to treatment A, the AUC_{0-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 concentra tion. The relative bioavailability as presented by the AUC, 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.

B.5.3 Part B

PROCEDURE

As the PK properties of the drug were not sufficient to support a BID dosing, modified release (MR) formula tions were developed. The food effect of these MR formu lations will be discussed in Part B. Specific requirements for the clinical development of MR formulations have been issued by USA FDA (1, 2) and by EU CPMP (3, 4).

The design of an exploratory bioavailability study on modified release drug products is presented below. For the design of this study, information from a recent bioavail ability study with other modified release products, from a Site of Absorption study and from a Modeling and Simulation experiment was used.

B.5.3.1 Protocol Outline

Comparison of Pharmacokinetics and safety of Modified Release formulations of 600 mg HMR123 with that of an immediate release formulation A single center, open label, crossover study in healthy men.

Table B.5-1

Summary of the pharmacokinetic parameters in plasma

	PK parameter geometric mean (min; max)				
	C_{max}	t_{max}[a]	$t_{½}$	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)

[a]For t_{max} median+(min; max) are presented

Table B.5-2

Summary of the point estimates of the treatment ratios or of the difference between treatments

PK parameter	Treatment	Point estimate of treatment ratio[a]	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_{0-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}[a] (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

[a] Point estimate of the treatment difference (h) is presented for t_{max}

B.5.3.1.1 Primary Objective

To compare the PK characteristics of modified release (MR) formulations of HMR123 with the PK of an imme diate release (IR) formulation of HMR123.

B.5.3.1.2 Secondary Objective

To assess the influence of food on the PK of MR formula tions of HMR123. In this chapter that deals with the food effect, only the secondary objective will be considered.

B.5.3.1.3 Study Design

The study was carried out in a single center, open label, single dose, four period crossover study design with two independent treatment groups.

Single oral doses of 600 mg HMR123 were given under fasting and under non fasting conditions. The order of treatments and of fasting and non fasting conditions was randomized in a four way crossover incomplete block design (Treatment Groups I and II). The washout periods between the administrations of study medication were at least 48 h each.

B.5.3.1.4 Inclusion Criteria

Healthy men aged 18 55 years and assessed as healthy based on findings in medical history, physical examination, blood pressure, pulse rate, and electrocar diogram (ECG) during screening.

B.5.3.1.5 Treatments

Treatment Group I

Treatment A: 600 mg HMR123 (one film coated tablet containing 200 mg+one film coated tablet containing 400 mg given together) as IR formulation under non fasting (NF) conditions (reference).

Treatment B: 600 mg HMR123 in MR formulation (matrix tablet 1) under fasting (F) and NF conditions.

Treatment C: 600 mg HMR123 in MR formulation (bilayer tablet 1) under F and NF conditions.

Treatment Group II

Treatment A: 600 mg HMR123 (one film coated tablet containing 200 mg+one film coated tablet containing 400 mg given together) as IR formulation under non fasting (NF) conditions (reference).

Treatment D: 600 mg HMR123 in MR formulation (matrix tablet 2) under F and NF conditions.

Treatment E: 600 mg HMR123 in MR formulation (bilayer tablet 2) under F and NF conditions.

MR tablet formulation 1 contains hydroxypropyl methyl cellulose, MR tablet formulation 2 contains carrageenan.

B.5.3.1.6 Pharmacokinetic Data

Concentration of HMR123 in plasma before and at predefined times after dosing.

EVALUATION

Bioanalytical data: Individual plasma concentrations of HMR123 were tabulated together with standard descrip tive statistics for each treatment. Individual and median profiles were presented graphically.

PK data: PK parameters were determined based on plasma concentrations of HMR123 using non compartmental procedures.

Primary PK measure: $AUC_{0-inf.}$

Secondary PK measures: C_{12h}, AUC_{0-12h}, AUC_{0-t}, AUC_{ext} (%), C_{max}, t_{max}, MRT, $t_{1/2z}$, t_{lag}.

The primary measure was subject to an analysis of variance (ANOVA) including sequence, subject nested within sequence (subject [sequence]), period, and treat ment effects. According to the treatment groups and fasting conditions, there were five realizations of the var iable 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 calculated for the ratios fasting/non fasting for each MR formulation.

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 MR formulations, each tested under fasting and non fasting conditions, and compares the results to the IR drug product as the reference formulation under non fasting conditions in two separate study groups. The bilayer tablets combine an IR component and an MR 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.

MODIFICATIONS OF THE METHOD

Recommendations exist to conduct in vivo studies first in an animal species before going to man. The pig is the recommended species because of the closest similarity in terms of physiology of the gastrointestinal (GI) tract, transit, or residence times in the specific segments. This approach has limited validity because modified release is primarily defined by the absorption properties of a drug. Absorption is influenced by the general composition, the sums of the physico chemical properties, the length, and residence times of each section of the GI tract, and 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.

B.5.4 Example

To illustrate the amount of data that can be obtained using the discussed study type, an overview of the pharmacokinetic results obtained from the study described above under "PROCEDURE" is presented below.

B.5.4.1 Results – Pharmacokinetics

The secondary PK objective in this study was to assess the influence of food on the pharmacokinetics of two MR formulations with HPMC (matrix and bilayer tablets 1, Treatments B and C, respectively), and two MR formulations with carrageenan (matrix and bilayer tablets 2, Treatments D and E).

B.5.4.1.1 Effects of Food (Secondary PK Objective)

No effect of food was recorded on AUC_{0-inf} 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 and their C_{max} values were more affected. C_{12h} values presented food consumption effects for all formulations, however, to a larger extent for the HPMC formulation than for the carrageenan tablets.

B.5.4.2 Pharmacokinetic Measures and Parameters

The model independent pharmacokinetic characteristics for HMR123 following single dose administration of the different treatments were calculated using non compartmental procedures. *Table B.5 3* gives the arithmetic means, standard deviations, and coefficients of variation, as well as the medians and ranges of the primary pharmacokinetic measure AUC_{0-inf}, and of the secondary measures $C_{12\,h}$, C_{max}, and MRT.

Table B.5 4 gives further arithmetic mean PK measures and parameters of HMR123 following oral single dose administration of HMR123.

In *Figs. B.5 1* and *B.5 2*, semilogarithmic presentations of the plasma concentration time versus time profiles are given.

B.5.4.3 Statistical/Analytical Issues

The primary parameter AUC_{0-inf} 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 ANOVA was performed separately for subjects in Group I and subjects in Group II.

Table B.5-3

Arithmetic means and standard deviations (SD) of the primary pharmacokinetic measure $AUC_{0-\infty}$, and of the secondary measures $C_{12\ h}$, C_{max}, and MRT following single dose administration of the different treatments

Treatment		AUC_{0-inf} (ng*h/mL)	$C_{12\ h}$ (ng/mL)	C_{max} (ng/mL)	MRT (h)
Group I					
B(NF)	Arithmetic mean	7,126.00	149.81	1,343.34	6.65
	SD	2,220.73	61.62	650.08	1.31
B(F)	Arithmetic mean	5,396.57	167.41	660.95	8.27
	SD	1,469.50	95.17	205.40	1.99
C(NF)	Arithmetic mean	9,264.27	110.70	1,955.25	4.80
	SD	2,147.19	53.02	472.25	0.79
C(F)	Arithmetic mean	9,195.10	148.35	2,319.81	5.24
	SD	1,930.40	81.09	598.63	1.13
Group II					
D(NF)	Arithmetic mean	10,194.24	68.02	3,226.19	3.90
	SD	1,681.87	18.34	794.93	0.64
D(F)	Arithmetic mean	9,020.77	113.47	1,858.81	5.10
	SD	1,963.15	44.71	355.81	1.36
E(NF)	Arithmetic mean	10,244.97	62.47	3,340.25	3.55
	SD	2,021.91	23.64	902.63	0.48
E(F)	Arithmetic mean	10,634.66	106.72	2,747.50	4.10
	SD	1,506.00	67.24	315.39	0.77

Table B.5-4

Mean pharmacokinetic measures and parameters of HMR123 following oral single dose administration of HMR123

Treatment	$AUC_{0-12\ h}$ (ng*h/mL)	t_{max} (h)	$t_{1/2z}$ (h)	t_{lag} (h)
Treatment Group I				
B(NF)	6,309.06	3.31	3.66	0.20
B(F)	4,182.51	2.72	4.86	0.11
C(NF)	8,743.51	2.34	3.01	0.06
C(F)	8,303.25	0.98	4.24	0.02
Treatment Group II				
D(NF)	9,908.92	2.20	2.73	0.11
D(F)	8,282.20	2.06	4.11	0.08
E(NF)	9,991.45	2.09	2.76	0.09
E(F)	10,052.60	1.08	3.25	0.02

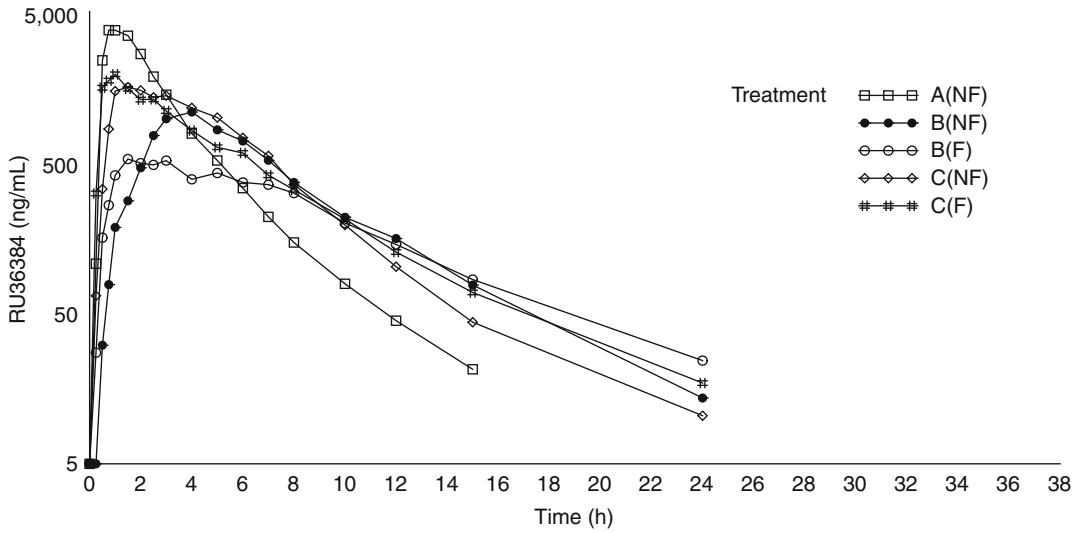

Figure B.5-1

HMR123 concentration in plasma versus time following treatments A(NF), B(NF), B(F), C(NF), and C(F) group I. Median plot log-linear scale

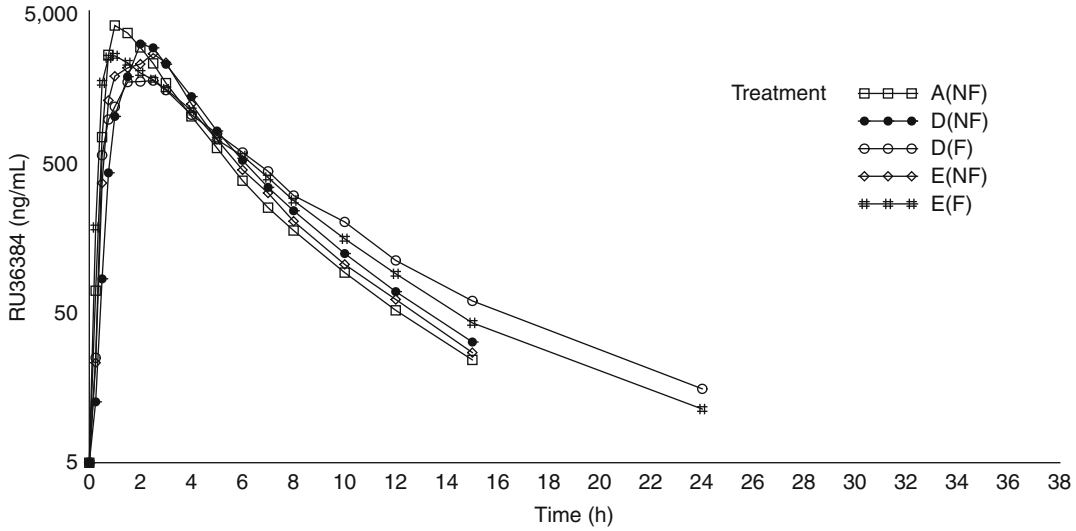

Figure B.5-2

HMR123 concentration in plasma versus time following treatments A(NF), D(NF), D(F), E(NF), and E(F) group II. Median plot log-linear scale

Point estimates and confidence intervals were calcu lated for the ratios fasting/non fasting for each MR formulation.

No significant effect of either period or sequence was found for the primary parameter AUC_{0-inf}, and for the secondary parameters C_{max} and $t_{1/2\lambda}$. Treatment effect was highly significant for all three PK parameters and subject effect was significant for AUC_{0-inf} and $t_{1/2\lambda}$ but not for C_{max}.

B.5.4.4 Hypothetical In Vivo Dissolution

Details on deconvolution and a critical assessment are presented in the chapter on formulations (pp. xxx).

REFERENCES AND FURTHER READING

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US FDA Guidance for Industry: Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlations. September 1997

US FDA Guidance for Industry: SUPAC-MR: Modified Release Solid Oral Dosage Forms. September 1997

B.6 Special Populations: Profiling the Effect of Obesity on Drug Disposition and Pharmacodynamics

James Gilmour Morrison

PURPOSE AND RATIONALE

Obesity is a global epidemic that is recognized by the World Health Organization (WHO) as the major health issue of the twenty first century, but is not yet recognized as a separate category of population for special consider ation by the FDA.

Excessive accumulation of body fat is associated with a number of chronic diseases including diabetes, cardio vascular diseases, and cancer and, as a result, can reduce life expectancy. Once considered a health issue of only wealthy nations, obesity is now more widespread and on the increase in poorer countries and younger people. A simple classification of overweight or obesity is the use of the body mass index or BMI, which takes a person's weight (in kilograms) and divides by the square of his or her height (in meters).

In contrast to elderly and pediatric populations, obe sity is still widely overlooked by drug developers. However, the physiological changes that occur in obesity can alter the metabolism and disposition of many drugs (Blouin et al. 1987; Blouin and Warren 1999; Cheymol 1993). For example, increases in the apparent volume of distribu tion and total body clearance have been reported for antibacterial agents (Dvorchik and Damphousse 2005; Bearden and Rodvold 2000).

Although much is known about obesity and its poten tial effects on drug disposition, surprisingly there is no universal dosing strategy for this population. In the age of individualized medicine, it is evident that one size fits all dosing is failing obese subjects, who are at risk of being under dosed. The best model to be used as a size descrip tor in obesity is still debated. Recent analysis suggests that dosing strategies based on lean body weight (LBW) is a better descriptor of the body composition for obesity than BMI or ideal body weight (IBW) (Han et al. 2007). Given the lack of clarity, it becomes even more impor tant to understand the profile of drug pharmacokinetics (PK) and pharmacodynamics (PD) in obese individuals and provide guidance in the labeled product. This is particularly important if the drug is being targeted for the comorbidities of overweight or obesity.

Regulatory guidance requires that the pharmacokinet ics 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 l7DA 1978). In some cases, an early assess ment of the drug in special populations can be helpful for future clinical development. European Agency for the Evaluation of Medicines Products (EMEA) (EMEA CPMPEWPl281196 1997) and US Federal Drug Adminis tration (FDA) guidances (US FDA 1996, 2002) recom mend that the pharmacokinetics of candidate weight control drugs is studied in obese subjects.

The availability of individuals who are obese (without complications) and are otherwise healthy, makes the early profiling of drug pharmacokinetics/pharmacodynamics in healthy obese subjects relatively straight forward. For example, an additional cohort of obese subjects could be considered in dose escalation studies when administer ing the drug to steady state. 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 devel oping drugs for metabolic diseases (e.g., obesity, diabetes), the advantage of an early switch to obese individuals is that one has a population closer to the final patient group than the healthy lean subject.

In late stage development, if population pharmacoki netic/pharmacodynamic analysis highlights a potential issue for development in obese or overweight subjects then a suitably powered special population study could be conducted at an appropriate therapeutic dose.

PROCEDURE

A typical exploratory study design for an early evaluation of the effects of obesity is presented for a highly lipo philic drug (XYZ1234). This study was designed to exam ine the pharmacokinetics parameters after repeated

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administration in a group of obese and lean subjects. The clinical doses proposed in this protocol were based on the maximal tolerated dose (MTD) after a single dose in lean healthy subjects of 0.6 mg. The first dose level proposed in the repeated administration to healthy lean subjects was 0.1 mg.

In view of the short half life of XYX1234 in lean sub jects of around 2 h at 0.2 mg, a BID regime was proposed.

B.6.1 Protocol/ Outline

A phase I, mono center, double blind, randomized, placebo controlled, sequential, repeated ascending oral dose.

B.6.2 Objectives

Primary**:** To assess the tolerability and the pharmacoki netic parameters of XYZ1234 after repeated dose admin istration in healthy lean male subjects and obese male subjects.
Secondary**:** To provide pharmacodynamic assessments of XYZ1234.

B.6.3 Study Design

In the healthy population, a total of 30 subjects, 6 subjects/ group, received XYZ1234 at dosages of 0.1, 0.2, 0.3, 0.4, or 0.5 mg, and 10 subjects received matched placebo (2 per dose group). Dosing duration was from Day 1 through Day 7 and the total duration of observation was from 2 to 5 weeks for subjects who completed the dosing regi men. In the obese population, a total of 12 subjects re ceived XYZ1234 at a dosage of 0.3 mg and 6 subjects received matching placebo. Dosing duration was from Day 1 through Day 21 and the total duration of observation was from 4 to 7 weeks for subjects who completed the dosing regimen.

Both the healthy lean and obese male populations were aged between 18 and 40 years. Obese male subjects were defined as individuals with a body mass index between $\geq$30 and $\leq$40. Inclusion criteria for healthy and obese sub jects included laboratory parameters within the following limits: hemoglobin ($\geq$11 g/dL), cholesterolemia ($\leq$3 g/L), triglyceridemia ($\leq$5 g/L), and fasting glycemia ($\leq$1.6 g/L).

Each subject received two capsules twice a day with 200 mL of non carbonated water: 7 a.m. in fasting condi tions and at 7 p.m. 2 h before dinner. On the last day of dosing subjects received two capsules in the morning only in fasting conditions.

EVALUATION

B.6.4 Pharmacokinetics

PK parameters were generated using non compartmental techniques (WinNonlin [Pharsight Corp.]) as follows:

- On Day 1
  - Maximum observed plasma concentration (C_{max}) and the time of its occurrence (t_{max})
  - Area under the plasma concentration versus time curve calculated using the trapezoidal method from time zero to the real time 12 h ($AUC_{0–12}$), or up to the last measurable sampling time if less than 12 h (AUC_{last})
- On Day 7 (lean) or 21 (obese)
  - C_{max} and t_{max}
  - $AUC_{0–12}$ or AUC_{last}
  - Terminal half life ($t_{1/2z}$) associated with the termi nal slope (z) determined according to the follow ing equation: $t_{1/2z} = 0.693/\lambda z$ where λz is the slope of the regression line of the terminal phase of the plasma concentration versus time curve. The half life was calculated by taking the regression of at least three points
  - Accumulation ratio based on area ($AUC_{0–12}$ Day 7/ $AUC_{0–12}$ Day 1)(R_{ac}).
  - Accumulation ratio based on C_{max} (C_{max} Day 7/ C_{max} Day 1), (R_{Cmax})
  - Pre dose (C_{trough}) concentrations on days 2, 3, 4, 5, 6, and 7; (and daily up to Day 21 in obese)
  - The time to achieve steady state determined using these trough levels
  - Clearance (CL_{ss}/F) and apparent volume (V_z/F), where possible

Descriptive statistics were calculated for each parameter.

Standard statistical tests for dose escalation trials were conducted but this analysis is not described here as it is covered extensively in other sections. However, for the comparison between obese and lean groups, an ANOVA was conducted on absolute and weight adjusted PK parameters.

B.6.5 Pharmacodynamics

As the main target indication was for the treatment of obesity, the primary pharmacodynamic evaluations were based on hunger satiety and nausea feelings esti mated using visual analogue scales (VAS). Secondary

pharmacodynamic endpoints related to pharmacological action of the drug that were evaluated, including gallbladder volumes assessed by ultrasonography, leptin, vasopressin levels in plasma, water imbalance estimated by the quantity of water intake and diuresis, and weight.

The descriptive statistics of the pharmacodynamic endpoints were summarized in tabular and graphical forms. Tests of treatment effect were also performed using a linear mixed effects model for repeated measures with fixed terms for treatment, time, and treatment by time effects and a random term for subject.

CRITICAL ASSESSMENT OF THE METHOD

The type of study described in this section broadly characterizes the impact of obesity on the disposition of the developmental drug, but assumes linear kinetics in obese subjects. A more comprehensive method may have been to complete a full dose range in obese subjects, particularly given that the compound had a poor exposure versus dose relationship.

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 exploratory study was specifically designed to evaluate any gross pharmacokinetic differences in obese versus lean subjects without being strictly powered for bioequivalence. Typically, this type of evaluation would be extended during the later clinical development by performing a population pharmacokinetic/pharmacodynamic assessment of the impact of weight on the disposition of the developmental drug during the phase IIb/III studies.

A suitably powered clinical study (special population study) would only be performed if such population PK/PD assessments raised significant differences and questions on lean versus obese subjects.

B.6.6 Example

To illustrate the type of data that can be obtained from healthy versus obese studies, a high level summary of the pharmacokinetic data relevant to this section is presented below.

B.6.6.1 Pharmacokinetics

B.6.6.1.1 Healthy Lean Subject Population

For illustration purposes, only data for the lean verus obese group at a common dose are presented in *Table B.6 1*. However, the pharmacokinetics results are described hereafter. Following twice daily oral dosing (0.1 0.5 mg b.i.d.) to healthy male subjects, XYZ1234 reached maximum observed plasma concentration (C_{max}) at approximately 4 h at steady state (*Fig. B.6 1*). Peak plasma

Table B.6-1

Mean pharmacokinetics of XYZ1234 in obese subjects versus matched lean subjects

Obese					Lean					
Parameters	*n*	Mean	SD	cv%	*n*	Mean	SD	cv%	t-test P value	ANOVA P value
$t_{1/2}$ (h)	11	3.52	4.38	124	6	12.2	21.7	177	NC	NC
T_{max} (h)	11	3.00	0.775	25.8	6	3.58	1.80	50.2	NC	NC
C_{max} (ng/mL)	11	0.777	0.271	34.9	6	0.977	0.315	32.2	0.190	0.200
AUC_{0-12} (h × ng/mL)	11	4.20	1.76	41.9	6	5.68	2.72	47.9	0.193	0.479
V_z (L/kg)	11	345	262	75.8	6	739	1,130	151	0.264	0.212
CL_{ss} (L/h/kg)	11	82.1	3.10	37.8	6	63.4	2.79	43.5	0.239	0.200
Weight normalized data (TBW)										
V_z (L/kg)	11	3.41	2.68	78.3	6	11.0	16.7	152	0.150	<0.001
CL_{ss} (L/h/kg)	11	0.802	0.282	35.2	6	0.947	0.444	46.9	0.419	0.004

Obese PK population $n = 11$, one subject excluded as suspected noncompliance (all levels <LLOQ).

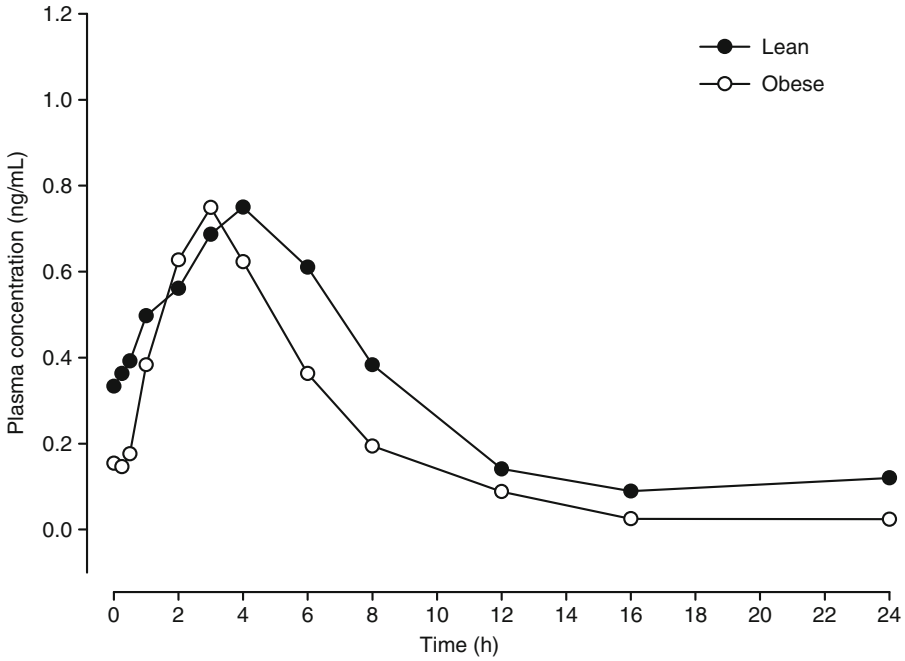

Figure B.6-1

concentrations displayed little or no increase with dose on Day 1 and a less than dose proportional increase on Day 7. Similarly, area under the plasma concentration versus time curve from time 0 to 12 h ($AUC_{0–12}$) increased in a less than dose proportional manner on both days (data not shown). Steady state plasma concentrations observed during repeated dosing (C_{trough}) was reached by Day 4 and associated with an approximate threefold rise in steady state exposure (C_{max} and $AUC_{0–12}$) compared to Day 1. Where assessable, the terminal elimination half life at steady state was generally around 12 h, although a secondary absorption was observed in some profiles.

B.6.6.1.2 Obese Subject Population

At a comparative dose (0.3 mg b.i.d.), there was no marked differences observed in the pharmacokinetics of XYZ1234 in obese compared to lean subjects following single dos ing, although, at steady state (Day 7 versus Day 21), there was a trend to a slightly higher C_{max} and $AUC_{0–12}$ in healthy lean subjects compared with obese (up to 35%). There was an apparent shorter half life in obese subjects (approximately 4 h compared to 12 h in healthy). Statis tically significant differences in PK parameters were only observed for CL_{ss}/F and V_z/F when normalized for total body weight.

B.6.6.2 Pharmacodynamics

B.6.6.2.1 Healthy Subject Population

A full statistical analysis of pharmacodynamic data was not conducted as the compound was terminated in devel opment. However, overall, no dose effect was observed for pharmacodynamic parameters evaluated in the study consistent with the poor exposure versus dose relation ship. Nausea VAS correlated moderately with the tolera bility of the product and no effects on hunger and satiety were observed. With regard to ultrasonography, signifi cant gallbladder contraction was observed at all dose levels versus the placebo. When changes from baseline were analyzed, significant decreases in body weight were observed from 0.2 mg b.i.d. XYZ1234 and higher versus placebo, but without dose effect. No effects on leptin and vasopressin or water balance assessments were observed.

B.6.6.2.2 Obese Subject Population

Nausea VAS correlated moderately with the tolerability of the product and no effects on satiety were observed. A significant decrease in hunger was observed versus pla cebo on Day 21. With regard to ultrasonography, signifi cant gallbladder contraction was observed for subjects

administered 0.3 mg b.i.d. XYZ1234 versus placebo on Day 21, and significant decreases in body weight were observed from Day 5 onward for subjects receiving XYZ1234. No effects on leptin and vasopressin or water balance assessments were observed.

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B.7 Special Populations: Renal Impairment

Gerard Sanderink · Gareth Shackleton · Jochen Maas · James Gilmour Morrison

PURPOSE AND RATIONALE

Kidney and liver are the main organs involved in the elim ination of drugs. Both have a metabolic and a direct excre tory capacity, although the first is predominant for drugs eliminated by the liver, while the most frequent mechanism of renal clearance is direct excretion of the unchanged drug or its circulating metabolites. In general, the elimination capacity of the kidney is lower than that of the liver, because of the smaller size and associated blood flow. Renal excre tion can be limited by the glomerular filtration rate (GFR) in case of passive excretion or by the transporter capacity of the total renal blood flow in case of active secretion.

Impaired renal function is a rather common condi tion, with an estimated 26 million people in the USA having chronic kidney damage (Coresh et al. 2007). Kidney func tion is well known to decrease with age, and in our expe rience mild or moderate renal impairment is the norm in study populations above 75 years.

It is therefore necessary to evaluate the potential impact of changes in renal clearance on drug pharma cokinetics in many cases, not only for prescribing infor mation, including dose adjustment, but also in order to conduct a clinical development program in large patient populations with adequate exclusion criteria guarantee ing safe drug administration.

The main rationale to evaluate the effect of renal impair ment is when a drug or its active metabolites are mainly eliminated by renal excretion, especially when a drug is intended to be given in patients with decreased kidney function like the elderly and has a narrow therapeutic mar gin that may warrant dose adjustment. However, impaired renal function can also affect nonrenal drug elimination, for instance, through decreased drug plasma protein binding because of low albumin levels. This is of relevance for drugs with high plasma protein binding (>90%) and that have a high hepatic extraction ratio (>0.7) (see also Chapter on protein binding considerations). Another indirect mecha nism is the inhibition of enzyme or transporter activity by uremic plasma.

For most drugs, the evaluation will focus on the effect of decreased glomerular filtration, but it should be kept in mind that in case of active renal secretion there is also a potential for drug drug interactions with transporter inhibitors like cimetidine (OCT transporters) or proben ecid (OAT and OATP transporters). Finally, the possibility that a drug is eliminated during hemodialysis is also to be considered.

Altogether, a study on the effect of renal impairment on drug pharmacokinetics may be warranted in many cases, and a FDA survey has shown that 71% of NDAs submitted during the period 2003 2007 for oral drugs included such a study, with only one third of them be cause of a predominant renal elimination pathway (Huang 2008).

Both the FDA and the EMEA guidances on renal imp airment (US FDA Guidance for Industry 1998; EMEA CHMP 2004) outline different approaches to study the effect of renal impairment on drug elimination. One approach is a population PK screen in large scale clinical trials, which allows to compare patients with reduced renal function with the typical patient for a given indica tion. Some limitations of this approach are that it does not address patients who are voluntary excluded from such studies, which is generally the case for severe renal impair ment. Also, some specific parameters like unbound drug fraction and circulating metabolites need to be included in the evaluation, and the sample size should provide suffi cient sensitivity. However, the approach may be very use ful to confirm the absence of a risk of renal impairment, especially when it is not easily feasible to conduct a specific study in renally impaired subjects without the clinical indication, like for instance anticancer drugs.

When a specific renal impairment study is conducted, this can be done according to a "full" design or according to a "reduced" or "staged" design. In the first case, all degrees of renal impairment are included in the study. In the reduced design, the effect of severe renal impairment in comparison to normal renal function is first evaluated. If there is no relevant effect, the information is complete. If not, the study can be completed with subjects with mild and moderate renal impairment. Of course, this approach is only of interest if no relevant effect is expected.

For a renal impairment study, subjects are classified according to their glomerular filtration rate (GFR) which is considered the best indicator of overall kidney function (NKG guideline). The current classification is as follows:

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.7,

Normal: GFR >80 mL/min
Mild impairment: 50 80 mL/min
Moderate impairment: 30 50 mL/min
Severe impairment: <30 mL/min

End stage renal disease patients (<15 mL/min) requiring hemodialysis are considered a separate group. With the more recent MDRD (modification of diet in renal disease) formula (see below) it has been proposed to change the limits for moderate impairment to 60 mL/min and for mild impairment to 90 mL/min.

Ideally, the GFR should be measured with a marker that has no other elimination pathways (tubular secretion, extrarenal) and does not undergo reabsorption and can easily be measured. The gold standards are exogenously administered inulin, iohexol, or ^{51}Cr EDTA with analysis being performed in both serum and urine. However, in general, the endogenous marker creatinine is used, and in routine clinical practice mostly by estimation of the renal function calculated from the serum creatinine (SCr) con centration. The most frequently used formula was establi shed by Cockroft and Gault (1976), based on a correlation analysis between SCr and actual creatinine clearance mea sured from serum and urine creatinine concentrations. This correction is necessary because SCr depends not only on renal excretion, but also on muscle mass and food intake:

$$\mathrm{GFR(mL/min)} = (140 - \mathrm{age})\ \mathrm{weight}/72\mathrm{SCr}\ (0.85\ \text{if female})$$

More recently, a more accurate formula has been proposed by the US National Kidney Foundation (1997) and a FDA advisory committee. The so called MDRD was established in a larger population and takes into account additional covariates. It is standardized on body surface area and the abbreviated formula (not taking into account serum albu min and urea) is (Levey et al. 2006):

$$\mathrm{GFR(mL/min/1.73m^2)} = 186(\mathrm{SCr})^{-1.154}(\mathrm{Age})^{-0.203}\ (0.742\ \text{if female})\ (1.210\ \text{if Black})$$

Both equations have limitations in patients with low mus cle mass or with vegetarian diets, are only to be used in adults, and can be biased by the fact that creatinine is also an OATPB1 transporter substrate.

PROCEDURE

B.7.1 Protocol

The pharmacokinetics of the low molecular weight hepa rin, enoxaparin, were evaluated in 12 healthy volunteers and 36 patients with mild, moderate, or severe renal imp airment (Sanderink et al. 2002). This open label, multi center, parallel group study was conducted at four centers. A total of 48 volunteers were enrolled in the study, 36 of whom had either mild, moderate, or severe renal impair ment according to the FDA and EMEA classifications (12 in each group) and 12 of whom were healthy volun teers, selected to match the overall age, weight, and gender distribution of the subjects with renal impairment. Sub jects were initially selected based on their creatinine clear ance calculated by the Cockroft Gault formula, which was then established more accurately during the study by collecting 24 h urine on day 4. Serum and urine creatinine levels were assessed, and creatinine clearance was esti mated using the following formula:

$$\frac{\text{Urine creatinine (mg/dL)} \times \text{24 h urine volume (mL)}/1,440\ \text{min}}{\sum[\text{SCr from days 1, 4, and 5 (mg/dL)}]/3}$$

In addition, eligible subjects were required to meet the following inclusion criteria: to be 18−75 years old, to have a body mass index (BMI) of 18−30 kg/m^2, and to have laboratory coagulation parameters within normal ranges.

Enoxaparin was administered once daily by subcuta neous injections at a dose of 40 mg for 4 days when the drug plasma exposure was anticipated to be at steady state.

B.7.2 Evaluation

Venous blood samples were taken over a 5 day period to determine anti factor Xa and anti factor IIa activities and the activated partial thromboplastin time. The schedule for blood sampling was as follows: day 1: pre dose, 30 min, 1, 1.5, 2, 3, 4, 6, 9, 12, and 16 h post dose; day 2: pre dose (i.e., 24 h post dose 1) and 3 h post dose 2; day 3: pre dose and 3 h post dose 3; day 4: pre dose, and 30 min, 1, 1.5, 2, 3, 4, 6, 9, 12, and 16 h post dose; day 5: 24, 30, and 36 h after dose 4.

Pharmacokinetic analysis was performed by a non compartmental approach with a full evaluation on day 1 after the first dose, and on day 4 the maximum observed activity (A_{max}), the time of maximum observed activity (T_{max}), the area under the plasma activity time curve extrapolated to infinity (AUC, only day 1), the area under the 24 h plasma activity time curve ($AUC_{(0-24)}$), the apparent terminal elimination half life ($t_{1/2\lambda z}$), and the apparent total body clearance (CL/F) were analyzed.

The differences between healthy volunteers and the three groups of patients with renal impairment were assessed on the logarithmic transformations of A_{max}, $AUC_{(0-24)}$, AUC, and CL/F for anti Xa activity, using

90% confidence intervals (CI) for log transformed ratios in pharmacokinetic parameters of patients with renal impairment versus healthy volunteers.

The relationship between total clearance and creatinine clearance was assessed using correlation and regression procedures.

The steady state was established by comparing values on days 2, 3, 4, and 5 for minimum observed activity (A_{min}) or A_{max}.

B.7.3 Critical Analysis

The study design is in agreement with the regulatory guidance, especially for the definition of the groups of renal impairment. A repeat dose regimen was chosen, because previous data had shown that steady state exposure was not well predicted by single dose pharmacokinetics. The 4 day duration was longer than necessary to achieve the steady state in patients with normal renal function, because it was anticipated that a prolongation of half life would occur in renal impairment, with a consequently longer time to reach steady state in these subjects. Nevertheless, the pharmacokinetics were also fully evaluated after the first dose in order to provide information for possible dose adjustment regimens. In contrast, the free fraction was not evaluated, because low molecular weight heparins act by binding to antithrombin III in plasma, and nonspecific binding is known to be low for LMWH. For an application of free drug evaluation in a renal impairment study, see the chapter on protein binding.

B.7.4 Modifications

A full design was implemented for this study, because an effect of renal impairment on enoxaparin was expected. It allowed to select a control group that matched the overall demographic distribution of the patients, which can be more difficult with a staged design, mainly because subjects with severe renal impairment tend to be younger than the other groups. In this study, the mean(+SD) of the severe renal impairment group was 49.2 ± 15.2 years versus 63.9 ± 7.4 and 62.4 ± 13.3 years in the mild and moderate groups, respectively. In the control group, the mean was 57.2 ± 14.3 years. Of notice, there was no individual subject by subject matching which would have needed 36 healthy volunteers instead of 12. This was given the fact that the means of the groups were to be compared, and not by a paired test. Interestingly, more healthy volunteers would not have increased the power of the study. The number of subjects of 12 per group was expected to allow to detect clinically meaningful differences. It is generally not possible to target a bioequivalence approach in special populations and many studies include only eight or even six subjects per group.

The dose regimen was the same in all groups, 40 mg once daily which is the prophylactic regimen, because higher doses of the drug were known to be well tolerated. This would not have been possible with the dose regimen for the treatment of deep venous thrombosis (1 mg/kg bid). For an investigational drug, it may sometimes be necessary to give a reduced dose, especially in the severely impaired group, for instance when the therapeutic margin is low or still unknown or the effect of renal function difficult to anticipate. In such cases, it is also possible to include the mild, moderate, and severe groups sequentially in order to select an appropriate safe dose for each group based on the finding in the previous group. An advantage can also be that it allows to compare directly the adjusted dose regimen to the reference regimen. But in any case, extrapolation to other doses remains problematic in case of non dose proportional pharmacokinetics. For a drug with dose and time independent pharmacokinetics, a single dose administration can be selected. An accurate evaluation of elimination half life and AUC_{inf} is in this case mandatory.

EXAMPLE

B.7.5 PK Study

The plasma pharmacokinetic profile on day 4 of the study described above is shown in *Fig. B.7 1* and the associated pharmacokinetic parameters in *Table B.7 1*.

The rate of absorption was not different among groups, as shown by a similar T_{max} (4 h on day 1 and 3 4 h on day 4). A_{max} was relatively high in the group with mild renal impairment on both days 1 and 4. Because of slower elimination, pre dose trough values on day 4 were increased with the degree of renal impairment, and A_{max} values in RI subjects became significantly increased after repeated dosing.

Exposure on day 4 was quite well predicted by day 1 pharmacokinetics in healthy volunteers (+10% accumulation). The accumulation in patients with severe renal impairment was 29%, suggesting some underestimation of AUC_{inf} and half life on day 1 in the groups with longer half lives because of a too short sampling duration (<24 h).

The mean log transformed anti Xa $AUC_{(0–24)}$ was 20% and 21% higher in mildly and moderately renally

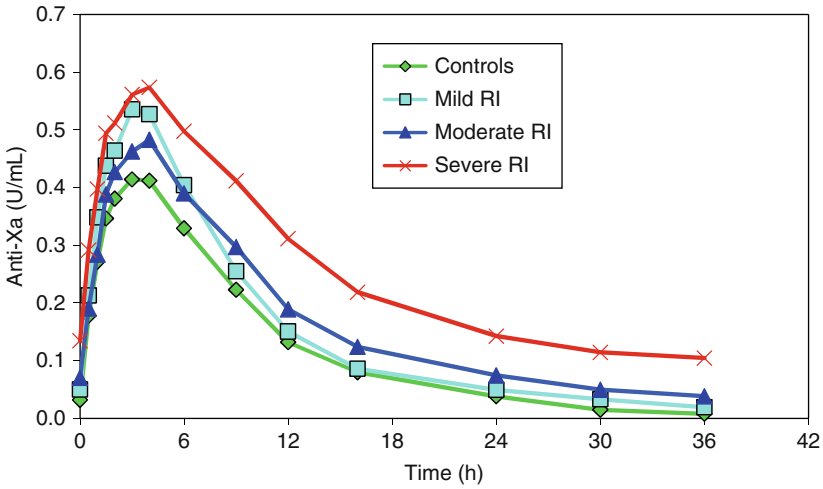

Figure B.7-1

Enoxaparin plasma anti-Xa pharmacokinetics on day 4 in renally impaired and normal subjects after 40 mg od dosing

Table B.7-1

Enoxaparin plasma anti-Xa pharmacokinetics parameters at steady state in renally impaired and normal subjects after 40 mg od dosing

	Controls (n = 12)	Mild RI (n = 12)		Moderate RI (n = 12)		Severe RI (n = 12)	
CrCL (mL/min) Mean ± SD range	120.7 ± 11.3 84 217	66.4 ± 2.8 51 81		38.5 ± 1.4 30 46		19.3 ± 2.0 5 28	
PK parameter	Mean (CV%)	Mean (CV%)	Ratio (90% CI) p-value	Mean (CV%)	Ratio (90% CI) p-value	Mean (CV%)	Ratio (90% CI) p value
$AUC_{(0-24)}$ (h IU/mL)	4.31 (26)	5.20 (32)	120.3 (99.8 145.0) 0.1035	5.53 (22)	120.9 (100.2 146.0) 0.0971	7.88 (36)	165.2 (136.8 199.4) 0.0001
A_{max} (IU/mL)	0.421 (26)	0.562 (29)	134.6 (111.9 161.9) 0.0088	0.497 (20)	111.7 (92.8 134.6) 0.3244	0.584 (30)	127.3 (105.6 153.3) 0.0341
A_{min} (IU/mL)*	0.036 (< 0.048)	0.046 (< 0.096)	 0.0225	0.071 (0.043 0.111)	 0.0001	0.132 (0.057 0.245)	 0.0001
T_{max} (h)*	3.0 (2.0 4.0)	3.0 (1.5 4.0)	 0.9029	4.0 (2.0 4.0)	 0.4373	4.0 (1.5 4.0)	 0.3725
$t_{1/2\lambda z}$ (h)*	6.87 (3.97 13.2)	9.94 (3.67 20.2)	 0.0117	11.3 (5.53 20.0)	 0.0011	15.9 (9.66 23.0)	 0.0001
CL/F (L/h)	0.98 (25)	0.87 (41)	83.1 (68.7 100.5) 0.1083	0.76 (22)	83.0 (68.5 100.5) 0.1093	0.58 (44)	60.8 (50.2 73.6) 0.0001

*, Median and range; , not calculated; <, below quantitation limit; **, exp(ln ratio)%; 90% CI, 90% confidence interval

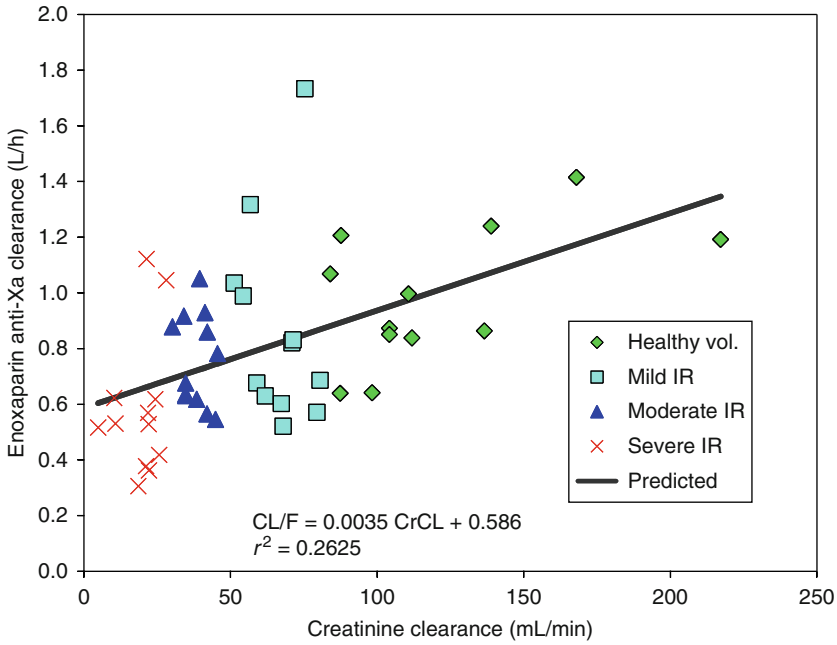

Figure B.7-2

Correlation between creatinine clearance and enoxaparin anti-Xa plasma clearance

impaired subjects than in normal volunteers (NS). In severe RI patients, mean $AUC_{(0-24)}$ was on average 65% higher on day 4 ($p < 0.001$).

The median apparent elimination half life increased significantly with the degree of renal impairment (6.87, 9.94, 11.3, 15.9 h).

On day 1, CL/F was similar between healthy volunteers and subjects with mild or moderate renal impairment (1.00, 0.99, and 0.90 L/h), but there was a statistically significant 27% difference between healthy volunteers and severe RI (0.73 L/h). In the absence of accumulation, apparent plasma clearance was similar on days 4 and 1 in healthy volunteers. In the other groups, estimations of CL/F tended to decrease over time. Differences across groups were therefore more pronounced on day 4: 17% in mild and moderate RI and up to 39% in severe RI based on mean log transformed values.

On day 4, anti Xa CL/F was linearly, although quite poorly, correlated with the degree of the renal impairment ($p = 0.0002$, $r^2 = 0.2625$). A more sigmoid relationship might have been more appropriate (*Fig. B.7 2*).

B.7.6 Population PK Screen

The effect of renal impairment was also evaluated in a population PK study at a higher dose (Bruno et al. 2003). Study TIMI 11A was a multicenter trial of the safety and tolerability of two doses of enoxaparin in patients with unstable angina and non Q wave myocardial infarction. The study population consisted of 630 patients with unstable angina or non Q wave myocardial infarction. The only exclusion criterion relevant for the analysis was creatinine ≥2.0 mg/dL. The median calculated creatinine clearance was 85.7 mL/min (5th 95th percentiles 41.2 152 mL/min). Fifty one patients had moderate renal imp airment (<50 mL/min), but only four had severe renal impairment (<30 mL/min). The effect of renal impairment was consistent with the PK trial (*Fig. B.7 3*).

B.7.7 Dose Adjustment

Although the number of subjects in each group was rather low in the pharmacokinetic study, the effect of renal impair ment was quite well estimated as confirmed by the popu lation PK study and the residual variability in each group was low. This is the best case to implement a dose adjust ment rather than individual monitoring and dose adapta tion. For a low clearance drug like enoxaparin, a decrease in clearance mainly translates into a prolongation of half life, so dose adjustment can be accomplished by a reduction in dose strength or prolongation of dosing interval. In the prophylactic setting and depending on the indication and country, enoxaparin is approved with a 40 mg once daily or a 30 mg twice daily regimen. The data from the PK study

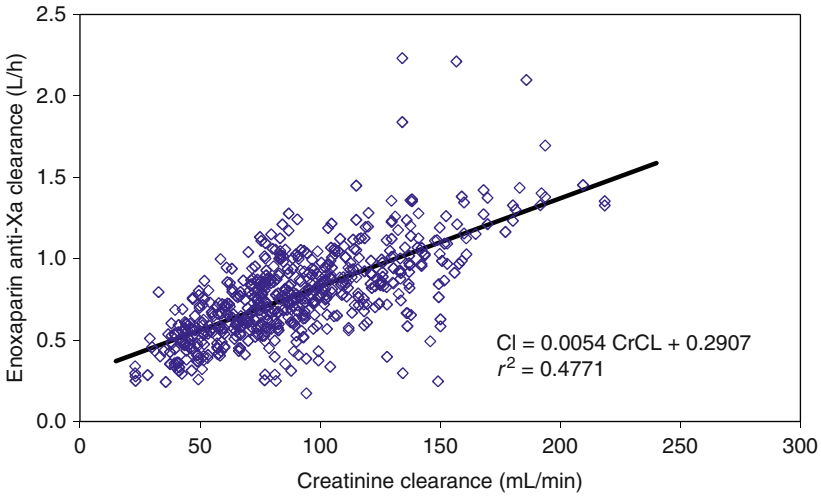

Figure B.7-3

Effect of renal function on enoxaparin anti-Xa plasma clearance in study TIMI-11A

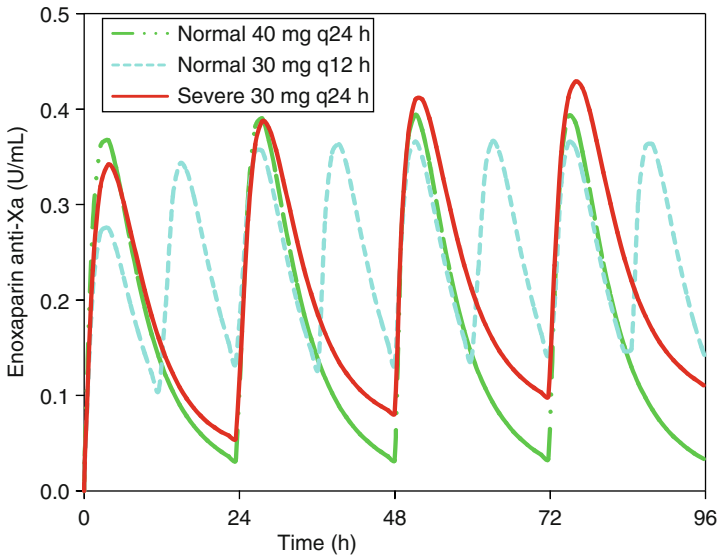

Figure B.7-4

Comparison of once-daily 30 mg enoxaparin dose regimen in severe renally impaired patients with standard prophylactic regimens in normal

were used to simulate different adjusted dose regimens in severe renal impairment, and it was concluded that 30 mg once daily would lead to early and steady state exposure parameters (A_{max}, A_{min}, and $AUC_{(0–24)}$) similar to that in subjects with normal renal function treated with either one of the standard regimens (*Fig. B.7 4*).

This dose adjustment recommendation is now part of the Lovenox®/Clexane® labeling in several countries, among them the USA (US FDA 2007). Similarly, a 1 mg/kg once daily dosing regimen is recommended for treatment of DVT in severe renally impaired patients, instead of 1 mg/kg bid or 1.5 mg/kg od.

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B.8 Special Populations: Hepatic Impairment

Gerard Sanderink

PURPOSE AND RATIONALE

Liver and kidney are the main organs involved in the elimination of drugs. Both have a metabolic and a direct excretory capacity, but liver is generally the main organ responsible for drug metabolism and also metabolite excretion. Direct biliary excretion occurs also, but is sometimes a futile pathway because of enterohepatic cycling (reabsorption). Because of a variety of processes involved in drug elimination by the liver, liver disease can affect the pharmacokinetics by several mechanisms, for instance, reduced metabolic enzyme activity (Frye et al. 2006), altered hepatic uptake or biliary excretion by transporters and more generally reduced liver blood flow (Verbeeck 2008). Major plasma proteins are synthesized by the liver and drug pharmacokinetics can be affected by decreased plasma protein binding. Liver disease can also affect other organs such as the kidney.

Both the FDA (2003) and EMEA CHMP (2005) have issued quite similar guidances for studies on the effect of hepatic impairment. For a new drug candidate the effect should be studied if the drug is likely to be administered in patients with hepatic impairment, if the condition is likely to affect the PK of the drug and if in that case it would be necessary to proceed with a dose adjustment in such patients. The FDA recommends a study to be performed if the liver contributes for more than 20% in the elimination of the parent drug or an active metabolite and for any drug with a narrow therapeutic range. An exception is made for drugs intended for single dose administration.

Moreover, for drugs with a high hepatic extraction ratio (>70%) and high plasma protein binding (>90%) the unbound drug pharmacokinetics should also be evaluated. In some cases the total drug concentration can mask an effect on the unbound drug concentration (see also chapter on protein binding considerations).

In contrast to renal impairment, and maybe because there are several mechanisms potentially involved in the effect of liver dysfunction on drug pharmacokinetics, it has proven difficult to find an adequate marker to classify liver disease with a good predictive value. Both guidances recommend the Child Pugh score as the preferred classification system, acknowledging its limitations and the need for further exploration. The Child Pugh score is a composite of five parameters, three biochemical (serum albumin, serum bilirubin, prothrombin time) and two clinical (encephalopathy and ascites) (*Table B.8 1*). The classification is A or mild hepatic impairment for a score of 5 6 points, B or moderate for a score of 7 9 and C or severe for 10 and more.

It is important to apply this score only for patients diagnosed with liver disease, because a score of 5 is the minimum value, even in the absence of any abnormalities. On the other hand, some parameters can also be changed in other clinical conditions. Even in patients with liver disease, the underlying condition can also affect the pharmacokinetics of drugs differently (e.g., alcoholic cirrhosis, cholestasis, viral hepatitis, liver metastases).

There are different approaches to study the effect of hepatic impairment on drug elimination. One approach is a population PK screen in large scale clinical trials, which allows to compare patients with liver disease with the typical patient population in the proposed indication. Some limitations of this approach are that it does not address patients that are voluntary excluded from such studies, which is generally the case for severe and sometimes also moderate hepatic impairment. Also, some specific parameters like unbound drug fraction and circulating metabolites may need to be included in the evaluation and the sample size should provide sufficient sensitivity. However, the approach may be very useful to confirm the absence of a risk of liver impairment, or to investigate the effect when it is not feasible to conduct a specific study in subjects without potential clinical benefit, for instance anticancer drugs (Bruno et al. 1998). In case of a risk, an alternative approach can be a dose escalation study in patients with liver dysfunction (Raymond et al. 2002).

When a specific hepatic impairment study is conducted this can be done according to a full design or a reduced design. In the first case all relevant degrees of hepatic impairment are included in the study. In the reduced design only the worst case is initially evaluated in comparison to normal subjects. If necessary, the study can be completed

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Table B.8-1

Child-Pugh score used for classification of liver disease

Parameter	Points		
	1	2	3
Serum albumin (g/dL)	>3.5	2.8 3.5	<2.8
Serum bilirubin (mg/dL)	<2	2 3	>3
Prothrombin time (*s* > control	<4	4 6	>6
Encephalopathy (grade)	None	1 or 2	3 or 4
Ascites	Absent	Slight	Moderate

with subjects from the intermediate group(s). In both approaches, the effect of severe hepatic impairment is often not performed, and thus moderate impairment is the worst case. Severe hepatic impairment then generally constitutes automatically a contra indication in the label ing. If there is an effect of moderate impairment and the mild condition has not been investigated, any restriction applicable to the first class can also be extended to the second.

PROCEDURE

B.8.1 Protocol

The antihistamine ebastine was a drug with a high first pass effect after oral dosing and the main circulating active metabolite, carebastine, was also partly eliminated by the liver. In addition, the metabolite was >99% bound to plasma proteins in normal subjects.

The effect of hepatic impairment on the pharmacoki netics of ebastine was evaluated in an open label, multi centre, parallel group study conducted at three centers (Lasseter 2004). Subjects were classified according to their Child Pugh score. Indocyanine clearance was also determined as a liver function test. Eight subjects with a Chid Pugh classification A and eight with classification B were treated with 20 mg ebastine once daily for 7 days. For safety reasons, four Child Pugh C patients (severe) were initially given only one single reduced dose of 10 mg. After evaluation of these patients, the option was to treat four more subjects for 7 days. Twelve healthy volunteers were selected to match the overall age, weight and gender distribution of the subjects with hepatic impairment.

EVALUATION

Venous blood samples for pharmacokinetic profiling of ebastine and carebastine in plasma were taken on day 1 (predose, and 0.5, 1, 1.5, 2, 3, 5, 8, 12, and 23.5 h postdose) and on day 7 (predose and 0.5, 1, 1.5, 2, 3, 5, 8, 12, 23.5, 48, 72, and 96 h postdose). Predose samples were also taken on days 3, 4, 5, and 6 to determine steady state achi evement. For Child Pugh C patients receiving a single dose, the sampling times were predose and 0.5, 1.0, 1 5, 2, 3, 5, 8, 12, 23.5, 48, 72, and 96 h postdose. Samples were analyzed by validated LC MS/MS methods with limits of quantifica tion of 0.05 ng/mL for ebastine and 1 ng/mL for carebas tine. For the active metabolite the unbound fraction was measured at 5 h, the anticipated T_{max}, using equilibrium dialysis.

Pharmacokinetic analysis was performed by a non compartmental approach with a full evaluation on day 1 after the first dose and on day 7 at steady state; the main parameters were C_{max}, T_{max}, C_{min}, AUC_{inf} (only day 1); AUC_{0-24}, $t_{1/2\lambda z}$ and the apparent total body clearance (CL/*F*). For the active metabolite, the unbound AUC_{0-24} was calculated by multiplying the total AUC_{0-24} by the unbound fraction at C_{max}.

The differences between healthy volunteers and the three groups of patients with hepatic impairment were assessed on ratios and 90% confidence intervals (CI) of log transformed pharmacokinetic parameters. Given the high variability of ebastine (>70%), the predefined no effect criteria was a <2 fold increase.

Indocyanine green was administered intravenously on day 2 of the study at 0.5 mg/kg. The relationship between drug exposure and indocyanine green clearance was assessed by a correlation procedure.

CRITICAL ANALYSIS

The study design is in agreement with the regulatory guid ances regarding the classification of subjects with hepatic impairment. A repeat dose regimen was chosen, because previous data had shown that steady state exposure was not well predicted by single dose pharmacokinetics. The 7 day duration was longer than necessary to achieve steady state in patients with normal liver function, in case a prolonga tion of half life would occur in hepatic impairment, with a consequently longer time to reach steady state. In order to mitigate any risk, only four subjects with Child Pugh class C were initially treated at half the therapeutic dose and only for 1 day. Therefore, the full PK was also evaluated on day 1 in the other groups for comparison. The free fraction was only evaluated at T_{max} of the active metabolite, because the methods were not sensitive enough for ebastine free frac tion at any time point and metabolite free fraction at C_{trough}.

MODIFICATIONS

A full design was implemented for this study, because an effect of hepatic impairment on ebastine or its metabolite was expected because of the high oral clearance for the parent drug and significant extraction ratio and protein binding of the metabolite. For many drugs only mild and moderate impairment are studied, because the drug is unlikely to be used in severe hepatic impairment and/or can be contraindicated. Also, pharmacokinetic studies in this patient population can be quite difficult to perform because of the poor overall health status and prognosis. In practice, it can take also much more time to recruit these patients compared to those from other groups.

A limited control group of 12 subjects that matched the overall demographic distribution of the patients was sel ected, rather than individual subject by subject matching which would have needed 24 healthy. This was justified by the fact that the means of the groups were to be compared, and not by a paired test. Actually, the first eight control subjects were recruited when 2/3 of the patients had been recruited and then the control group was completed at the end of the study. Healthy volunteers were selected in a way that for each demographic variable (age, weight), there were eventually two controls in each quartile of the distribution and also with a similar gender distribution.

The number of subjects of eight per hepatic impair ment group was expected to allow to detect clinically meaningful differences. It is generally not possible to tar get a bioequivalence approach in this special population to exclude any effect.

For a drug with dose and time independent pharma cokinetics a single dose administration could have been selected. An accurate evaluation of elimination half life and AUC_{inf} is in that case mandatory.

For a discussion regarding options for plasma protein binding in this kind of studies see also the chapter on protein binding considerations.

EXAMPLE

B.8.2 PK Study

The results of the study described above did not show a significant impact of hepatic impairment on ebastine and its metabolite total drug pharmacokinetics (*Fig. B.8 1*).

At steady state after 20 mg once daily dosing, ebastine AUC was on average 86% higher in mild and 51% in moderate hepatically impaired patients, which was con sidered not clinically relevant given the high variability. For the active metabolite carebastine, which had much higher exposure levels than the parent drug, the AUC_{0-24} in mild and moderate impaired patients represented 84 and 94%, respectively, of that in normal controls, suggesting a similar formation rate and turnover. The free metabolite concentrations were also similar in these two groups, so no effect on free drug exposure was observed either (*Table B.8 2*).

In the severe hepatic impairment group, the exposure after a 10 mg single dose was 27% of that of parent drug and 40% for the metabolite. This administration was well

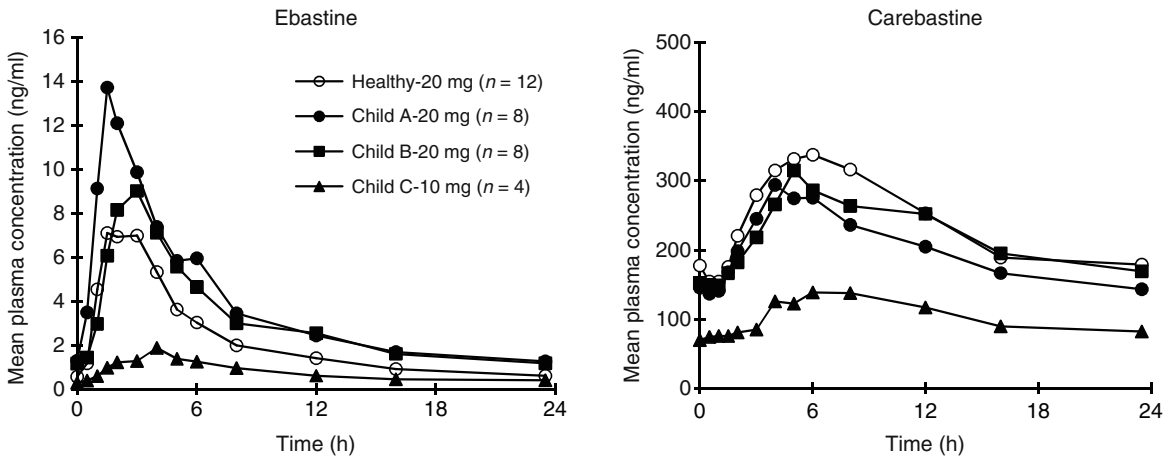

Figure B.8-1

Ebastine and metabolite pharmacokinetics on day 7 after repeat dosing in subjects with hepatic impairment and healthy controls. Adapted from Lasseter (2004)

Table B.8-2

Ebastine total drug and carebastine total and free metabolite exposure on day 7 after 20 mg once daily dosing (10 mg in Child-Pugh C)

	Ebastine	Carebastine		
	AUC	Total AUC	Free fraction (%)	Free AUC
Healthy volunteers (n = 12)	51.9 (65)	5,608 (40)	0.54 (97)	30.7 (85)
Child-Pugh A (n = 8)	89.5 (70)	4,661 (40)	0.55 (74)	26.7 (90)
Child-Pugh B (n = 8)	73.5 (73)	5,191 (43)	0.67 (65)	32.1 (64)
Child-Pugh C (n = 4)	18.2 (64)	2,443 (15)	1.84 (66)	46.1 (72)

Mean (CV%). $AUC_{0\ 24\ h}$ in ng h/mL

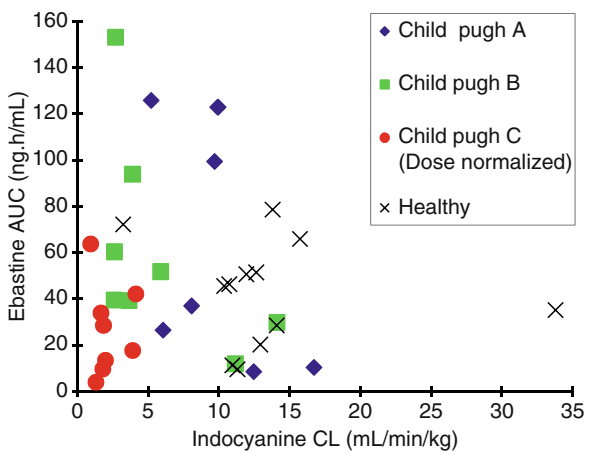

Figure B.8-2

Correlation between ebastine AUC_{0-24} on day 7 and indocyanine clearance in subjects with hepatic impairment and healthy controls

tolerated so four other subjects were treated at the same dose for 1 week. Total parent drug and metabolite exposure were 37 and 47% of that in healthy volunteers, respectively, close to what would be expected given the lower dose. However, the free metabolite fraction was about threefold higher, and thus the free metabolite AUC was in fact slightly higher than in the control group (*Table B.8 2*).

Indocyanine clearance decreased with Child Pugh score, although values were overlapping between the groups, showing that this parameter is driven by different aspects of liver function. However, no negative correlation between ebastine AUC and indocyanine clearance could be established either (*Fig. B.8 2*).

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B.9 Special Populations: Protein Binding Aspects

Gareth Shackleton

PURPOSE AND RATIONALE

It is generally considered that only unbound drug in the plasma elicits a pharmacological response. Therefore, it follows that unbound concentrations of drug should correlate better with safety and efficacy than total plasma concentrations when the free fraction is subject to change.

Clinical populations requiring special investigation, as outlined elsewhere in this volume, often present with different plasma protein concentrations than the rest of the clinical trial population. This is particularly true in comorbid hepatic and renal insufficiency, and may also be the case in pregnancy, the elderly, different racial groups as well as in patients suffering other comorbid diseases, such as cancer, arthritis, Crohn's and other immune diseases.

In hepatic impairment, the concentration of albumin in plasma can be reduced by up to 50% in severe impairment (Ko et al. 1991) and that of α_1 acid glycoprotein by 60% in moderate impairment (Lau et al. 1997). Consequently, unbound drug concentrations may be different in hepatic impairment than in healthy subjects, as seen with tiagabine (Lau et al. 1997). Of greater concern is the fact that total plasma concentration and unbound plasma concentration may not be altered by hepatic impairment in the same way or to the same extent. This is exemplified with salicylate, for which total plasma concentrations are similar in cirrhotic subjects than in control healthy subjects, while unbound plasma concentrations are higher in the cirrhotic subjects (Roberts et al. 1982). For some drugs, particularly those with a narrow therapeutic index, this may necessitate dose adjustment or contraindication in clinical practice.

Consequently, according to the US FDA (2003) guidance for industry, pharmacokinetic studies in hepatically impaired subjects with compounds that are highly cleared by the liver (extraction ratio >0.7) and that are extensively plasma protein bound (>90%), should include sampling to assess unbound fraction at least at trough and maximum plasma concentrations. Similarly, in the EMEA Guideline on the Evaluation of the Pharmacokinetics of Medicinal Products in Patients with Impaired Hepatic Function (EMEA CHMP 2005), it is stated that "if the drug or metabolite exhibits a high extent of plasma protein binding, the pharmacokinetics should be described and analyzed with respect to the unbound concentrations of the drug and active metabolites in addition to the total concentration."

The regulatory guidance is similar, but not entirely consistent, for the conduct of studies in patients with renal impairment. According to the US FDA (1998b) guidance, for drugs and metabolites exhibiting relatively extensive plasma protein binding (defined as being greater than 80% bound), pharmacokinetics should be analyzed with respect to unbound concentrations as well as total plasma concentrations. This guidance goes on to describe that although unbound plasma concentrations should be measured in every plasma sample drawn for pharmacokinetic measurement, measurement of a limited number, or even a single sample, is acceptable if binding is independent of drug concentration, metabolite concentrations, and other time varying parameters. The difficulty for the experimenter with this scenario is what level of evidence is required to satisfy the prerequisites for the more limited sampling and analysis regime. The guidance goes further, in recommending that if the effect of renal impairment on pharmacokinetics is studied using a population pharmacokinetic approach, then this should also be performed by measurement of unbound concentrations when appropriate (i.e., when plasma protein binding >80%). Unlike the US FDA, the EMEA CHMP (2004) guidance does not offer a threshold level of protein binding, but is otherwise in general agreement with the US FDA guidance. Like the US FDA, the EMEA suggests that analysis of a limited number of samples may be allowable if binding has been demonstrated to be concentration independent. However, the EMEA guidance recognizes that an in vitro study, using plasma from a dialysis patient immediately before dialysis, may be sufficient if no difference in plasma protein binding is observed compared to healthy control subjects.

The US FDA (1977, 1998b) guidance for the clinical evaluation of drugs in infants and children recommends that protein binding is investigated in all age groups. In vitro studies covering therapeutic blood levels can generate

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the relevant information on extent, linearity, and binding to specific proteins recommended by these guidance documents. The EMEA CHMP (2007) noted that "potential differences in plasma protein binding (in all age groups of the paediatric population) should be considered."

The US FDA guidance of 1977 for the evaluation of drugs in infants and children extends the recommendation for in vitro studies to pregnancy. However, in its later guidance on pharmacokinetic studies in pregnancy, the US FDA (2004) recommends ex vivo experiments in the same way as that described for studies in renally impaired patients.

Overall, the regulatory recommendation is for the measurement of unbound plasma concentrations ex vivo in pharmacokinetic studies in renally and hepatically impaired subjects, while in vitro studies are considered adequate in other special populations. This is largely consistent with a seminal paper on the topic by Benet and Hoener (2002). Based on basic pharmacokinetic principles, supported with a survey of recently marketed drugs, Benet and Hoener demonstrated that consideration of plasma protein binding for dose adjustment is generally unnecessary. Exceptions were parenterally administered drugs with a high clearance or orally administered drugs with a high non hepatic clearance. The scientific basis behind more thorough investigations in renal and hepatic impairment lies in the potential for concomitant changes in plasma protein binding and intrinsic clearance in these populations, which may result in unpredictable changes in total and unbound exposure as observed with salicylate (Roberts, 1983), zileuton (Awni et al. 1995), and phenytoin (Gugler et al. 1975).

Because of the relative importance of protein binding considerations in renal and hepatic insufficiency, the rest of this chapter will focus on these areas. A protocol for protein binding measurement as part of a clinical pharmacokinetic study in hepatically impaired subjects is described. This protocol was designed to be consistent with the regulatory guidance, although other approaches would have been possible, and are discussed. Example data derived from this clinical study design is provided.

PROCEDURE

The design of protein binding measurements in a clinical study with investigational drug X001 in hepatically impaired subjects is described below. For simplicity, only those elements relevant to the evaluation of unbound plasma concentrations are presented.

PROTOCOL

This study was designed "to investigate the pharmacokinetic parameters of X001 when given via oral route to subjects with mild and moderate hepatic insufficiency and healthy volunteers."

The study was a single center, open label, single dose study with three groups. Two groups of eight subjects each were selected with Childs Pugh scores of either 5 6 (mild hepatic impairment) or 7 9 (moderate hepatic impairment). Further groups of 16 subjects were healthy volunteers matched for age, weight, and gender with the hepatically impaired subjects. Each subject received a 20 mg dose of X001 with 100 mL water. Blood samples (6 mL) were taken for pharmacokinetic assessment at 0 (pre dose), 1, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 168, 336, 504, 672, 1,056 h post dose. An additional 14 mL blood was taken at 1, 2, 3, 4, 12, and 24 h to determine plasma protein binding. Blood samples were collected into tubes containing EDTA anticoagulant, and kept on ice no longer than 3 h prior to preparation of plasma. Plasma samples were stored frozen at −20°C, analyzed within 6 months and subjected to no more than three freeze thaw cycles. To investigate plasma protein binding, plasma was subjected to equilibrium dialysis on triplicate aliquots of plasma at each of the six timepoints in each subject. Dialysis equipment was comprised of 1 mL Teflon cells (Dianorm), 10 kDa high permeability cellulose dialysis membrane (Dianorm). The cells, containing plasma in one half cell and 67 mM Sörensons phosphate buffer pH 7.4 in the other, were incubated at 37°C for 4 h, with rotational mixing at 12 rpm. The weights of plasma and buffer added to the cells and removed from the cells after the incubation were recorded. Dialysates and retentates were stored frozen prior to measurement of X001 in plasma, retentate, and dialysate by specific validated LC MS/MS methods. The lower limit of quantification (LOQ) for the plasma and retentate assay was 1 ng/mL, while the LOQ for the dialysate assay was 5 pg/mL.

EVALUATION

The free fraction (f_u) was calculated as follows:

$$f_u = 1 - \frac{[(C_r - C_d) \times (m_r/m_p)]}{[(C_r - C_d) \times (m_r/m_p) + C_d]}$$

where C_r is the X001 concentration in plasma retentate (pg/g), C_d the X001 concentration in plasma dialysate (pg/g), m_r the mass of the retentate at the end of the incubation (g), and m_p is the mass of the plasma added to the cell at the start of the incubation (g).

Since the free fraction was found to be constant over time, a mean free fraction was calculated for each subject and used to calculate pharmacokinetic parameters of unbound X001 AUC_{0-t}, where t represented the last evaluable timepoint for free fraction measurement.

CRITICAL ANALYSIS

The study design is consistent with the regulatory guid ance, in that the plasma protein binding was measured ex vivo in samples obtained after administration of the study drug to volunteer patients. Samples were taken for protein binding measurement over a dosing interval as suggested by the US FDA (2003) guidance. Equilibrium dialysis is a time consuming, labor intensive, and low throughput technique that would be difficult to conduct on a large number of samples, such that in this case only six time points were to be evaluated per subject. In vitro data, usually generated during preclinical development, can be used to assess the likelihood that concentration dependent binding would occur over the peak and trough plasma concentration range. The likelihood that peak and trough measurements can be made also needs to take into account the achievable LOQ of the bioanalytical assays. In this case, six timepoints were considered necessary.

Anticoagulants, in particular heparin anticoagulants, have been shown to affect plasma protein binding (De Smet et al. 2001). A preliminary assessment of the influence of anticoagulants on the protein binding of the candidate drug would be generally recommended. Similarly, plasti cizers in vacutainers have been shown to affect the binding of drugs to α_1 acid glycoprotein (Sager and Little 1989), and for drugs known to bind to this protein, this should be ruled out.

The sampling regime specifies a maximum of 3 h storage of blood on ice prior to preparation of plasma, a maximum of 6 months storage of plasma prior to equilibrium dialysis, and subjected to less than three freeze thaw cycles. This implies prior knowledge of the protein binding behavior of the candidate drug during storage, gained during prelim inary studies.

Equilibrium dialysis was conducted under relatively standard conditions. A 4 h equilibrium time is often suf ficient to achieve equilibrium, but should be checked in a pilot study. Weighing the samples so that corrections for fluid shift between the half cells could be calculated would be expected to generate more accurate results than if ass umptions were made that the volumes in the half cells remained constant during equilibration. Due to complex sample handling procedures, equilibrium dialysis is better suited to in vitro experiments than to the kind of ex vivo experiment described here. The triplicate measurement of protein binding would greatly add to the time and resource required to conduct the experiment, but it would seem like a reasonable precaution to mitigate against cell leakages, spilt samples, or contamination.

Modern LC MS/MS instruments have much improved sensitivity and make this kind of experiment possible. Given that the aim of the experiment is to assess free fraction at peak and trough plasma concentrations, it is reasonable to expect that the dialysate assay should be capable of measur ing the free drug concentration at a total plasma concentra tion equivalent to the LOQ of the plasma assay. In this case, the LOQ of the dialysate assay (5 pg/mL) would be sufficient to assess the plasma protein binding over the whole range of measurable total plasma concentrations for a drug with a free fraction of 0.5% or greater. A drug with more extensive plasma protein binding would require an even more sensi tive dialysate assay. As can be seen from the example data, the LOQ of the dialysate assay was not strictly adequate, but a lower LOQ would likely have been prohibitively difficult to achieve.

MODIFICATIONS

The guidances from both FDA and EMEA recommend that protein binding should be evaluated ex vivo at a minimum of peak and trough plasma concentrations in samples taken from patients administered the test compound. However, when plasma protein binding is known to be independent of concentration, presence of metabolites or other time varying factors (meals, circadian rhythms, and concomi tant medications) then an in vitro study is acceptable. The experimenters should therefore satisfy themselves that these criteria have been demonstrably met. Obtaining samples from hepatically or renally impaired subjects for in vitro plasma protein binding studies may be most easily obtai ned pre dose from the clinical trial subjects. Protein bind ing can then be assessed in the same individuals used for pharmacokinetic evaluation. Ensuring good quality results is more manageable from an in vitro study as the study design can be simplified, not least by the use of radiolabe led compound. Usually this would entail ^{14}C labeled com pounds, but tritiated compounds may also be suitable and can improve limits of quantification.

Modern bioanalytical LC MS/MS is capable of highly sensitive, automated analysis but it requires good valida tion of assays for every analyte to achieve accurate and reproducible results. In contrast, radioanalysis is higher throughput and does not need to be validated for every analyte. So an alternative to LC MS/MS analysis in the study described would be to spike radiolabeled material in to the ex vivo, post dose plasma samples as a radiotracer for protein binding measurements. However, in principle, the radiolabeled compound should represent a small per centage of the total compound in the sample so as not to alter the equilibrium between bound drug and free drug. This would significantly raise the lower LOQ, and would be unlikely to be suitable to achieve the goals of the study described above.

Equilibrium dialysis is a time consuming, labor intensive, complicated process compared to the other widely used method of ultrafiltration. Despite ultrafiltration being a rapid, simple methodology, that is generally well suited to such ex vivo experiments, it is not suitable for compounds that stick to the filters as this results in an underestimation of the free fraction.

Finally, six samples were taken for plasma protein binding measurement in order to evaluate protein binding over the dosing interval. In fact, regulatory guidance calls for a minimum of peak and trough samples, so the sample number could be reduced. However, there is a risk that measurements in trough samples may not be achievable due to limits of sensitivity (as was the case in this example) and so taking additional samples is advisable, even if they are not subsequently analyzed. In addition, if protein binding does alter between peak and trough it is difficult to adequately calculate an unbound AUC over the dosing interval without intermediate sample times.

EXAMPLE

The results given in *Tables B.9 1* and *B.9 2* are from the study design described in the previous sections illustrating the results that can be obtained and some of the challenges associated with this kind of experiment.

Prior to conducting the clinical study, an intensive study was conducted to validate the sample collection procedures, ensuring that they had minimal effect on plasma protein binding. *Table B.9 1* shows the main results from this study conducted with ^{14}C labeled X001. The X001 free fraction in vitro varied with sample collection and storage conditions, and from experiment to experiment, ranging from 0.025 to 0.045 in control experiments, depending to a large extent upon the blood donor. However, the conditions shown in *Table B.9 1* gave the optimum control of X001 free fraction. It cannot be said that the sample collection conditions will have no effect on the free fraction measured in the clinical trial, but that the effect of specifying these sample conditions will have kept bias and variability as low as possible while making the experiment achievable in practice and allowing the detection of variation in free fraction between control and hepatically impaired subjects.

It will be noted from the results in *Tables B.9 1* and *B.9 2* that the free fraction was approximately 0.04%, that is, ten times lower than the free fraction expected to be measurable at the trough plasma concentration with a dialysate assay LOQ of 5 pg/mL. Consequently, it was only possible to measure plasma protein binding at 1, 2, 3, and 4 h post dose in all subjects, with measurement possible at 12 h post dose in only a few subjects. Hence, it was not possible to entirely meet the recommendations of existing regulatory guidances.

However, from the measurements that were made it was clear that protein binding did not change over time during 1 12 h post dose. This facilitated the calculation of unbound pharmacokinetic parameters. It can be seen from *Table B.9 2* that mild and moderate hepatic impairment did not significantly alter plasma protein binding or total and unbound exposure (C_{max} or AUC).

Table B.9-1

Effect of sample collection parameters on plasma protein binding of [^{14}C]-X001

Experimental conditions	Free fraction (%) (n = 6)	Difference from control (%)
Using EDTA as anticoagulant[a]	0.039 ± 0.003	11.4
Blood collection in vacutainers[b]	0.03 ± 0.006	20
Blood storage time on ice for 3 h prior to plasma preparation[c]	0.036 ± 0.004	2.9
Three freeze-thaw cycles[c]	0.040 ± 0.002	11.1
Six months storage at 20°C[c]	0.039 ± 0.001*	24.6
f_u in icteric samples (n = 12 subjects)[d]	0.045 ± 0.009	21.6

[a]Control value = 0.035 ± 0.003 determined in serum

[b]Control value = 0.025 ± 0.002 determined in plasma collected with EDTA anticoagulant into conventional polypropylene blood tubes

[c]Control value at t = 0 h timepoint for the relevant experimental work, with fresh plasma collected into EDTA vacutainers = 0.035 ± 0.001 (blood storage), 0.031 ± 0.001 (frozen storage), or 0.045 ± 0.003

[d]Free fraction in non icteric samples (n = 6 subjects) = 0.037 ± 0.007

All experiments were performed at a [^{14}C] X001 concentration of 500 ng/mL

*n = 5

Table B.9-2

Pharmacokinetics of X001 in mild and moderate hepatic impairment compared to matched healthy control subjects

	Mild hepatic impairment (n = 9)	Healthy matched control (n = 8)	Moderate hepatic impairment (n = 8)	Healthy matched control (n = 8)
C_{max} (ng/mL)	118 ± 51.6	120 ± 39.7	119 ± 64.7	124 ± 41.9
AUC (ng h/mL)	6,120 ± 1,310	5,790 ± 1,930	4,550 ± 1,490	5,100 ± 1,800
f_u [a] (%)	0.047 ± 0.004	0.047 ± 0.020	0.050 ± 0.006	0.048 ± 0.012
$C_{max,u}$ (pg/mL)	55.6 ± 24.2	56.4 ± 31.9	58.4 ± 30.7	58.7 ± 22.5
$AUC_{u,0-24}$ (pg h/mL)	405 ± 160	393 ± 99	449 ± 221	471 ± 150
$AUC_{u,0-4}$ [b] (pg h/mL)	145 ± 75	146 ± 79	162 ± 94	140 ± 51

[a] f_u was independent of total concentration or time, so was averaged across timepoints for each subject to calculate unbound pharmacokinetic parameters

[b] AUC to the last common timepoint at which unbound concentrations could be measured, that is, 4 h post dose

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B.10 The Human ADME Study

Ulrich Kuerzel · Volker Krone · Manfred Zimmer · Gareth Shackleton

B.10.1 Introduction and Guidelines

Sophisticated in silico (Matter and Schmider 2006) and humanized *in vitro* models of drug absorption (Mertsch 2006) and drug metabolism (Dudda and Kuerzel 2006b), in combination with animal data (Gupta and Atul 2000; Inskeep and Day 1999), provide excellent predictions of absorption, distribution, metabolism, and excretion (ADME). Nevertheless, a clinical "reality check" of these predictions is needed with a human ADME study (hADME study). This study, also known as a human mass balance study, is comprised of the administration of the NCE in radiolabeled form in order to monitor the compound and its metabolites (Beumer et al. 2006; Roffey et al. 2007; Dalvie et al. 2000; Pool 1999 and references cited therein, Deroubaix and Coquette 2004).

Approximately three quarters of the top 200 prescribed drugs in the United States in 2002 are removed from the body primarily by metabolism, one third via the kidney, while biliary clearance of unchanged drug plays only a minor role (Williams et al. 2004), underlining the need to investigate drug metabolism. Based on this knowledge, the potential for drug metabolites to interact with the target receptor and contribute to efficacy, or off target and cause toxicity (Liebler and Guengerich 2005; Stevens 2006) needs to be investigated. Similarly, the potential involvement in drug drug interactions (US FDA 2006; EMEA 1997; Tucker et al. 2001) as a victim (Dudda and Kuerzel 2006a) or perpetrator (Dudda and Kuerzel 2006b) should be elucidated. Furthermore, the knowledge of clearance pathways may trigger the investigation of drug transporters involved in the excretion of drug and metab olites via the kidneys or the liver (Eisenblaetter, see chapter on "Relevance of Transporters in Clinical Studies" in this book).

hADME studies preferentially use compounds labeled with C 14. Labeling with tritium (H 3) provides in many cases less stability, and requires a "wet/dry" comparison of the samples to distinguished free tritiated water from drug and its metabolites. Similar precautions and tests for in vivo stability of the labeled compound are necessary if other nuclides are used as radiolabel. For example, radioiodine is often used for the labeling of peptides and proteins but has the disadvantage of the potential loss of the label liberating free iodine into the body.

The administration of radiolabeled compounds in clinical studies is highly regulated to ensure the safety of the enrolled subjects (Beumer et al. 2006 and literature cited therein). The recently published draft US FDA guid ance from the radioactivity drug research committee on "human research without an investigational new drug application" describes the prerequisites for the adminis tration of a radiolabeled drug (US FDA 2009). Special emphasis is given to the radioburden to which each indi vidual is subjected based on the dosimetry calculation, as explained later in this chapter in detail.

In contrary to the level of regulatory detail on the technical aspects of the radiolabeled hADME study, the objectives of this type of study are not specifically detailed in regulatory guidelines but must be deduced from general guidelines (EMEA 1988) or from those dedicated to other topics such as drug interaction (US FDA 2006) or related preclinical studies (EMEA 1994; US FDA 2008).

It is generally accepted that the scope of metabolism investigations is limited to small molecules rather than biologicals. The draft US FDA drug interaction guidance states that "Classical biotransformation studies are not a general requirement for the evaluation of therapeutic biologics (ICH guidance *S6 Preclinical Safety Evaluation of Biotechnology Derived Pharmaceuticals*), although certain protein therapeutics modify the metabolism of drugs that are metabolized by the P450 enzymes," referring to the respective document from the International Conference of Harmonization ICH, 1997 adopted by the European agency (EMEA 1998).

PURPOSE AND RATIONALE

As described above, a complete understanding of the hADME properties of a drug is of critical importance for the safety and efficacy of a new chemical entity (NCE) and thus for the approval of a drug intended for the treatment of humans.

A question of particular relevance is whether human metabolites are adequately exposed to the animal species

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used in toxicity testing to ensure safety. This was articulated in a paper published by a multidisciplinary committee together with proposals on how to deal with it from the point of view of needs and feasibility (Baillie et al. 2002). The discussion around a threshold of abundance beyond which a human metabolite should be evaluated for its occurrence and safety in toxicological test species triggered a series of publications (Hastings et al. 2003; Baillie et al. 2003; Davis Bruno and Atrakchi 2006; Guengerich 2006; Smith and Obach 2006; Humphreys and Unger 2006) together with practical strategies for industry (Luffer Atlas 2008). The Center for Drug Evaluation and Research (CDER) of the US Food and Drug Administration (US FDA 2008) has published its position on this point, recommending that a metabolite exceeding 10% of parent compound in plasma in human at steady state should have similar levels of exposure in toxicological test species. Disproportionate and especially unique human metabolites are of particular concern in this respect, as shown in the decision tree published in the guidance and reproduced in *Fig. B.10 1*.

A typical hADME study, as illustrated in this chapter, includes an overall balance of excretion of the administered radioactivity (mass balance), 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. Plasma protein binding can also be addressed on a case by case basis but is not covered in this chapter. The discussion of the procedure starts with a description of the dosimetry calculations required to determine a safe dose to humans.

PROCEDURE

B.10.2 Dosimetry

Planning clinical studies with radiolabeled test substances requires an estimation of the bodies' exposure to radioactivity (dosimetry) to balance the safety of the subjects receiving the radiolabel with administering adequate radioactivity to ensure the objectives of the study can be met.

The estimation of the so called committed effective dose (CED; The radioburden on the basis of the radioactive dose, not to be confused with the gravimetric dose of the drug) that may be expected in healthy human adult volunteers after administration of a radiolabeled test compound is derived from both quantitative whole body autoradiography (QWBA) and mass balance studies in animals (Zimmer 2006; Krone 2006). Dosimetry involves

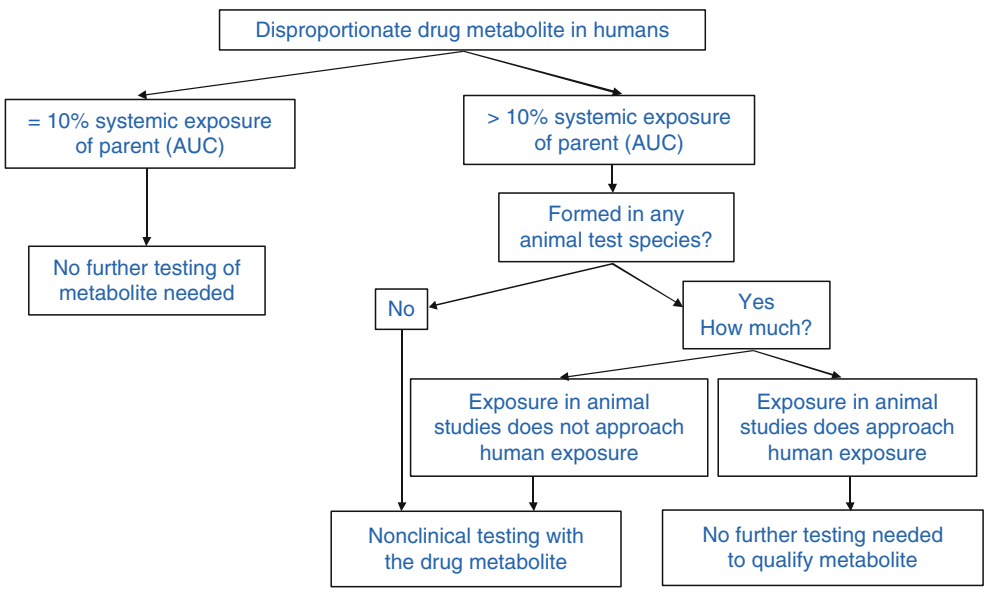


Figure B.10-1
Decision tree for safety testing of metabolites (Reproduced from US-FDA 2008)

taking these measurements of the exposure of tissues and organs to radioactivity during its residence in the body, accounting for the energy of the radioactive decay processes and the sensitivity of different tissues to radioactivity and extrapolating to humans.

Dosimetric calculations are carried out in accordance with internationally recommended procedures and most recent recommendations issued by the WHO (World Health Organization) and the ICRP (International Commission on Radiological Protection).

B.10.2.1 Dose Categories

In order to estimate the risk/benefit ratio of a study with radiolabeled substance in humans, the potential benefit to society (by increase in knowledge) must be weighed against the potential harm to the exposed individual.

The WHO has defined categories with different levels of risk depending on the dose of radiation received by the subjects (WHO 1977). *Table B.10 1* shows the risk categorization, modified by ICRP taking into account more recent changes in the risk assessment (ICRP 1993a), since its initial release.

The lowest risk (*category I*) is of the order of one in a million (it should be noted that the risk is the total detriment from the exposure, namely, the sum of the probability of fatal cancer, the weighted probability of nonfatal cancers, and the probability over all succeeding generations of serious hereditary disease resulting from the dose). The corresponding dose region is less than 100 μSv, which is the amount of dose delivered by natural background radiation within a few weeks (ICRP 1993a).

At the other extreme, the highest risk (*category III*) includes risks of the order of one in a thousand or greater. The corresponding dose region is tens of mSv or more which is greater than the current annual dose limit for occupational exposure.

To justify investigations involving doses or risks in category III, the benefit would have to be substantial and usually directly related to the saving of life or the prevention or mitigation of serious disease.

In between, *category II* is subdivided into IIa and IIb, including risks of the order of one in a hundred thousand to one in ten thousand. In dose terms, it includes the annual doses received by essentially all radiation workers in the course of their normal jobs and the annual doses received by members of the public from the totality of sources to which they are exposed (excluding some extreme exposures to radon). To justify risks in category IIa, the benefit will be related to increases in knowledge, leading to health benefit, whereas in category IIb, the benefit will be more directly aimed at the cure or prevention of disease (ICRP 1993a; IRPA 2003).

For comparison purpose, the radiation burden from a few common diagnostic X ray examinations is listed in *Table B.10 2* (ICRP 1993b).

B.10.2.2 Dosimetry Calculation

B.10.2.2.1 Raw Data

The concentrations of radioactivity in organs and tissues are listed as "percent of administered dose." It is necessary to define rules to handle values that fall below the limit of quantification (BLQ), for example

- If more than one value is below the LOQ, the first one is replaced by the LOQ value and the remaining values are fixed to BLQ.
- If at least one value below LOQ is measured between values above LOQ, all the values are set to the LOQ value.

Table B.10-1

Risk categories depending on radiation dose

Level I of risk	Risk category (probability of detriment)	Corresponding effective dose range (adults)[mSv]	Level of societal benefit
Trivial	Category I ($\sim 10^{-6}$ or less)	<0.1	Minor
Minor to intermediate	Category II		Intermediate to moderate
	IIa ($\sim 10^{-5}$)	0.1 1	
	IIb ($\sim 10^{-4}$)	1 10	
Moderate	Category III ($\sim 10^{-3}$ or more)	>10	Substantial

These rules are described in *Table B.10 3*, with the original data shown in the table on the left and the data used in dosimetry calculations shown on the right with the replaced values highlighted.

B.10.2.2.2 Absorbed Dose

In the time interval of interest, the average energy absorbed per unit mass in the target region, the mean absorbed dose D_{mean}, is equal to the product of five terms:

$$D_{mean} = \tilde{A} \cdot nE \cdot \Phi \cdot m^{-1}$$

where

- $\tilde{A}$ is the number of nuclear transformations in the source region during the time interval of interest [Bq s] ≡ 1.
- n is the mean number of ionizing particles per nuclear transformation [Bq^{-1} s^{-1}] ≡ 1.
- E is the mean energy per particle [kg Gy] ≡ *J*.
- nE is the mean energy emitted per nuclear transformation [Bq^{-1} s^{-1} kg Gy] ≡ *J*.
- Φ is the fraction of the particle energy emitted by the source region and imparted to the target region, called the *absorbed fraction*.
- m^{-1} is the reciprocal of the mass of the target region [kg_{man}].

For C 14, the mean emitted energy (nE) amounts to $7.92 \cdot 10^{-15}$ J (ICRP 1983). For β radiations of low energy like those from ^{14}C the absorbed fraction (Φ) amounts to "1" (except for GIT content and urinary bladder where it was fixed to "0.5"). Also, the target region coincides almost entirely with the source region. Hence, computation of the absorbed dose requires only to estimate $\tilde{A}$, the number of nuclear transformations occurring in the target during the time interval of interest.

B.10.2.2.3 Calculation of $\tilde{A}$

The value of $\tilde{A}$ is estimated from the area under the curve (AUC) of the tissue concentrations of the radioisotope

Table B.10-2
Radiation burden from common diagnostic X-ray examinations (ICRP 1993b)

Examination	Effective dose [mSv]
Chest	0.04
Skull	0.1
Mammography (screen film)	0.1
Thoracic spine	1.0
Abdomen	1.2
CT head	1.8
Lumbar spine	2.1
CT pelvis	7.1
CT abdomen	7.6

Table B.10-3
Replacement rule for values below limit of quantification

Percent dose					Percent dose				
Time (h)	Adrenals	Liver	Testis	Thyroid	Time (h)	Adrenals	Liver	Testis	Thyroid
0	0	0	0	0	0	0	0	0	0
0.5	0.022	0.0925	0.008	0.003	0.5	0.022	0.0925	0.008	0.003
1	0.081	1.34	0.334	BLQ	1	0.081	1.34	0.334	0.001
3	0.139	2.19	0.256	0.002	3	0.139	2.19	0.256	0.002
6	0.153	2.23	0.201	0.002	6	0.153	2.23	0.201	0.002
12	0.090	1.02	0.099	BLQ	12	0.090	1.02	0.099	0.001
24	0.022	0.872	0.003	BLQ	24	0.022	0.872	0.003	BLQ
48	0.005	0.451	BLQ	BLQ	48	0.005	0.451	0.020	BLQ
72	0.002	0.113	BLQ	BLQ	72	0.002	0.113	0.020	BLQ
168	BLQ	0.041	BLQ	BLQ	168	0.001	0.041	0.020	BLQ
336	BLQ	BLQ	0.003	BLQ	336	BLQ	0.020	0.003	BLQ
LOQ	0.001	0.020	0.002	0.001	LOQ	0.001	0.020	0.002	0.001

over time (percentage of dose versus time), using the trapezoidal rule.

$$AUC_{0\ \infty} = AUC_{0\ n} + C_n/\lambda_z$$

where

- AUC_{0-n} is the area under the curve from the time of administration up to the limit of quantification (LOQ), or to the last available data point.
- C_n is LOQ or the percentage of dose at the last available data point.
- λ_z is the first order rate constant that is derived from regression analysis of the calculated terminal half life ($t_{1/2}$).

The unit [percentage of dose] is transformed to [fraction of dose]:

$$\tilde{A} = \frac{AUC_{0\ \infty}}{100};$$

Finally, ($D_{mean} = \tilde{A} \cdot nE \cdot \Phi \cdot m^{-1}$) estimates the mean absorbed dose per Bq.

B.10.2.2.4 Gastrointestinal Tract and Urinary Bladder

Gastrointestinal tract (GIT) and urinary bladder may be irradiated by both the dose related to tissue ($\tilde{A}_{tissue}$, as above) and the dose related to content ($\tilde{A}_{content}$), such that the total is a combination of both:

$$\tilde{A} = \tilde{A}_{tissue} + \tilde{A}_{content}$$

$\tilde{A}_{content}$ can be calculated in consideration of the mean residence times in man given in ICRP 62 + 78 (ICRP 1993c, 1997) and as given in *Table B.10 4*, and in consideration of the excretion information obtained from the mass balance study in rats after oral dose.

$$\tilde{A}_{content} = MRT \cdot 3{,}600 \cdot F$$

where

- $MRT \cdot 3{,}600$ is the mean residence time transformed to [s].
- F is the fraction excreted via GIT or via urine.

B.10.2.2.5 Coefficients of Safety

In order to avoid underestimations of radiation burden, the following assumptions have to be made:

- When no data are available for one of the radiosensitive organs defined by the ICRP, the radioactivity concentration observed in the most irradiated tissue is assigned, unless other data (like blood values assuming that all organs and tissues were perfused) allow a better fit.
- Elimination half life is calculated using the last two or three data points, depending on which results in a higher value.
- To consider a possible impact of the radiolabeled test compound on the esophagus wall during swallowing, MRT is set to 0.25 h.
- The mean absorbed dose in GIT and urinary bladder related to content is calculated with $\Phi = 0.5$ assuming that the radioactivity is located on the surface of the wall and only 50% of the radiated dose is able to penetrate into the tissue (*Fig. B.10 2*).
- Taking into account that the radiation emitted by radiocarbon is of low energy and has a range of approximately 0.25 mm in water, this approach may be accepted as "conservative."

Table B.10-4
Mean residence time of gastrointestinal content (ICRP 1993c, 1997)

Section of GIT; bladder	Mean residence time (h)
Esophagus	0.25
Stomach	1
Small intestine	4
Upper large intestine	13
Lower large intestine	24
Urinary bladder	4

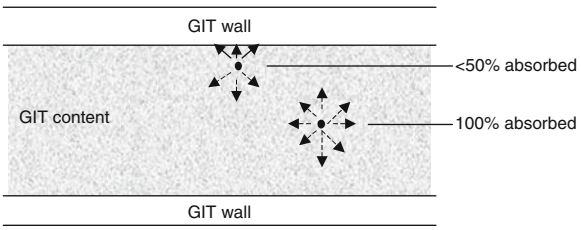


Figure B.10-2
Absorbed dose in the wall related to radioactivity located in the lumen of GIT and urinary bladder

- The fraction excreted (F) is fixed to "1" for esophagus and stomach when the dose is given orally (for i.v. dose F = "0") and is normalized to 100% for the remaining GIT sections and urinary bladder (even if the excretion balance is not completed).

$$F_{urine} = \frac{\%excreted_{urine}}{\%excretetd_{urine} + \%excreted_{feces}};$$

$$F_{feces} = \frac{\%excreted_{feces}}{\%excretetd_{feces} + \%excreted_{urine}}$$

- D_{mean} in red bone marrow is derived from D_{mean} in bone marrow applying the correction factor "2" [D_{mean} in bone marrow · 2], assuming a 1:1 ratio for red and yellow marrow and that the radioactivity is completely located in the radiosensitive hematogenic tissue (ICRP 1974).
- As the QWBA study is typically performed in male animals, D_{mean} in breast is derived from the organ dose calculated in adipose tissue.
- D_{mean} in bone surface is derived from D_{mean} in bone applying the correction factor "40" [D_{mean} in bone · 40] assuming that the radioactivity is completely located in the radiosensitive surface layer and $weight_{surface\ layer}$ corresponds to 1/40 $weight_{total\ bone}$ (ICRP 1974).

B.10.2.2.6 Equivalent Dose

The biological effectiveness is dependent on the type and energy of radiation, therefore radiation weighting factors have been selected by the ICRP (Table B.10 5; ICRP 1990).

The equivalent dose in tissue H_T is given by the expression:

$$H_T = \sum_R w_R \cdot D_{T,R}; [Sv]$$

where

$D_{T,R}$ is the absorbed dose averaged over the tissue or organ T, due to radiation R.

w_R is the radiation weighting factor for radiation R.

As the radiation weighting factor for electrons amounts "1," the mean absorbed dose corresponds to the equivalent dose when radiocarbon is used as radiolabel.

B.10.2.2.7 Effective Dose

The relationship between the probability of stochastic effects and equivalent dose also varies with the organ or tissue irradiated. Taking this varying radiosensitivity into account, the effective dose corresponds to the equivalent dose in organ or tissue multiplied by the specific weighting factor (Table B.10 6; ICRP 1990).

The values have been developed from a reference population of equal numbers of both sex and a wide range of age. In the definition of effective dose they apply to workers, to the whole population and to either sex.

Table B.10-5

Weighting factors for different types and energy of radiation (ICRP 1990)

Type an energy range	Radiation weighting factor, w_R
Photons, all energies	1
Electrons and muons, all energies	1
Neutrons, energy <10 keV	5
10 100 keV	10
>100 keV to 2 MeV	20
>2 20 MeV	10
>20 MeV	5
Protons, other than recoil protons, energy >2 MeV	5
Alpha particles, fission fragments, heavy nuclei	20

Table B.10-6

Mandatory tissues for dosimetric calculations (ICRP 1990)

Tissue or organ	Tissue weighting factor, w_T
Gonads	0.20
Bone marrow (red)	0.12
Colon (lower large intestine)	0.12
Lung	0.12
Stomach	0.12
Bladder	0.05
Breast	0.05
Liver	0.05
Esophagus	0.05
Thyroid	0.05
Skin	0.01
Bone surface	0.01
Remainder	0.05

For purpose of calculation, the remainder is composed of the additional tissues and organs listed in Table B.10 7.

The list includes organs that are likely to be selectively irradiated. Some organs in the list are known to be susceptible to cancer induction. If other tissues and organs subsequently become identified as having a significant risk of induced cancer they will then be included either with a specific W_T or in the additional list constituting the remainder. The latter may also include other tissues or organs selectively irradiated (*cursive*) by a particular radioactive compound.

In those exceptional cases in which a single tissue or organ listed in Table B.10 7 receives an equivalent dose in excess of the highest dose in any of the 12 organs for which a weighting factor is specified, a weighting factor of 0.025 should be applied to that tissue or organ and a weighting factor of 0.025 to the average dose in the rest of the remainder as defined above (ICRP 1990).

B.10.2.2.8 Committed Effective Dose

Finally, the committed effective dose (CED) E, is the sum of the weighted equivalent doses on all the tissues and organs of the body.

The contribution of all additional tissues to CED is calculated as a mass weighted mean (MWM):

$$\mathrm{MWM} = \frac{mean(weight_{organ} \cdot D_{organ})}{mean(weight_{organ})}$$

where

$\mathrm{weight}_{\mathrm{organ}}$ corresponds to the weight of the organs or tissues listed for the ICRP Reference Man.

D_{organ} is the equivalent dose for each additional organ or tissue.

Table B.10-7

Additional tissues for dosimetric calculations (ICRP 1990)

Adrenals	*Myocardium*
Adipose tissue	Pancreas
Blood	*Pituitary gland*
Brain	*Prostate*
Eyes (uvea/retina)	Small intestine
Upper large intestine	Spleen
Kidney	Thymus
Muscle	Uterus

The effective dose is given by the expression (ICRP 1990):

$$E = \sum_T w_T \cdot H_T; [\mathrm{Sv}]$$

where

H_{T} is the equivalent dose in organ or tissue.

w_{T} is the tissue weighting factor for each organ or tissue.

B.10.3 Compound Supply

Compound supply for radiolabeled human studies is described in the chapter entitled "Synthesis of Radiolabeled Compounds for Clinical Studies" of this book (Atzrodt and Allen).

B.10.4 Clinical Study Design

The design of a typical hADME study for a candidate drug (XYZ1234) is presented below as a protocol outline. 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.

B.10.4.1 Study Title

Excretion balance and pharmacokinetics after administration of 10 mg [^{14}C] XYZ1234 to healthy male subjects. Single center, open label, nonrandomized, and single oral dose study

B.10.4.2 Primary Objectives

- To determine the excretion balance and systemic exposure of radioactivity after oral administration of [^{14}C] XYZ1234 to humans
- To determine the pharmacokinetics of XYZ1234 and its contribution to overall exposure of radioactivity
- To collect samples in order to determine the metabolic pathways of XYZ1234 and identify the chemical structures of the main metabolites. (It should be noted that metabolism investigations can exceed 12 months from sample collection while the summary of the clinical trial report is expected to be available within 1 year of the end of the trial (European commission 2005).

Therefore, it is recommended that a separate metabolite profiling and identification study plan is conducted and reported separately from the clinical study report covering radio , pharmacokinetics, and mass balance evaluation.)

B.10.4.3 Study Design

A single center, open label, single administration of XYZ1234 (10 mg) as an oral solution in fasted healthy male volunteers.

Subjects enter the clinical site on the morning one day before dosing and remain at the study site at least 7 days after dosing.

B.10.4.4 Sample Size

Healthy male subjects aged 30 55 years will be selected from the panel of volunteers recruited by the CRO.

It is planned that six subjects will be enrolled in the study. Four subjects must complete the study (no replace ments unless more than two dropouts).

To ensure that six subjects will be dosed on Day 1, at least eight subjects found to be eligible after screening visit will be hospitalized in the unit on Day 1.

B.10.4.5 Treatments

After overnight fasting, a single oral dose of ^{14}C XYZ1234 (10 mg, 3.7 MBq) will be administered as a solution.

B.10.4.6 Inclusion Criteria

Demography

1. Healthy male subjects, between 30 and 55 of age inclusive
2. Body weight between 50.0 and 95.0 kg inclusive, Body Mass Index between 18.0 and 28.0 kg/m^2

Health Status

3. Certified as healthy by a comprehensive clinical assess ment (detailed medical history and complete physical examination)
4. Normal vital signs after 10 min resting in supine posi tion: 95 mmHg $<$ systolic blood pressure $<$ 140 mmHg; 45 mmHg $<$ diastolic blood pressure $<$ 90 mmHg; 40 bpm $<$ heart rate $<$ 100 bpm
5. Normal standard 12 lead ECG after 10 min resting in supine position; 120 ms $<$ PR $<$ 220 ms, QRS $<$ 120 ms, QTc $\leq$ 430 ms
6. Laboratory parameters within the normal range unless the Investigator considers an abnormality to be clini cally irrelevant for healthy subjects; however, serum creatinine and hepatic enzymes (AST, ALT) should be strictly below the upper laboratory norm; creati nine clearance (acc. to Cockroft Gault formula) must be $\geq$50 mL/min

Regulations

7. Having given written informed consent prior to any procedure related to the study
8. Covered by Health Insurance System where applica ble and/or in compliance with the recommendations of National Law in force relating to biomedical research
9. Not under any administrative or legal supervision

B.10.4.7 Exclusion Criteria

Medical History and Clinical Status

1. Any history or presence of clinically relevant cardio vascular (including signs of arrhythmia/tachycardia and family or personal QT prolongation), pulmonary, gastrointestinal, hepatic, renal, metabolic, hematolog ical, neurological, psychiatric, systemic (affecting the body as a whole), ocular, or infectious disease; any acute infectious disease or signs of acute illness
2. Frequent headaches and/or migraine, recurrent nau sea, and/or vomiting (more than twice a month)
3. Blood donation within 1 month before administration
4. Symptomatic hypotension, whatever the decrease in blood pressure, or asymptomatic postural hypoten sion defined by a decrease in SBP equal to or greater than 20 mmHg within three minutes when changing from the supine to the standing position
5. Presence or history of drug hypersensitivity, or allergic disease diagnosed and treated by a physician
6. History or presence of drug or alcohol abuse (alcohol consumption $>$ 40 g/day)
7. Smoking more than five cigarettes or equivalent/day, unable to stop smoking during the study
8. Excessive consumption of beverages with xanthine bases (>4 cups or glasses/day)

Interfering Substance

9. Any medication (including St. John's Worth) within 14 days before administration, or within five times the elimination half life or pharmacodynamic half life of that drug, whichever the longest

Biological Status

10. Positive reaction to any of the following tests: Hepatitis B surface (HBs) antigen, anti Hepatitis C virus (anti HCV) antibodies, anti human immunodeficiency virus (HIV)1 antibodies, anti HIV2 antibodies
11. Positive results on urine drug screen (amphetamines/metamphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, opiates)
12. Positive alcohol test

Regulations

13. Failure to give written informed consent prior to any procedure related to the study

Specific to the Study and the Compound

14. Any history of orthostatic dysregulation (neurocardiogenic syncope, postural orthostatic tachycardia syndrome)
15. Predicted poor metabolizers for CYP2D6, CYP2C9, and CYP2C19
16. Subject with specific dietary habits, such as vegan or vegetarian
17. Subject with irregular bowel habits (more than 3/day or less than 1 every 2 days)
18. Subject undergoing dental care or presenting dental caries
19. Subjects who are occupationally exposed to radiation
20. Participation in a trial with ^{14}C radiolabeled medication in the 12 months preceding the study
21. Use of radiopharmaceuticals or radionuclides in therapeutic or diagnostic procedures in the 12 months preceding the study (except dental radiography and plain X rays of the extremities)
22. Consumption of grapefruit and commercialized orange juice within 3 days before study drug administration

B.10.4.8 Discharge Procedures

The subjects will remain in the unit for a minimum of 7 days post dose (until T168H). After this period, collection of urine and feces will be stopped and all subjects will be collectively released if

- The combined cumulative excretion of radioactivity in urine, feces, and expired carbon dioxide exceeds a mean of 90% of the administered dose
- And being not less than 85% in any one individual

If by Day 8, the release criteria are not met in all subjects, they will be kept in the clinical unit

- Until release criteria are met for all subjects
- Or until Day 15 (whatever comes first)

If the criteria are still not met on Day 15 in all subjects, they will be released, but have to return weekly to the clinical unit for collection of additional blood samples and single 24 h collections of urine and feces

- Until the cumulative total of urinary and fecal 24 h excretion of radioactivity drops below 0.5% of the administered dose
- Or for a maximum of 4 additional weeks (whatever comes first)

The study period will not exceed 7 weeks after dosing (including End of Study visit). Subjects will collect urine and feces for a 24 h period ending on Days 22, 29, 36, and 43. From Day 22, subjects for which excretion of radioactivity drops below 0.5% of administered dose could be released individually. Subjects will return to clinic within 7 days after the last excreta collection or on the last follow up visit (Day 43) for the End of Study visit.

B.10.4.9 Duration of Study

A minimum stay of 8 days in clinic for sample collection (from Day 1 until morning of Day 8) is mandatory, but could be prolonged up to Day 15 if the release criteria are not met in all subjects.

In case that the release criteria are not met after 15 days of excreta collection, weekly visits (up to Day 43) will be planned for additional 24 h collections of excreta, depending on the radioactivity assessment. The study period will not exceed 7 weeks (including End of Study visit, excluding screening period) after dosing.

B.10.5 Analytic Instrumentation and Methods Used During Preparation, Conduct, and Evaluation of hADME Studies

Various analytical methodologies are used throughout the course of a hADME study from preparation of the study

medication through to the analysis of samples collected in the study. *Table B.10 8* gives an overview on these methods and the area within a hADME study in which they are applied.

B.10.5.1 Metabolic Profiling

Metabolic profiling is defined here as the separation of a drug from its metabolites for the purpose of detection, quantitation, and identification (section Structure Elucidation). A well performed metabolic profiling experiment should provide a comprehensive view of the total drug related material in a sample, and which of those components are quantitatively the most important. Typical samples analyzed are plasma, urine, and feces, but in certain circumstances, other biofluids could be analyzed, such as cerebrospinal fluid, saliva, semen, and, more rarely, tissue biopsies. Interpretation of the metabolic profiles of all samples allows the overall metabolic disposition of a drug to be determined.

In the beginning, a choice must be made as to which samples will be analyzed. While samples are collected at many timepoints, all of which could be analyzed, the nature of metabolic profiling experiments, as described below, favors a reduction of sample numbers. This could be achieved by selecting specific samples for analysis (Pieniaszek et al. 1999) or by pooling samples from different subjects (Affrime et al. 2000) or pooling timepoints from an individual subject. The latter is more common (Cook et al. 2003; Roffey et al. 2003; Rodrigues et al. 2003; Bu et al. 2004).

For plasma, one approach therefore is to analyze a C_{max} sample and a pool of plasma that is representative of the AUC of total radioactivity from each individual subject. Such a pool can be made following the procedure described by Rodrigues et al. (2003). Additional timepoints may be analyzed to make an estimate of the half lives of various metabolites. ile it may not be possible, or necessary to measure the half life of individual metabolites accurately, some assessment of the half life compared to unchanged drug is valuable.

With urine and feces, the aim is to profile samples representative of the whole dose administered to the volunteers. This could be done by pooling from each sample collection over the course of the study (7 14 days of urine and feces). However, this would likely dilute the drug related materials and make the analysis more complicated. Therefore, a compromise may be necessary, pooling together just early samples containing the majority of the dose a target of 90 95% of the dose could be appropriate in most cases, but 85% may be acceptable (Roffey et al. 2003).

After pooling, the next step in a metabolic profiling experiment is to extract all of the drug related material out of the sample. The aim of this step is to generate an "extract" that contains 100% of the drug related material in a form that is appropriate for the next step: resolution of this material into its component parts. In most cases, this is performed by reverse phase high performance liquid chromatography (RP HPLC; Snyder and Dolan 2006) although other analytical techniques can also be used such as thin layer chromatography (TLC; Wu et al. 1995), gas chromatography (GC; Mori et al. 1984), and capillary electrophoresis (CE; Schieferecke et al. 1998). For RP HPLC, the sample should be in a small volume of liquid, preferably containing a high proportion of water. Typically, the extraction step will involve concentrating the drug related material, reducing the sample volume from several milliliters to a few hundred microliters.

For plasma, the extraction method may simply involve precipitation of plasma proteins with organic solvent (e.g., acetonitrile, methanol), followed by evaporation of the supernatant to concentrate the sample and reduce or remove the organic solvent. Strong acids can be used as an alternative to organic solvents, but are generally a last resort because of the risk of artifacts due to metabolite degradation. As a general rule, the mildest conditions possible should always be used.

The extraction procedure may be even simpler for urine samples (and other largely aqueous biofluids). In some cases, when renal clearance is high and high concentrations of radioactivity are therefore present, urine can be analyzed directly. With no extraction step, it is guaranteed that 100% of the drug related material will be analyzed. More commonly, some concentration of the sample is necessary. This can sometimes be achieved by simply freeze drying a sample and reconstituting it in a small volume of HPLC buffer. A better cleanup of the sample, to remove endogenous components, can be achieved by liquid liquid extraction with an immiscible solvent or solid phase extraction with a solid adsorbing material (Kostiainen et al. 2003). However, both of these options can introduce a degree of selectivity into the extraction process, such that the extract is no longer representative of the drug related material in the original sample.

Extraction from feces can be even more challenging and is necessary when drug and its metabolites are excreted via biliary secretion (or are not absorbed). Simple mixing of a fecal homogenate with an organic solvent (e.g., acetonitrile, ethyl acetate) followed by centrifugation to remove solids can be effective. However, it is rarely so

Table B.10-8

Overview on analytical methodological approaches used in a hADME study

Area	Samples/matrix to be analyzed	Purpose	Instruments/methods	If not considered appropriately, possible drawbacks	Remarks
Dos metry	• An ma t ssue d str but on • Routes and amount of excret on n an ma s	Ca cu at on of effect ve rad at on dose	• Quant tat ve who e body autorad ography • L qu d sc nt at on count ng (LSC)	Expos ng vo unteers on study to a h gher r sk than cons dered appropr ate Subopt ma [a] rad oact v ty dose m ght be app ed	See sect on Dos metry
Character zat on of rad o abe ed drug substance	F na batch to be used for formu at on	• Qua ty contro of drug for adm n strat on • Stab ty determ nat on • Determ nat on of the spec f c rad oact v ty[b] (as bas s for formu at on work,[c] b oana yt cs,[d] and mass ba ance ca cu at ons)	• Ana yt ca HPLC w th UV[e] and rad o detector[f] • Standard ana yt ca techn ques as used for non- abe ed drugs n other c n ca stud es depend ng on drug spec f c t es (e.g., Kar F scher t trat on, on chromatography, etc.) • LSC	mpur t es and poor content or ack of stab ty w have an mpact on metabo c prof ng and overa compar son of the drug/metabo te rat o Th s s part cu ar y cr t ca when the by-product can be formed a so by b otransformat on mpact ng on cons derat ons of metabo te exposure (US-FDA 2008) An naccurate spec f c rad oact v ty (e.g., caused by hygroscopy, or water oss, counter on exchange, so vate format on or d s ntegrat on, etc.) can cause an error n the dose ca cu at on, drug quant tat on n samp es, and poor mass ba ance	Keep mpur t es as sma as poss b e: rad ok net cs can be s gn f cant y a tered by mpur t es w th a sma vo ume of d str but on and/or a ong ha f- fe

Table B.10-8 (Continued)

Area	Samples/matrix to be analyzed	Purpose	Instruments/methods	If not considered appropriately, possible drawbacks	Remarks
Character zat on of formu at on[g]	F na formu at on (random y samp ed)	• Guarantee reproduc b ty by fu character zat on of formu at on parameters • Qua ty contro • Stab ty • Tota rad oact v ty • Un form ty of mass/ content • M crob o og ca safety	See "Character zat on of rad o abe ed drug substance" above P us: so d state character zat on (n case of so d formu at on): by XRPD[h]; part c e s ze d str but on (Cou ter counter, m croscopy,...); spec fic surface determ nat on (e.g., BET[i]) Standard m crob o g ca cu t vat on	Reproduc b ty m ght be d sputed (e.g., n case of so d state character zat on m ss ng) See "Character zat on of rad o abe ed drug substance" above	Whenever a character zat on of so d-state propert es s not poss b e, one shou d cons der to adm n ster the drug as a so ut on
Determ nat on of app ed exact dose	Back-measurement of dose conta ners, sp ed formu at on, w pes, straws (n case of so ut on adm n stered), ...	Ensure qua ty of mass ba ance	LSC[j]	Mass ba ance mpacted by prec s on of dose determ nat on, (n terms of poss b e underest mat on or overest mat on of ba ance) An ncomp ete ba ance can tr gger quest ons regard ng ong ha f- fe, unfavorab e d str but on and retent on n t ssues, or rrevers b e b nd ng	The exact dose adm n stered s fundamenta to the outcome of the study
Rad oana ys s, Rad ok net cs	Standard: B ood, p asma, ur ne, feces, exp red a r	Mass ba ance, routes of excret on, compar son w th drug PK, b ood–p asma d str but on of rad oact v ty	LSC[j], AMS (acce erated mass spectrometry)	An ncomp ete ba ance can tr gger quest ons regard ng ong ha f- fe, unfavorab e d str but on and retent on n t ssues, or rrevers b e b nd ng	Depend ng on the rad oact ve dose app ed, the ntended m t of quant tat on and the ha f- fe expected, the appropr ate ana yt ca method has to be chosen, whether th s s convent ona LSC, or a so-ca ed ow- eve LSC or even AMS

B oana ys s of drug	Standard: P asma, ur ne	Compar son w th other c n ca stud es and for compar son w th rad oana ys s to est mate the amount of metabo tes formed	The standard, va dated b oana yt ca assay used a so for other c n ca stud es, most probab y today: HPLC-MS/MS	f rad oact v ty concentrat ons, drug concentrat ons, and metabo c prof ng resu ts are ncons stent, exp anat ons are necessary	
Metabo c prof ng	Standard: P asma, ur ne, feces	Overv ew of number and amounts of metabo tes to corre ate w th exposure eve s n tox co og ca spec es	HPLC w th n- ne rad odetect on (w th so d or qu d sc nt ator[f]) or fract onated co ect on of the chromatogram accord ng to retent on t me nterva s and measurement v a LSC (on ne, off- ne) or even AMS	The cho ce of an appropr ate HPLC system s cruc a to avo d super mposed metabo tes – wh ch cou d ead to overest mat on of the exposure of a metabo te, w th an mpact on safety assessment (US-FDA 2008)	See sect on Metabo c Profi ng
Metabo te structure e uc dat on	Standard: p asma, ur ne, feces	Structura character zat on of metabo tes, f na prove of dent ty to metabo tes dent f ed n tox co og ca spec es	HPLC-MS (w th the poss b ty to fo ow severa fragmentat on steps) MS for mo ecu ar formu a determ nat on HPLC and NMR n case of ncomp ete c ar f cat on w th MS techno og es	Enant o- or d astereomer or const tut ona somers shou d be conf rmed or exc uded as far as poss b e to avo d add t ona safety cons derat ons or even safety ssues	MS cannot exc ude the super mpos t on of structura y s m ar metabo tes w th s m ar or dent ca retent on t me w thout hav ng reference compounds ava ab e. To obta n a re evant reference metabo tes n t me s not a ways poss b e. Therefore, so at on and pur f cat on w th ana ys s by NMR and LSC m ght offer an a ternat ve source of reference metabo tes See sect on Structure E uc dat on

[a] Suboptima in terms of achieving the objectives of the study due to imitations of instrumentation used for mass ba ance, profi ing, and structure e ucidation.
[b] Radioactivity (measured in Bq or μCi) re ating to a certain amount of ana yte (e.g., per g, mL or mo).
[c] For the exact ratio of drug amount to radioactive dose.
[d] Know edge of the isotope distribution pattern is crucia for accurate quantification of the drug.
[e] Determination of re ated impurities and content determination by ca ibration against reference standard.
[f] Overview of scinti ation counting techniques and recent advances in radioactivity detection; for HPLC, see Kiffe et a . (2008); iquid radiochromatogrphy techniques, see Zhu et a . (2007).
[g] Types most probab y used: powder in the bott e either to prepare a so ution or a suspension, capsu e formu ations, ready to use so utions, individua y prepared suspensions.
[h] X-Ray powder diffractometry to determine crysta modification or amorphism.
[i] For examp e, by N2 gas adsorption isotherms.
[j] Examp e of standard instrument and hints for samp e preparation see Krone (2006).

straightforward and other approaches, as outlined above, need to be used alone or in combination. Combining extraction methods may be effective, but as a general rule, extraction methods should be kept as simple as possible to avoid nonspecific losses, degradation, and extraction of specific components. It is often the case that the efficiency of extraction from feces is lower than optimal and an extraction efficiency of >85% has to be considered sufficient.

Following extraction, the next step is to separate all of the drug derived components from each other so that they can be quantified individually. Typically, this is achieved by RP HPLC with gradient elution (Snyder and Dolan 2006), meaning that the chromatographic stationary phase is less polar than the mobile phase and that the polarity of the mobile phase decreases over the course of the chromatographic analysis. In this way, more polar metabolites, such as drug conjugates, generally elute from the stationary phase first with progressively less polar metabolites eluting sequentially. The aim is to separate all detectable metabolites from each other so that they can be quantified by radioanalysis.

Radioanalysis for metabolic profiling is conducted either online with the chromatographic separation or off line following collection of the HPLC eluent in timed fractions. Online radiodetectors are the fastest option but do not always have the necessary sensitivity, particularly with solid scintillant detection cells. Sensitivity can be improved with liquid scintillant flow cells in which the HPLC eluent is mixed with a liquid scintillation cocktail as it passes out of the HPLC column and into the detection cell. Off line detectors are even more sensitive but have a major drawback of reduced resolution and low throughput. For off line detection, fractions must be taken with sufficient frequency such that no more than one metabolite is contained within a given fraction. This means taking timed fractions of the HPLC eluent every 10 30 s, depending upon the complexity of the metabolism over a metabolic profile that may typically be in excess of 60 min long. Therefore, 96 well format radiodetectors, such as Topcount, have become popular for metabolic profiling as they combine sensitivity with the ability to collect and count a large number of fractions within a reasonable time frame. Liquid scintillation counting can also be used following collection of a suitably sized fraction, at the expense of some resolution of components. Ultimate sensitivity is provided by converting the fractions to elemental carbon and analyzing by accelerator mass spectrometry (AMS; Lappin and Stevens 2008).

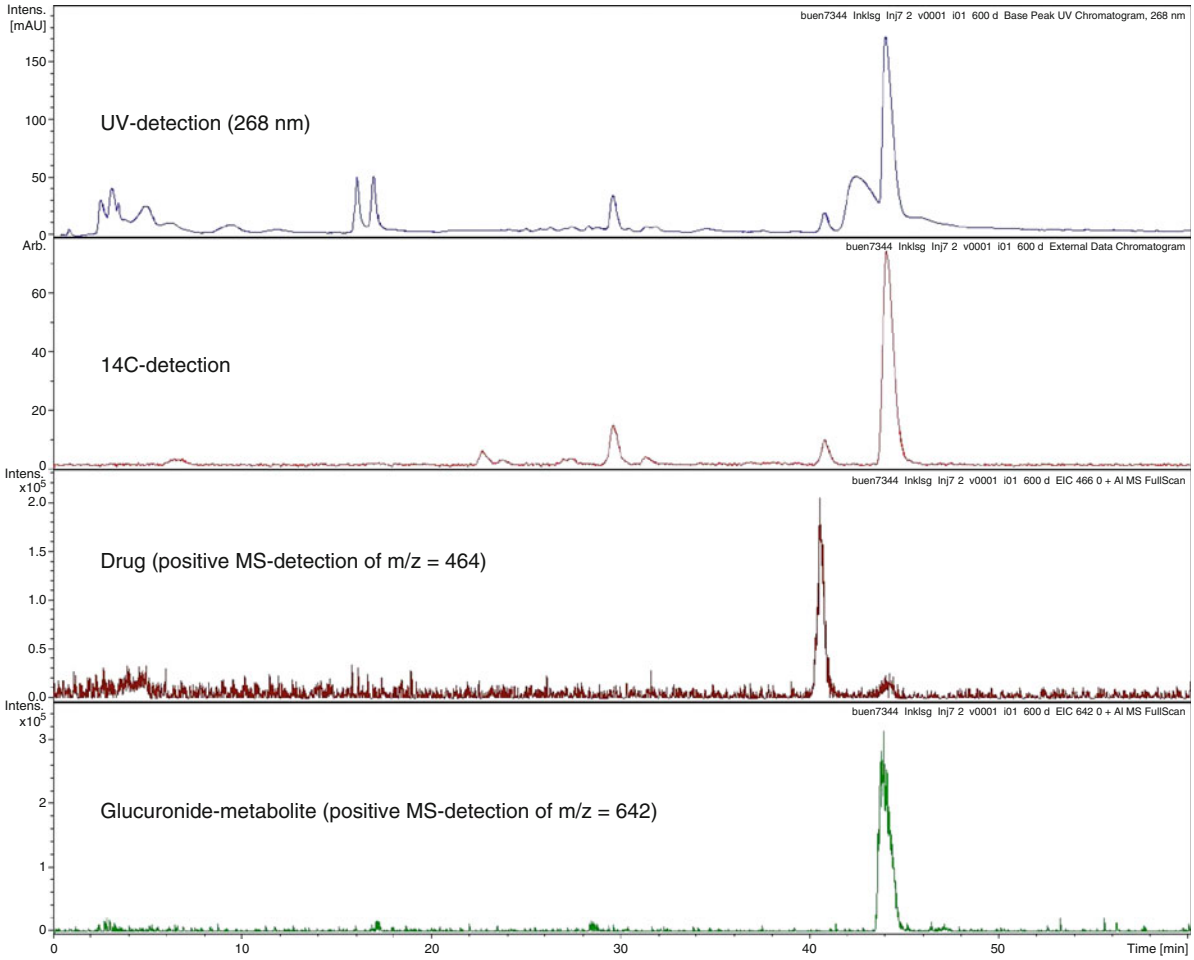
B.10.5.2 Structure Elucidation

Liquid chromatography hyphenated with mass spectrometry (LC MS) has become the standard first tier technique for metabolite structure elucidation. Metabolites detected (via radioactivity) by metabolic profiling (section Metabolic Profiling) can be directly investigated by MS in the same chromatographic run either online, by splitting off a proportion of the HPLC eluent, or off line by collecting fractions for separate analysis.

Selectivity (via mass of drug and specific fragmentation) and sensitivity (assuming sufficient ionization) of modern mass spectrometers have led to this preference for LC MS as a starting point for structure elucidation. (An overview of the different MS and HPLC techniques, as well as sample preparation is given by Kostiainen et al. 2003. A practical example including most of the described analyses is described also by Obach et al. 2005). Comparing the mass spectra of the metabolites with the mass spectrum of the unchanged drug provides a first level of structural information on metabolites. In the example shown in *Fig. B.10 3*, a glucuronide is proposed based on the mass difference to unchanged drug and demonstrated to be the major metabolite. Subsequently, a skillful selection of fragmentation experiments performed with Iontrap technology, to sequentially fragment the metabolite, can pinpoint which parts of the metabolite are unchanged, which parts are altered, and which types of metabolic reaction have been involved, according to the obtained mass differences.

This is illustrated in the example shown in *Fig. B.10 4*. The MS fragmentation steps give a good indication that the main metabolite is in fact a glucuronide by the neutral loss of mass 176 from the molecular ion with m/z 642 to the fragment with an m/z 464. Subsequent fragments were the same as observed for unchanged drug (202, 220, and 300).

Access to TOF MS (time of flight mass spectrometry; a method of mass spectrometry in which ions are accelerated by an electric field. The respective flight time depends on the mass to charge ratio of the particle), Orbitrap (ions move in rings that oscillate along a central spindle. The frequency of these harmonic oscillations is inversely proportional to the square root of the mass to charge ratio.), or FTICR (fourier transform ion cyclotron resonance mass spectrometry; determination of the mass to charge ratio (m/z) of ions based on the cyclotron frequency of the ions in a fixed magnetic field) offers very precise determination of the mass of a molecule that is complimentary to the information provided by ion traps,



After protein precipitation and extraction the UV-chromatogram (top) indicates a relatively clean matrix. The radiochromatogram specifically detects the drug-related compounds. In parallel the MS scans following the mass of the drug and the mass of an assumed glucuronide indicated the glucuronide as the main metabolite in the sample investigated.

Figure B.10-3

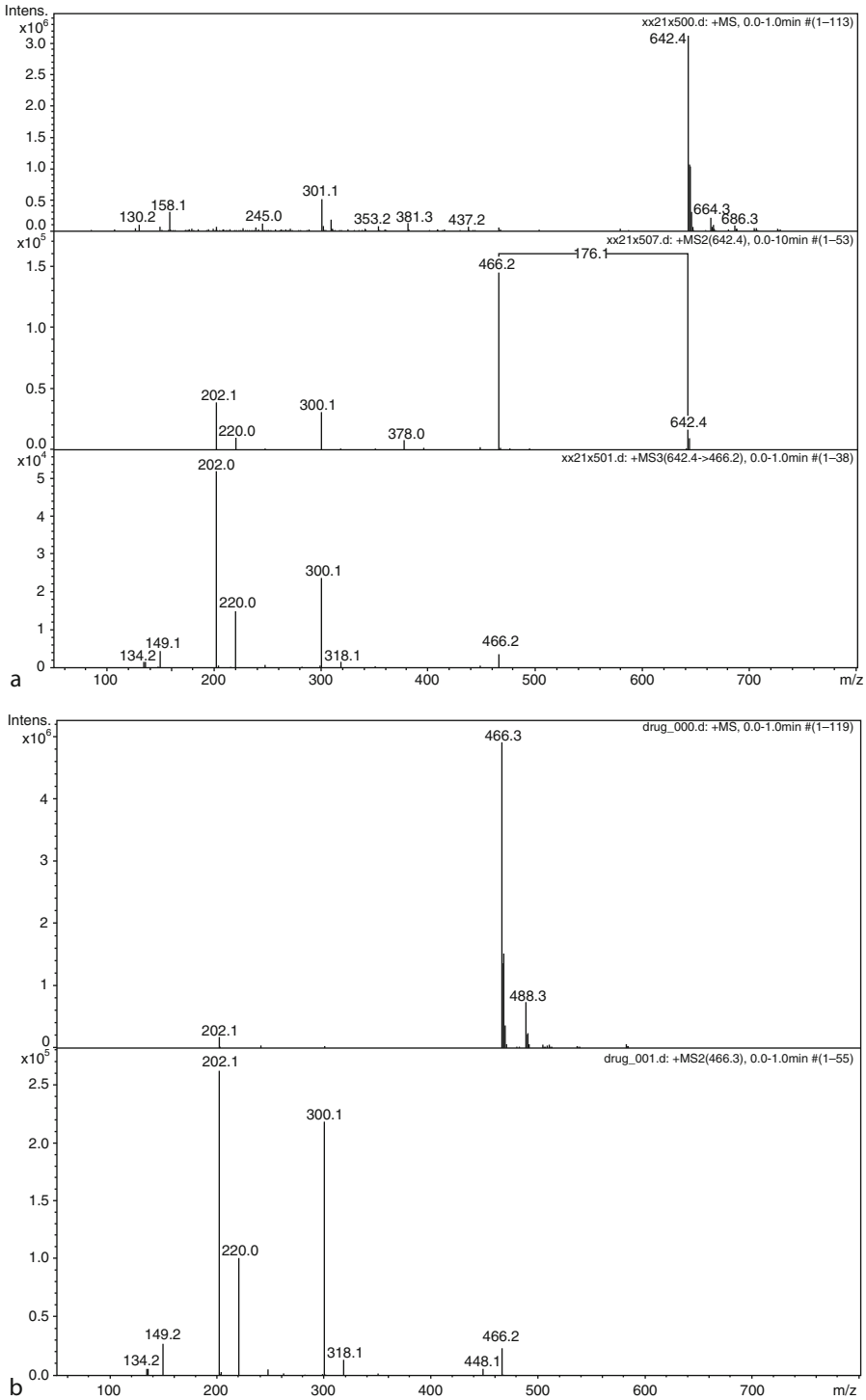
Identification of a glucuronide as the main metabolite in matrix

allowing the elemental composition of metabolites and their fragments to be reduced to a few possibilities or even assigned unequivocally.

For example, structure elucidation of another glucuronide using MS mode for the exact mass determination of a metabolite (395.07968 U) and the MS^2 mode (fragmentation of the m/z 395 signal in the negative ion mode) allowed the molecular formula to be assigned (*Fig. B.10 5*).

The molecular formulas as given in *Table B.10 9* were proposed based on the exact mass. The first formula in the table ($C_{18}H_{19}O_8S$; delta 0.42 ppm) was consistent with the putative glucuronide. The elemental composition fitted closely with that expected from the drug (e.g., number of carbon and sulfur atoms), and the mass difference observed between the measured mass and the theoretical mass of $C_{18}H_{19}O_8S$ was the lowest, making this the most likely structure.

These mass spectrometry experiments can be very efficiently conducted in line with radioanalysis and are often sufficient as final structural information particularly when reference standards are available for comparison or



(a) Putative glucuronide; MS^3: m/z 642=>464=>202/220/300.

(b) Original drug; MS^2: m/z 464=>202/220/300.

Figure B.10-4

Comparison of MS fragments obtained from the glucuronide

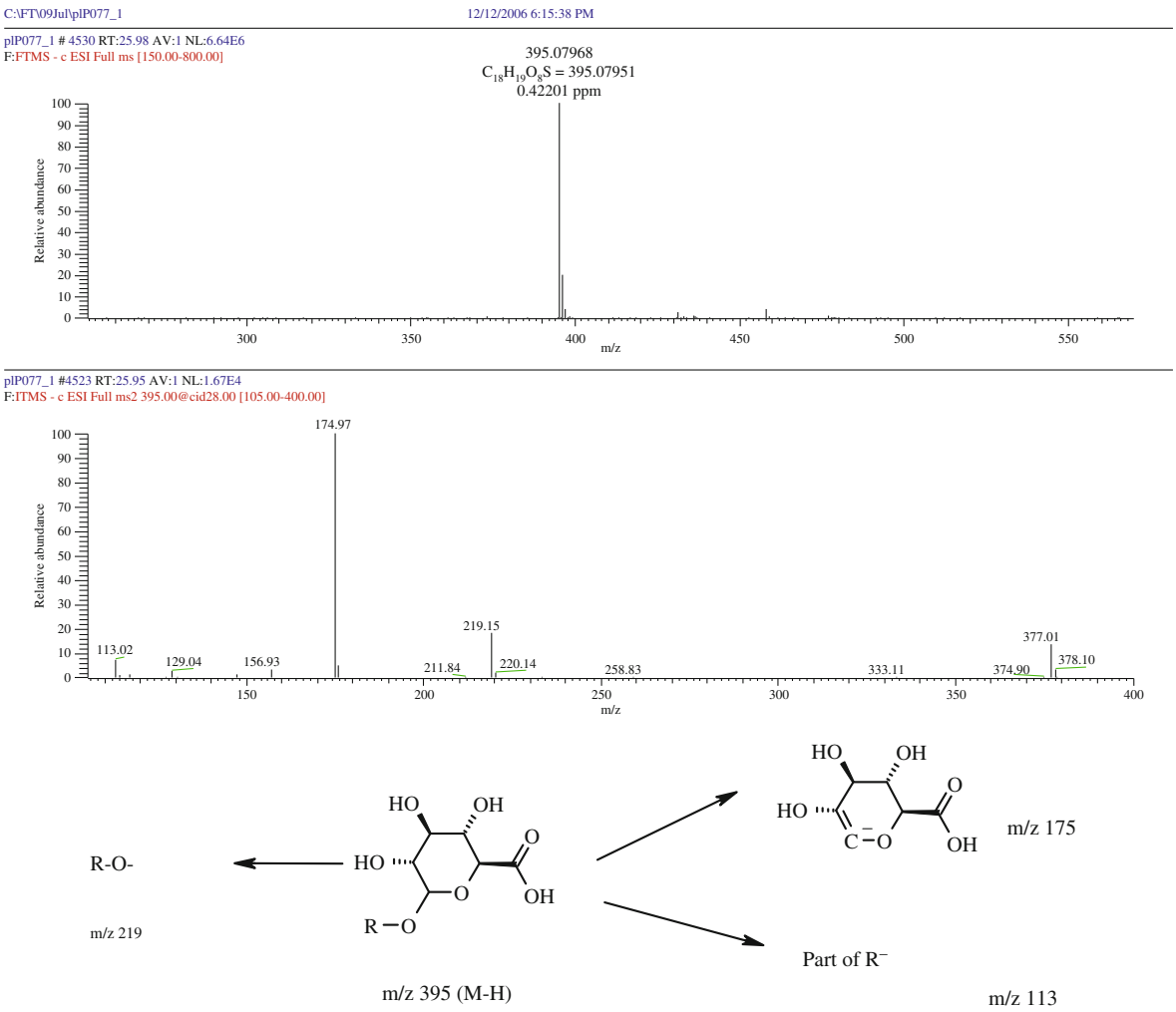


Figure B.10-5

Exact mass determination of a metabolite, fragmentation in the MS^2 mode

Table B.10-9

Proposed compositions based on exact masses

m/z	Theoretical mass	Delta (ppm)	Composition
395.07968	395.07951	0.42	$C_{18}H_{19}O_8S$
	395.07817	3.82	$C_{16}H_{17}O_7N_3S$
	395.08154	4.72	$C_{13}H_{21}O_7N_3S_2$
	395.08289	8.11	$C_{15}H_{23}O_8S_2$
	395.07549	10.6	$C_{13}H_{19}O_{10}N_2S$
	395.08422	11.5	$C_{16}H_{19}O_4N_4S_2$

the metabolite has been previously proven in animal species used in toxicology studies. For example, the loss of a methyl group in a molecule bearing a methoxy group can often be definitively identified by mass spectrometry without recourse to other techniques.

However, despite high selectivity and sensitivity of mass spectrometers, both features have their limitations. HPLC fractions already obtained during metabolic profiling with off line radiodetection may already provide a sufficiently clean sample with chromatographically separated metabo lites to improve sensitivity compared to online LC MS of unfractionated samples. Nevertheless, it may be necessary

to develop additional chromatographic and concentration steps to resolve sensitivity issues. Insufficient HPLC separation or insufficient selectivity in terms of possible isomers also requires additional effort. For example, of particular relevance to drug metabolism studies, mass spectrometry cannot always discriminate between different positions of hydroxylation, especially aromatic hydroxylations. In such a case, nuclear magnetic resonance investigations (NMR) are most helpful (Also as an independent second analytical method to minimize risk of misinterpretation). The disadvantage of the additional NMR experiments is the amount of analyte to be isolated and cleaned up (The progress of purification from a several thousand fold excess of endogenous compounds can be followed nicely in 1D 1H spectra. Purified metabolite fractions, controlled by NMR, also provide a basis for more detailed, reliable MS investigations. During HPLC, coeluting metabolites/isomers can be finally identified) (normally in the µg range, dependent on the type of NMR instrumentation and experiments) (Common NMR experiments for metabolite structure elucidation that can be performed, for example with a Bruker DRX 600 ultrashielded and a 1.7 mm cryo microprobe; ^{1}H ^{13}C ^{15}N inverse with z gradient (1.7mm CP TCI) are: 1D proton spectra; 1D selective and HH TOCSY (total correlation spectroscopy); 1D selective and HH NOESY (Nuclear Overhouser Enhancement Spectroscopy); J resolved; HH COSY (correlation spectroscopy); HH ROESY (Rotating frame Overhouser Enhancement Spectroscopy); ^{1}H ^{13}C and ^{1}H ^{15}N HSQC (Heteronuclear Single Quantum Coherence); ^{1}H ^{13}C and ^{1}H ^{15}N HMBC (Heteronuclear Multiple Bond Coherence)). Exhaustive fractionation, isolation, and cleaning steps (starting in many cases with preparative HPLC, followed by semipreparative and analytical HPLC steps with various eluent gradients or with specific isocratic conditions) are necessary (*Fig. B.10 6*). Normally, the amount of metabolites necessary for these investigations cannot be isolated from the small plasma samples usually available for bioanalysis or metabolism (in the range of a few milliliters) (An exception is described by Dear et al. 2008 using 30 100 mL of human plasma using unlabeled drug). Thus, separation, isolation, and cleaning of metabolites are usually from urine and feces samples. The purified, structurally identified, and even (by NMR elucidation) quantifiable and certifiable metabolite fractions can be used as a reference for the plasma profiling of the hADME study and to prove the existence of the metabolite in the plasma of animal species used for safety testing.

Since NMR is the preferred spectroscopic method to determine proximity relations, following typical structural information can be gained which are normally not accessible via MS:

- Position of hydroxylation in aliphatic chains, aliphatic, and/or aromatic ring systems
- Position of phase II metabolite substituents (e.g., in case of several options for the position of a glucuronide or the type of connection to the glucuronide for instance, in case of transacylation)
- Structural rearrangement as seen sometimes in case heterocycles are affected by metabolism (example described by Doss et al. (2004) using MS and NMR to prove the rearrangement of a piperazine containing drug to two metabolites consisting of ring contracted imidazoline moieties instead)
- Indication for isomerization
- Steric position of the new substituent caused by metabolism

For example (*Fig. B.10 7*), several metabolites with a glucuronide group at different positions of a pyranose moiety of the drug could be structurally assigned only by NMR spectroscopy (mainly by heteronuclear muliple bond coherence experiments).

The hyphenation of HPLC directly to NMR (LC NMR) has not become as important as LC MS for metabolite structure elucidation. As described above, the amounts and the purity of metabolites requiring several consecutive HPLC steps are often handled more favorably with the separate techniques. However, some examples exist in literature (even hyphenating LC NMR MS) mostly with drugs of low molecular weight and concise structure (e.g., Shockcor et al. 2000).

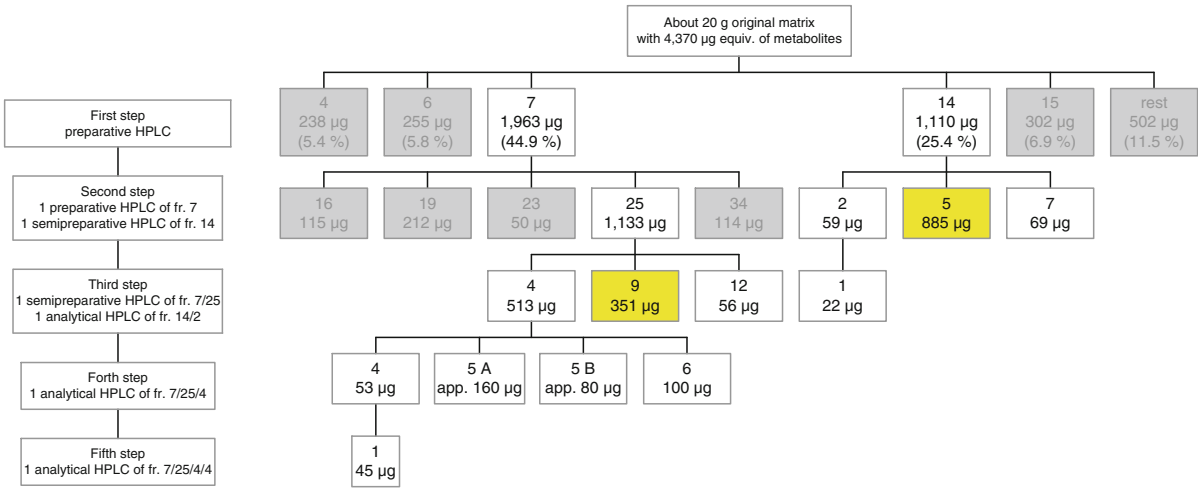
It should be noted that valuable additional information regarding metabolite structure elucidation can be obtained by enzymatic cleavage reactions (such as β glucuronidase or sulfatase) or by hydrogen deuterium exchange to get knowledge about the number of exchangeable hydrogen atoms.

EVALUATION OF PHARMACOKINETICS, RADIOKINETICS, MASS BALANCE, AND METABOLIC PROFILING

Taken in part from Frick et al. (2006).

The following pharmacokinetic data are derived from the hADME study:

- Radioactivity administered orally and radioactivity recovered in whole blood, plasma, urine, feces, and expired air



The numbers at top of the respective boxes symbolize the fraction number (specific for each HPLC step) analyzed at least for the radioactive content and thus, containing the information on quantity. The percent-values given in the scheme correspond to proportion of drug related material in the collected fractions. Although the first profile (first step) resulted in only five main metabolite signals (besides additional small peaks amounting to 11.5% in total), the subsequent separation steps applied on the two initial main fractions (fr. 7 + 14) disclosed several additional metabolites coeluting in the first chromatographic step. Only two metabolites out of the scheme (marked in yellow, No. 5 and 9) were assessed as relevant regarding the examined matrix (due to their amount in the range of or higher than 10% of the original radioactivity of the sample with regard to some loss during clean up). However, the white boxes indicate metabolites of interest because of comparable metabolite retention times with metabolites considered relevant in human plasma and therefore these metabolites were also identified by NMR. The fractions in the dark grey boxes were not investigated further due to their retention times indicating no relevance for the plasma profile comparison.

Figure B.10-6

Example of a fractionation process scheme

- Concentrations of unchanged compound and (if appropriate) metabolite(s) in plasma and urine
- Metabolic profiles: number of, and radioactivity attributable to, each metabolite (or at least each radiochromatographic peak) in plasma, urine, and feces
- Structures of observed metabolites (if possible)

Due to the small sample size, all results are only presented descriptively for the different bioanalytical and pharmacokinetic data calculated: number of relevant observations, geometric mean, geometric standard deviation, arithmetic mean, standard deviation, coefficient of variation, median, minimum, and maximum.

If measured radioactivity for plasma, blood, and urine is related to the weight of a sample, as is often the case, the results are reported on a volume basis for comparison to bioanalytical data. To make the conversion, a constant specific gravity of blood (1.05 g/mL), plasma (1.03 g/mL), and urine (1.02 g/mL) can be assumed for all subjects. Radioactivity measurements are reported in terms of concentration of radioactivity (dpm/mL or Bq/mL) and as concentration of drug equivalents (µg equivalents per mL sample [µg eq/mL]) calculated from the specific radioactivity of the original compound (Bq/µg).

All metabolites are characterized using retention times and mass spectrometric data when possible. In order to track metabolites throughout the various metabolism studies, a consistent metabolite nomenclature is required. Typically this may involve an arbitrary number (1 to *n*) in the order of the chromatographic elution. A suffix letter can be used to name co eluting metabolites or to identify metabolites not named in a previous study, for example, M2b.

Sample extraction recoveries are calculated using measured radioactivity in samples and sample extracts. Recoveries are expressed as percentage of radioactivity in the samples prior to extraction. Extraction losses, if they can be reasonably considered as nonspecific, are often not taken into account in the final data. However, if recovery is particularly poor and apparently specific, it may be necessary to account for recovery.

In radiochromatograms, peaks are quantified using peak area integration. Quantitative evaluation of

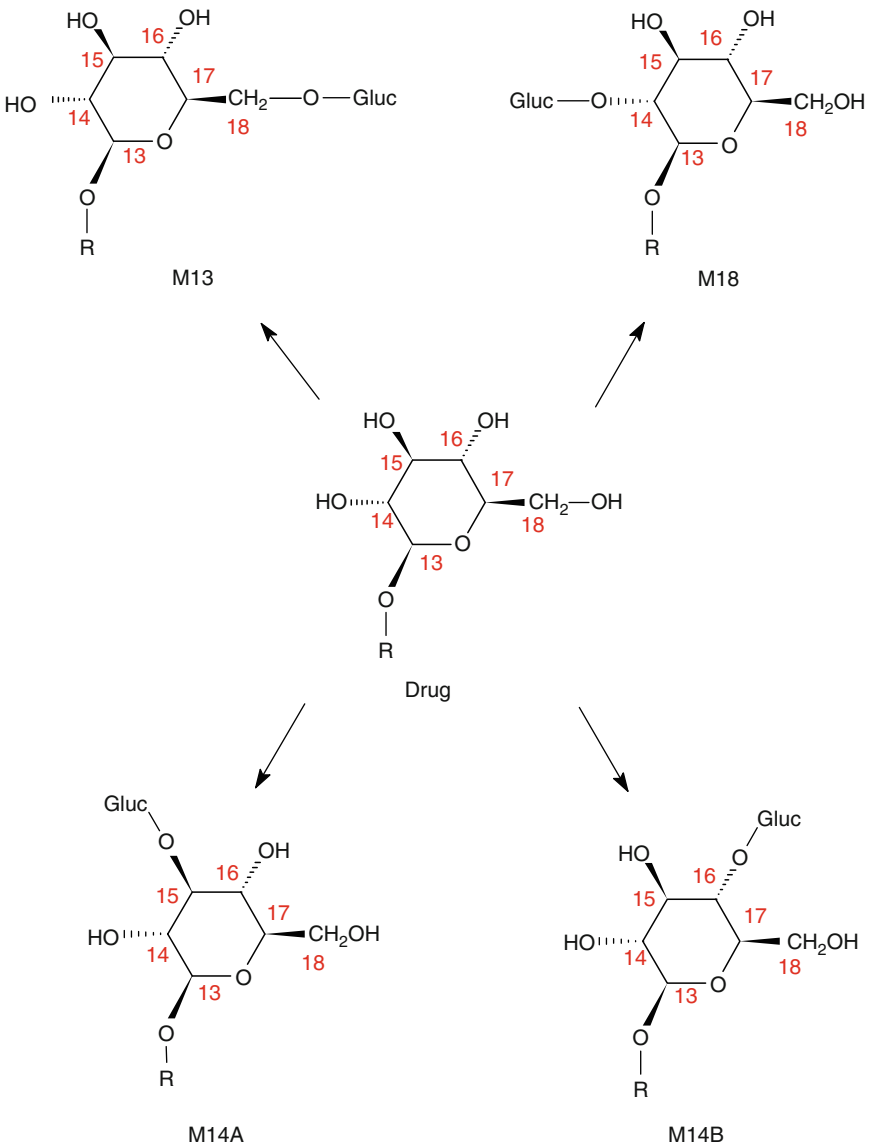


Figure B.10-7
Different glucuronide positions of a drug pyranose-moiety assigned by NMR-spectroscopy

unchanged compound and metabolites in plasma and excreta are expressed as a percentage of radioactivity in each matrix (% metabolite), calculated as follows:

$$\%\_metabolite = \left[\frac{Metabolite\_Area\_counted}{Total\_peak\_detected}\right] \times 100$$

where

Metabolite Area counted is the peak area.

Total peak detected is the total radioactivity associated with discrete peaks within the chromatogram.

In addition, the time course of major plasma metabolites is determined from the plasma concentration at each measured timepoint, calculated as follows:

$$Metabolite\_Concentration = \frac{\%\_metabolite \times Total\_plasma\_radioactivity\_concentration}{100}$$

where

Total plasma radioactivity concentration is the mean concentration of total radioactivity in plasma at each measured timepoint.

Quantitative evaluation of unchanged compound and metabolites in urine and feces as well as in the sum of urine and feces are expressed as a percentage of the administered dose for each compound in each excreta (% dose), calcu lated as follows:

$$\%\_dose = \left[\frac{Metabolite\_Area\_counted}{Total\_peak\_detected}\right] \times \%\_dose\_excreta$$

where

Metabolite Area counted is the peak area.
Total peak detected is the total radioactivity associ ated with discrete peaks within the chromatogram.
% dose excreta is the percentage of the administered dose excreted during the selected period.

A limit of detection (percentage of radioactivity or percentage of dose) is estimated for each sample analyzed, based on a signal to noise ratio of 3.

CRITICAL ASSESSMENT OF THE METHOD

Roffey et al. (2007) defined the objectives of a human mass balance study as follows:

1. Is the proposed clearance mechanism sufficiently supported by the identities of the drug related mate rials in excreta, so as to provide a complete under standing of clearance and potential contributors to interpatient variability and drug drug interactions?
2. What are the drug related entities present in circula tion that are the active principals contributing to pri mary and secondary pharmacology?
3. Are there findings (low extraction recovery of radiola bel from plasma, metabolite structures indicative of chemically reactive intermediates) that suggest poten tial safety issues requiring further risk assessment?
4. Do questions 2 and 3 have appropriate preclinical support in terms of pharmacology and safety pharmacology?

For the predictiveness to the market situation factors such as dose, age, food conditions (fasted, with food), special population (e.g., poor/extensive metabolizers), and of course the limitation in subject size in this study are of importance. However, the hADME study does not stand alone in the submission dossier. The information retrieved from this study together with the pharmacoki netic data on parent compound and perhaps important metabolites from all other clinical pharmacokinetic stud ies (including dose effect, drug drug interaction, food effect, patients with kidney and liver impairment, etc.) complete the picture on the ADME characteristics of the drug.

The study described in this chapter contains a large number of exclusion criteria that are specific to the hADME study. Some are related to the use of radioactivity and ensuring that annual dose limits are not exceeded. Others are related to improving the quality of the study results; for example, regular bowel habits and good dental health (particularly for solution formulations when liquid can become trapped in cavities) can both impact on the quality of the mass balance data. Yet others are related to minimizing variability in drug metabolism (excluding poor metabolizers, specific dietary habits, concommitant medication, and grapefruit intake), which may be unreal istic in the target population, but considering the small number of subjects in the study, it is necessary to reduce external sources of variability. Even so, significant variabil ity in metabolic profiles can occur (see section Exam ples, Example 3: Metabolic Profiling, and *Fig. B.10 13*).

The completeness of mass balance found in the hADME study is of special importance in the evaluation and interpretation of the study. Roffey et al. (2007) argue not to follow a strict balance to be achieved in human mass balance studies. They performed a literature survey of 171 drugs, resulting in a mean overall recovery of 89% ± 11%. Potential causes of low mass balance, discussed in the article, were non covalent tissue sequestration, affinity for phospholipids, binding to specific proteins in tissues, binding to melanin, other mechanisms of non covalent sequestration such as disulfide bond formation with pro teins, covalent binding, and compliance issues.

A comprehensive review of mass balance studies in the field of anticancer drugs giving detailed recommendations on design, conduct, and evaluation of this type of study was published by Beumer et al. (2006). In this article a mass balance of 90% was regarded as a target value to take into account all analytical variation in addition to the factors described by Roffey et al. (2007).

The target balance has a direct influence on the dis charge criteria defined in the study protocol. The higher the expected recovery, the stricter the criteria that have to be applied, that is, the longer subjects have to be kept in the clinical unit for continued sample collection. This also influences the willingness of subjects to enroll on the study. Thus, mass balance and practicality of the study can be counteractive components.

To obtain information on the absorption of an orally administered drug is only possible in rare situations, for example, if a second arm following intravenous administration is included into the study. This, however, is rarely the case. Thus, only estimations of oral absorp tion are possible, for example, based on the amount of renally excreted radioactivity as compared to the overall

dose as a minimum extent of absorption to be shown by the study.

A critical factor in performing the hADME study is the timing of the study. The US FDA guideline on metabolites in safety testing (US FDA 2008) expresses the need to perform metabolite exposure assessment and following toxicological tests of metabolites potentially needed before large scale clinical trials. Although the assessment of both, abundance of a metabolite in humans and its exposure ratio versus the animals species used in toxicological trials does not require a radiolabeled human study, only this study is able to guarantee that no major or even unique human metabolite have been overlooked.

Thus, the timing of the hADME study in clinical development is affected and these studies are increasingly being performed as early as possible in clinical development (Deroubaix and Coquette 2004) not only to evaluate the major human metabolites in the context of metabolite exposure in animal toxicity species.

In addition, the study can help developers to understand safety and efficacy results in the target patient population or special populations and can support the prediction and management of drug interactions and high risk populations. These advantages of conducting the study early have to be balanced against possible uncertainties related to the therapeutic dose (preferably within a factor of 3 versus the dose used in the hADME study) 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

As discussed above, the design of the clinical study does not cover all types of conditions and populations that the drug is designed for but should be regarded as a "probe study" to investigate the ADME properties under exemplary conditions in combination with the highest likelihood to achieve its goals.

Thus, the study is typically conducted as a phase I study in healthy volunteers, usually all male with normal liver and kidney function and a restricted age range. Subjects are often screened to ensure only extensive metabolizers are enrolled (at least for CYP2D6, CYP2C9, and CYP2C19), and the study is usually conducted under fasting conditions. Each of these parameters can be modified, or additional cohorts included, on a case by case basis to make the study more predictive of the market situation, but this must be carefully considered from a regulatory and drug development perspective. For example, a drug designed specifically for a female only indication may warrant a hADME study in females, but the exposure of women of child bearing potential to radioactivity has to be considered (postmenopausal subjects may be an alternative as in Vos et al. 2002) as should the design of the QWBA study supporting dosimetry.

The use of a solution instead of a tablet or capsule might be appropriate in order to achieve higher oral absorption and, thus, higher plasma exposure with the benefit to analyze and identify drug metabolites easier at higher plasma levels although under conditions less close to the market situation.

For ethical reasons, cytotoxic compounds, for example, in oncology (Beumer et al. 2006) require a hADME study to be performed in patients instead of healthy subjects, which brings another hurdle in the organization of the study.

Analytical alternatives exist in the application of AMS analyses as described above or in the application of stable isotopes (Mutlib 2008).

In the context of establishing plasma exposure of metabolites in order to support the considerations of animal versus human metabolite exposure, the hADME study, even under single dose administration, plays a critical role. In order to gain the necessary information earlier, strategies exist on the basis of nonradioactive determinations and metabolite explorations (e.g., Luffer Atlas 2008). Nevertheless, the conduct of a conventional radioactive hADME study validates the predictiveness and completeness of the metabolite information obtained by other means and appears still as an indispensable tool in drug development.

B.10.6 Examples

B.10.6.1 Example 1: Dosimetry

This example shows the procedure of dosimetric calculations using digitized autoradiograms obtained after administration of radiolabeled test compound to a laboratory animal (*Figs. B.10 8 B.10 10*, *Table B.10 10*).

B.10.6.2 Example 2: Study Design and Kinetic and Mass Balance Results

To illustrate the type of data that can be obtained, a high level summary of the pharmacokinetic results obtained from the study described above under *Procedure* is presented below (taken from Frick et al. 2006).

(i) *Balance of excretion*: On average, 34.6% of the administered radioactivity was excreted in urine and 60.6%

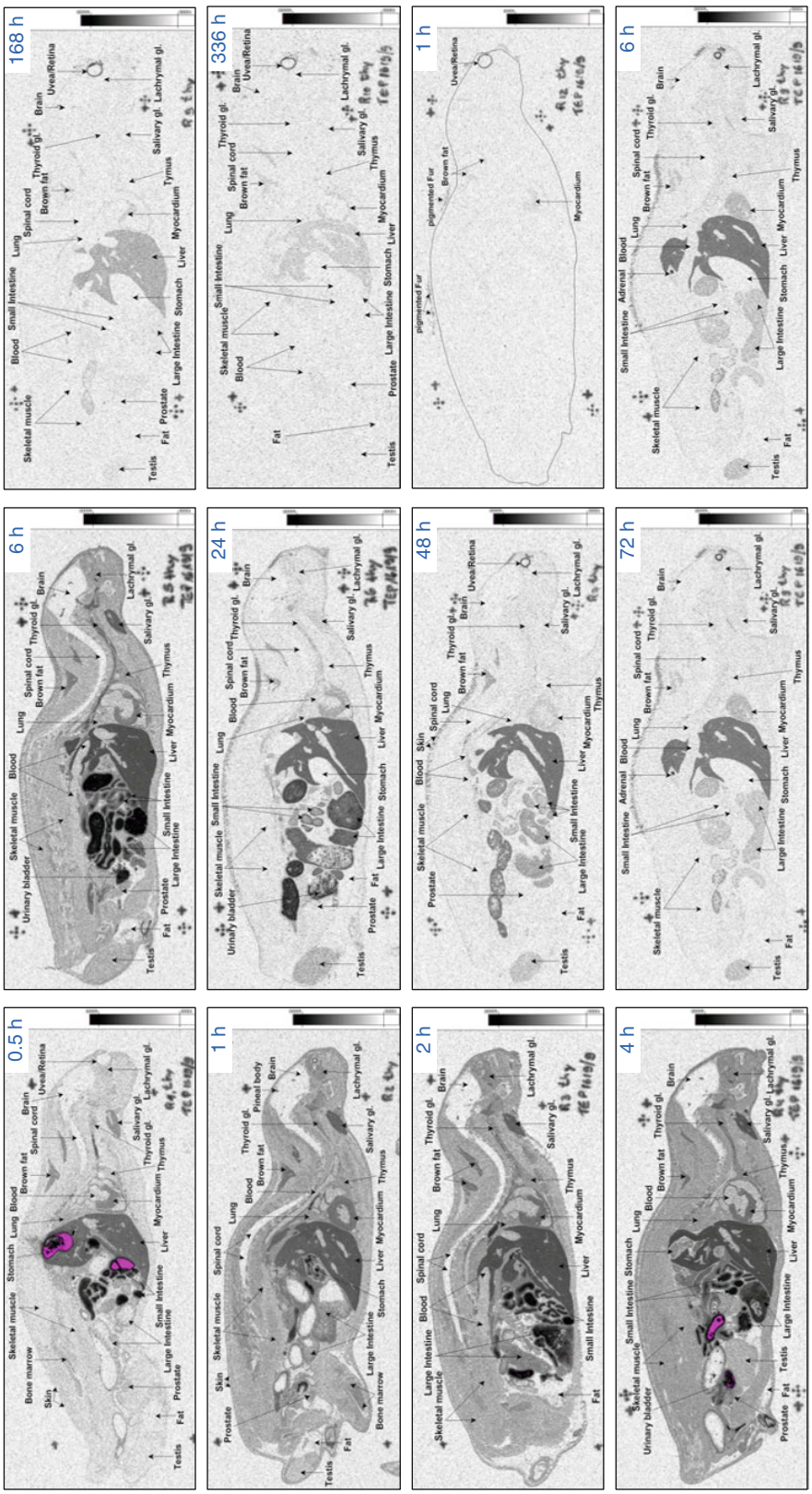
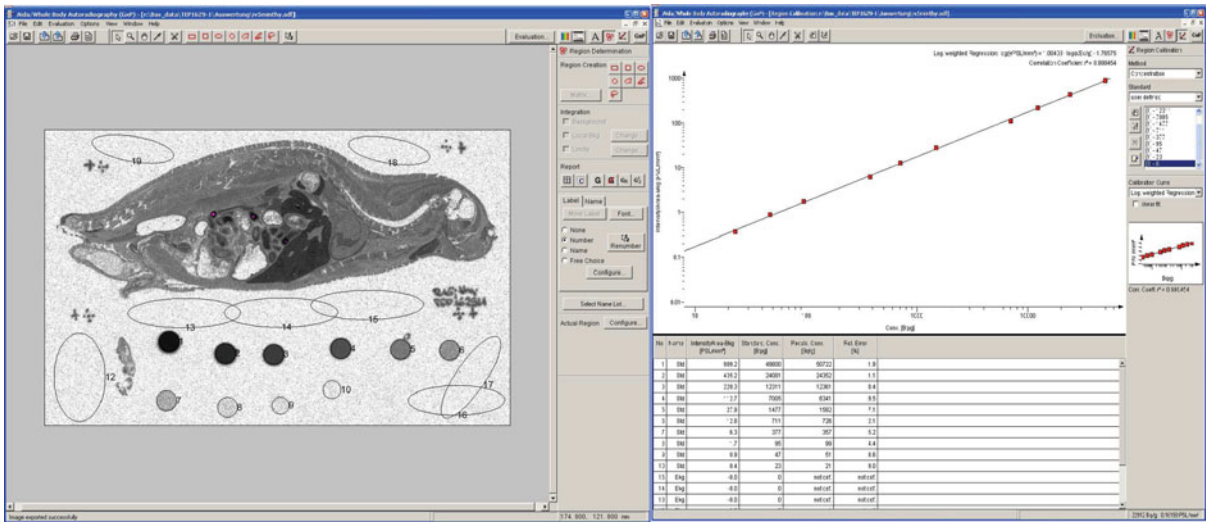


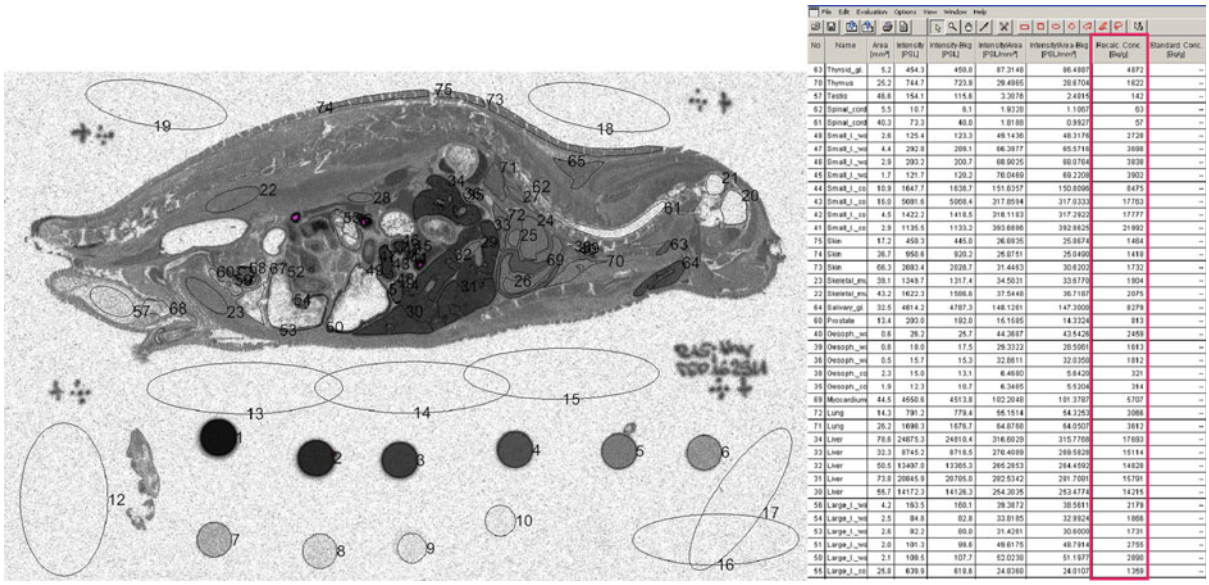
Figure B.10-8
Distribution pattern of radioactivity after oral dose (Long Evans rat)



Standards with defined radioactivity concentrations (circles beneath the animal; Bq/g) allow a link between the signal of the analysis system (greyscale; PSL/mm²) and the radioactivity concentration. Together with the known specific radioactivity of the labelled compound the concentration can be calculated in organs and tissues.

Figure B.10-9

Calibration of the digitized autoradiogram



Calculation of the concentrations of the radiolabelled compound and/or its metabolites.

Figure B.10-10

Analysis of the digitized autoradiogram

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 elim inated in the urine in a form different than XYZ1234 or XYZ5678, as one or several other metabolites.

Table B.10-10

Calculation of the committed effective dose (CED) expected in man after administration of 1 MBq of the radiolabeled test compound

Calculation of CED in man					
Listed tissues	**D (μSv)**	**Weighing**	**Eff dose (μSv)**	**Eff dose (mSv)**	
Testis	6.826	0.20	1.3651		
Lower large intestine (colon)	2,541.044	0.12	304.9253		
Lung	0.126	0.12	0.0151		
Red bone marrow	0.885	0.12	0.1062		
Stomach (gastric wall + content)	57.715	0.12	6.9258		
Bladder	0.227	0.05	0.0114		
Breast	0.116	0.05	0.0058		
Liver	0.574	0.05	0.0287		
Esophagus	91.389	0.05	4.5694		
Thyroid	0.066	0.05	0.0033		
Bone surface	6.724	0.01	0.0672		
Skin	1.531	0.01	0.0153		
MWM of additional tissues	**2.202**	0.05	0.1101		
CED per MBq			**318.1487**	**0.318**	
Additional tissues	**D (μSv)**	**Weighing**	**Eff dose (μSv)**	**Mass (man, kg)**	**D * mass**
Adrenals	0.273	0.05	0.0136	0.01400	0.0038
Brain	0.102	0.05	0.0051	1.40000	0.1429
Myocardium	0.228	0.05	0.0114	0.33000	0.0752
Kidneys	0.497	0.05	0.0249	0.31000	0.1541
Pancreas	0.885	0.05	0.0442	0.10000	0.0885
Prostate	1.147	0.05	0.0574	0.01600	0.0184
Spleen	0.430	0.05	0.0215	0.18000	0.0774
Uveal tract	127.200	0.05	6.3600	0.00075	0.0954
Gut wall	151.368	0.05	7.5684	0.64000	96.8756
Thymus	1.659	0.05	0.0829	0.02000	0.0332
Adipose tissue	0.116	0.05	0.0058	13.50000	1.5661
Blood	0.199	0.05	0.0100	5.50000	1.0949
Pituitary gland	1.843	0.05	0.0922	0.00060	0.0011
Salivary gland	0.442	0.05	0.0221	0.10000	0.0442
Muscle	0.359	0.05	0.0180	28.00000	10.0646
			Mean	**3,341**	**7,356**
		Mass weighted mean		**MWM =**	**2,202**

The calculation is based on the QWBA data and on excretion data coming from a mass balance study in rat (percentage of radioactivity excreted via urine and feces). The effective doses obtained in organs and tissues are estimated using defined organ and tissue weights from both rat and human.

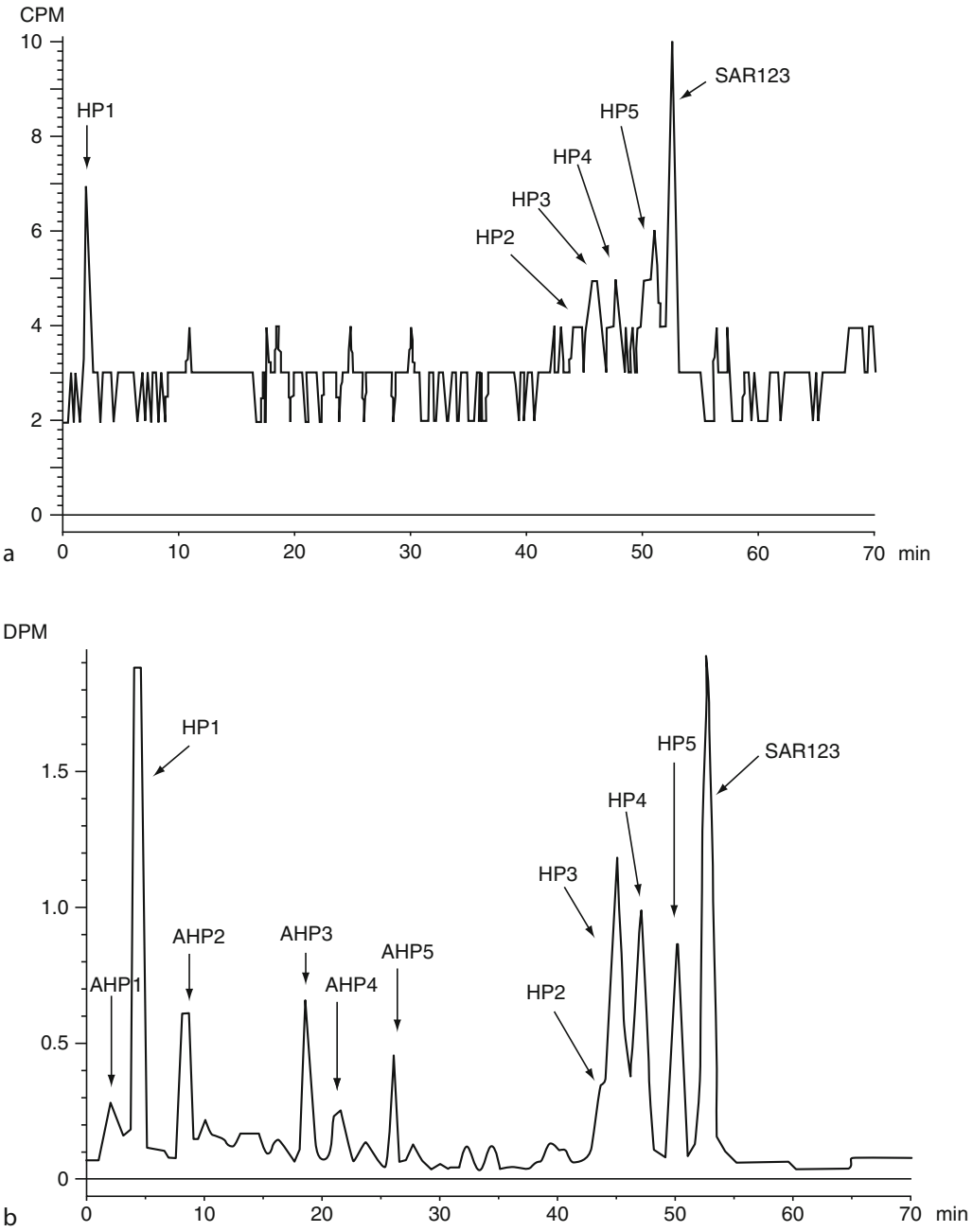


Figure B.10-11

Plasma metabolic profiles generated using (a) Topcount NXT or (b) AMS as a radiodetector

(ii) *Pharmacokinetic parameters in plasma and urine*: ^{14}C XYZ1234 solution was rapidly absorbed from the gastrointestinal tract. The peak radiocarbon concentration in plasma averaged 1.61 µg eq/mL and occurred between 0.5 and 4 h. AUC_{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 approxi mately 80% of the plasma concentrations. C_{max} = 1.33 µg eq/mL and AUC_{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. AUC_{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 nonrenal clearance, which was tenfold higher (42 mL/min). In contrast, the renal clearance of the metabolite XYZ5678 averaged 144 mL/min.

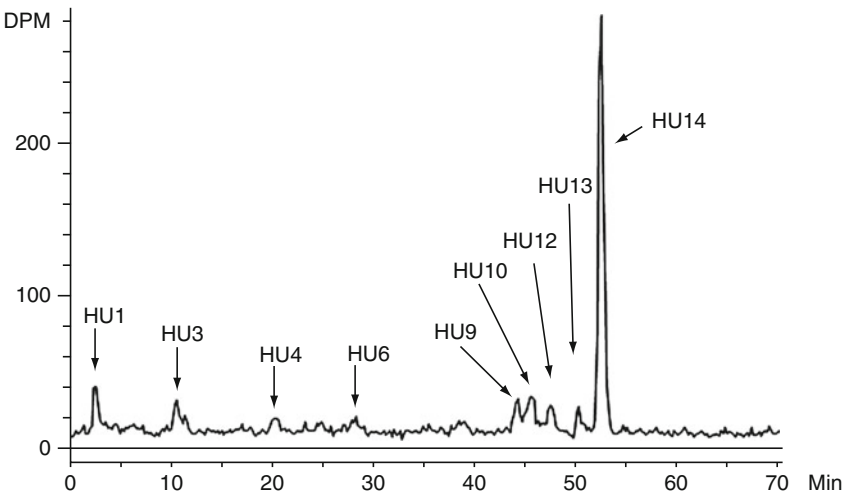
The C_{max} of XYZ1234 and XYZ5678 summed was 99% of the C_{max} of radiocarbon pharmacokinetic in plasma. The t_{max} for radiocarbon and for XYZ1234 were similar, the t_{max} of XYZ5678 appearing a few hours later. The difference in average urinary excretion between the sum of XYZ5678 and XYZ1234, and the radiocarbon, showed that 14.1% of the drug was elim inated 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.

B.10.6.3 Example 3: Metabolic Profiling

This example shows a typical experiment, including the application of AMS, to illustrate application of specific methodology to metabolic profiling as outlined in general terms below:

(i) *Sample collection:* Six healthy male volunteers were administered a single 50 mg (2.5 MBq) dose of ^{14}C SAR123 after an overnight fast. Plasma samples were collected at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, and 48 h and then every 24 h up to 336 h post dose. Urine was collected at 0 3, 3 6, 6 12, 12 24, and every 24 h thereafter up to 168 h post dose, while fecal samples were collected every 24 h up to 168 h post dose.

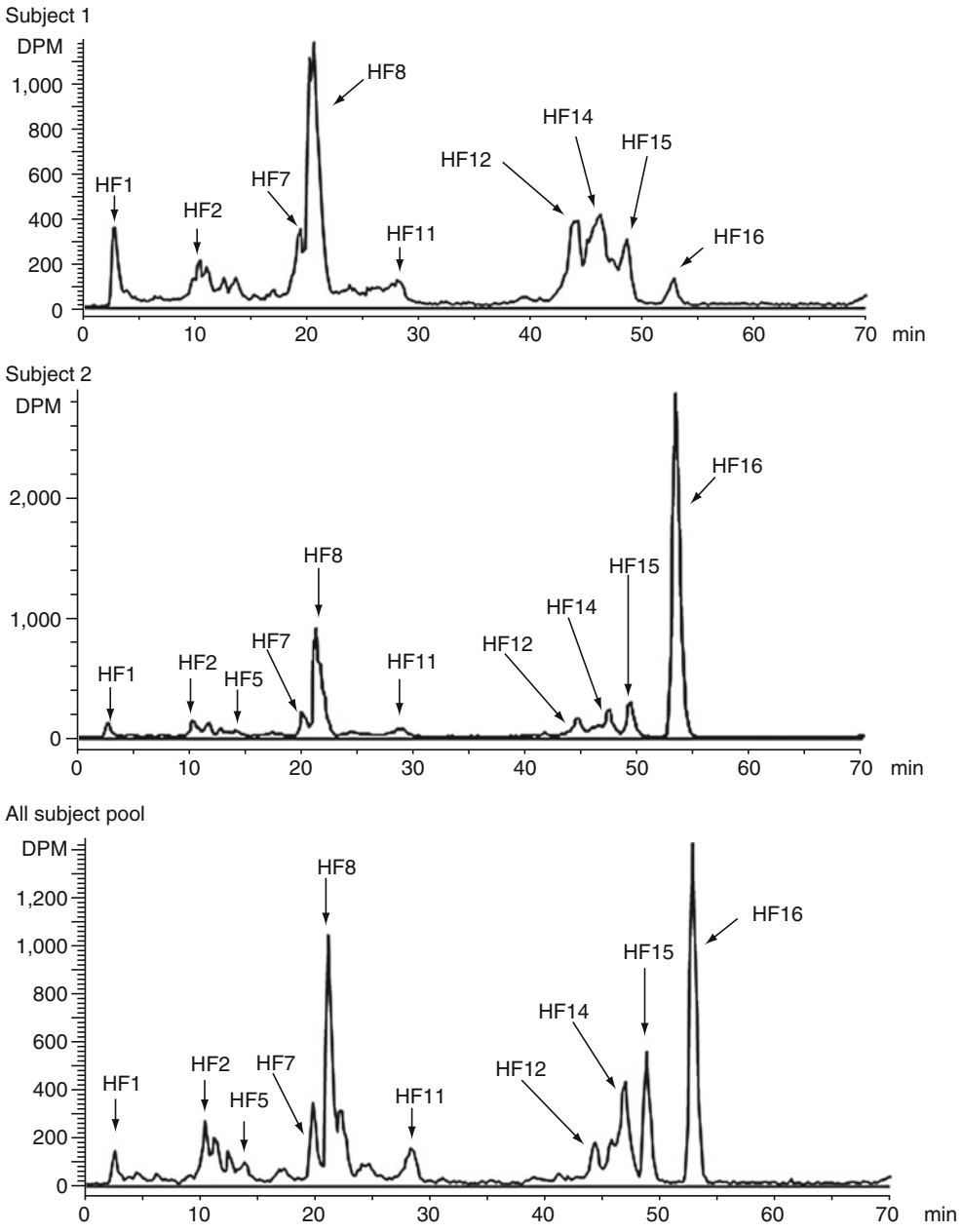
(ii) *Sample analysis (plasma)*: Plasma pools were prepared by mixing equal volumes of plasma from each subject at each sample time where plasma radioactivity con centration was above the LOQ (2, 6, 12, and 24 h). Aliquots (5 10 mL) were diluted with an equal vol ume of ammonium formate buffer (20 mM, pH3) and freeze dried. The freeze dried material was extracted three times with 10 mL methanol, the extracts combined and evaporated to dryness under nitrogen. The residue was reconstituted in 0.3 mL methanol, and 0.1 mL was analzsed by reverse phase gradient HPLC with off line radiodetection by



HU14 was identified as unchanged SAR123 by co-chromatography and LC-MS/MS.

Figure B.10-12

Representative radiochromatographic profile of pooled human urine following oral administration of ^{14}C-SAR123 to six healthy male subjects



HF16 was identified as unchanged SAR123 by co-chromatography and LC-MS/MS

Figure B.10-13

Radiochromatographic profiles of human fecal homogenate following oral administration of ^{14}C-SAR123 to six healthy male volunteers

Topcount NXT spectrometer or AMS. Extraction efficiency ranged between 73% and 91%, depending upon the timepoint. Plasma concentrations of radioactivity were too low to accurately quantify any metabolites as shown in *Fig. B.10 11a*, and metabolites representing 5% plasma radioactivity (approximately 30% parent compound concentration) were below the limit of detection. AMS was able to detect at least five additional metabolites (*Fig. B.10 11b*) improving the limit of quantitation

to include metabolites representing <10% parent compound: meeting the expectations of the FDA guid ance (US FDA 2008).

(iii) *Sample analysis (urine):* Urine was mixed with 0.1 volume methanol to avoid nonspecific losses by binding to plastic vessels. Urine pools were prepared for each subject to represent approximately 95% (range 95.6 98.2%) of the total urinary excretion of radioactivity (1.4% ± 0.7 % administered dose). Aliquots (20 mL) were freeze dried and reconstituted in 5 mL ammonium formate buffer (20 mM, pH 3) and applied to a solid phase extraction media (reverse phase, C8). The SPE media was washed with ammo nium formate buffer containing 5% and 15% aceto nitrile and drug related material eluted with buffer containing 75% acetonitrile. The extract was evapo rated to dryness, reconstituted in approximately 0.2 mL 50:50 (v/v) methanol: ammonium formate buffer (20mM, pH9.5), and 0.1 mL analyzed by reverse phase gradient HPLC with off line radiodetection by Topcount NXT spectrometer (*Fig. B.10 12*). Recovery of radioactivity by this procedure was 90.6% ± 6.2 % (mean ± SD). More than 20 components were detected in urine extracts, although only 14 could be reliably quantified because of the low concentration of radioactivity (LOQ 1% total radioactivity or 0.01% administered dose). The main component in urine was unchanged drug representing 43.9% of the radioactivity in the sample (0.63% administered dose).

(iv) *Sample analysis (feces):* Feces were homogenized with 10% methanol in water. Fecal homogenate pools were prepared for each subject, to represent a target of approximately 95% (range 97.4 99.2% in the case) of the total fecal excretion of radioactivity (88.8% ± 5.6 % administered dose). Aliquots (1 g) were extracted four times with two volumes of acetontrile. The combined extracts were evaporated to dryness under nitrogen and reconstituted to a final radioac tive concentration of approximately 600,000 dpm/ mL prior to analysis of 0.05 mL aliquots by reverse phase gradient HPLC with off line radiodetection by Topcount NXT spectrometer (*Fig. B.10 13*). Recovery of radioactivity by this procedure was 97.7% ± 11.4% (mean ± SD). Twenty components were detected in feces, with 16 representing more than 1% of the administered dose (mean of six sub jects). Considerable variability was observed in the metabolic profile between subjects as shown in the representative chromatograms of subjects one and two in *Fig. B.10 13*. On average across the six subjects, unchanged drug represented 17.7% of the fecal radioactivity (16.3% of the dose), with only one metabolite representing more than 10% of the dose and three others representing more than 5% dose.

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B.11 Synthesis of Radiolabeled Compounds for Clinical Studies

Jens Atzrodt · John Allen

B.11.1 Introduction

During the development of new drugs the candidate's pharmacokinetic (PK) properties and the absorption, distribution, metabolism, and elimination (ADME) characteristics have to be evaluated first in vitro, then in animals and finally in humans (Caldwell et al. 1995; Roffey et al. 2007). The objectives of human ADME studies are to evaluate mass balance data and, most important, to confirm that the metabolism of the drug is similar to what was described in animal species (Deroubaix and Coquette 2004). In order to keep track of the drug molecules throughout the body and excreta even after their transformation into different metabolites, the administration of radiolabeled drugs is considered essential (Marathe et al. 2004; Dalvie 2000). Usually, ^{14}C is the label of choice for most drug candidates since it can be introduced into a metabolically stable position in the backbone of the compound, the detection is easy and in case of combustion of samples the produced $^{14}CO_2$ can be nicely absorbed quantitatively (see *Scheme B.11 1*) (Beumer et al. 2006). Generally, ^{3}H labeled drugs can be prepared more easily and quickly than their ^{14}C counterparts. On the other hand, the ^{3}H label is often less biologically stable and it is more difficult to predict its metabolic stability and therefore one always needs to bear in mind the potential risk of the in vivo formation of $^{3}H_2O$ (Dueker et al. 1998). The latter is highly toxic and can be distributed in the whole body, which makes radioactivity measurement and quantification even in animal studies more difficult. Therefore, ^{3}H labeled drug candidates are usually administered less frequently to humans and only if the specific activity of the ^{14}C compound is not sufficiently high enough for the planned investigations, for example, in case of high molecular weight and/or very low dose drugs. For large complex biological molecules, such as proteins, antibodies, etc. a ^{3}H or ^{14}C labeling by a total synthesis approach could be extremely difficult or even impossible and hence, an iodination with $^{125}I_2$ or a ^{125}I precursor could be an alternative approach (Dewanjee 1992). However, the structural changes caused by an additional iodine atom in the molecule have to be considered and both materials (iodinated and non iodinated) tested for bioequivalence. Other potential radioactive isotopes are ^{33}P and ^{35}S but compared to ^{14}C, these isotopes were much less frequently applied for labeling of drug candidates.

Apart from human ADME studies, short lived radionuclides such as ^{11}C, ^{18}F are used for positron emission tomography (PET) (Cherry 2001) to study, for example, drug passage over the blood brain barrier and selective accumulation in critical organs, receptor occupancy, dose response or tumor metabolism, and proliferation rates (Rösch 2003). However, labeling syntheses with these short lived isotopes require specific considerations, which are not the subject of this chapter.

B.11.2 General Aspects to be Considered for the Synthesis of ^{14}C-Labeled Compounds

B.11.2.1 Technical Considerations for ^{14}C-Labeling

Carbon 14 has many of the properties of an ideal tracer nuclide for human ADME studies (Catch 1961). Because of its very long half life (5,730 years), it is unnecessary to correct for decay and ^{14}C labeled compounds can be prepared and stored for a long period if radiolytical decomposition can be reduced (see Sect. B.11.2.2). Carbon 14 decays to nitrogen 14 with the emission of a β particle (maximum energy: 156 eV; average energy: 49 eV). This emission is sufficiently energetic to make measurement at moderate specific activity fairly simple, but weak enough to make shielding unnecessary. The range of these soft β particles is about 15 16 cm in air and 0 2 mm in a solid medium. This means even at very high specific activities ^{14}C labeled compounds can be safely handled in standard glass vessels and conventional lab equipment if reasonable radiation safety precautions are taken. However, in most countries working with radioactive materials requires strict reporting, licenses and/or authorizations

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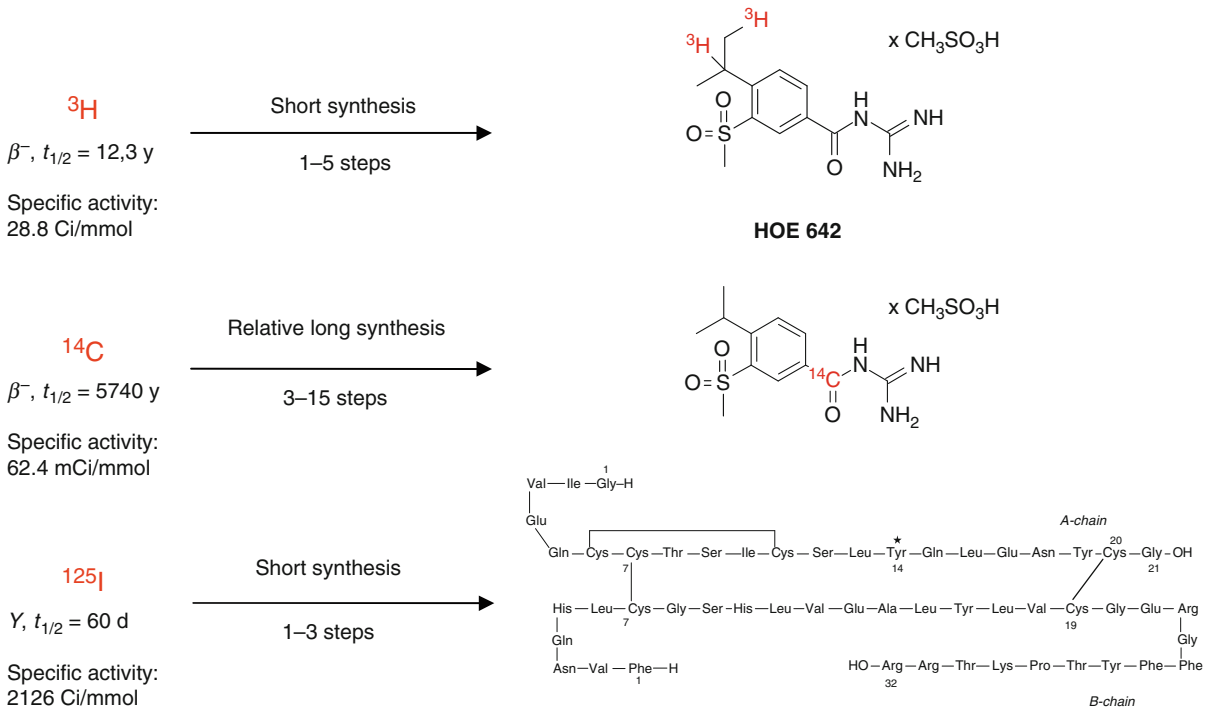


Scheme B.11-1
Radioactive isotopes used for labeling of drug candidates

from authorities. Local regulations may be different or stricter with respect to handling radiation, to contain ment equipment, and to lab facilities.

B.11.2.2 Synthetic Considerations for ^{14}C-Labeling

In general, any organic compound that can be synthesized can be labeled, but specific labeling of more complica ted molecules maybe difficult and expensive. Usually, a target directed total synthesis approach is required for ^{14}C labeling of drug development candidates. For the right choice of the labeling position, different aspects have to be considered carefully. The position of the label should be away from sites that are chemically unstable or from sites of metabolic attack to ensure the label is kept in the main metabolic fragment. In cases where high speci fic activities are required for the planned study, a double ^{14}C labeling or alternatively ^{3}H labeling needs to be con sidered because the maximum achievable specific activity for single ^{14}C labeled organic compound is 62.4 mCi/ mmol (see also *Scheme B.11 1*) (Schulte 1966).

On the other hand, the development of a labeling synthesis is dependent on the availability of suitable precursors, the length and complexity of the synthesis pathway, the reliability of the process as well as radiation safety aspects, for example, avoidance of volatile reaction components if possible.

B.11.2.3 Planning of a ^{14}C-Synthesis

The reaction pathway developed for ^{14}C synthesis should satisfy the following criteria (Raaen et al. 1968).

(a) Introduce the ^{14}C label as near as possible to the last step of the synthesis.
(b) Introduce the ^{14}C label after stereochemical resolution.
(c) Introduce the ^{14}C label in a known position.
(d) Ensure adequate specific activity and high radio chemical purity of the product.
(e) Provide high radiochemical yields.
(f) Consider synthetic efforts for unlabeled precursor synthesis.

No special laboratory equipment is required, but prepara tion techniques may differ from conventional synthetic work especially when volatile radioactive precursors are handled or volatile intermediates and/or side products

are expected. Further difficulties may increase as the scale of the reactions is reduced, for example, yield, impurities, solvent content, and crystallization.

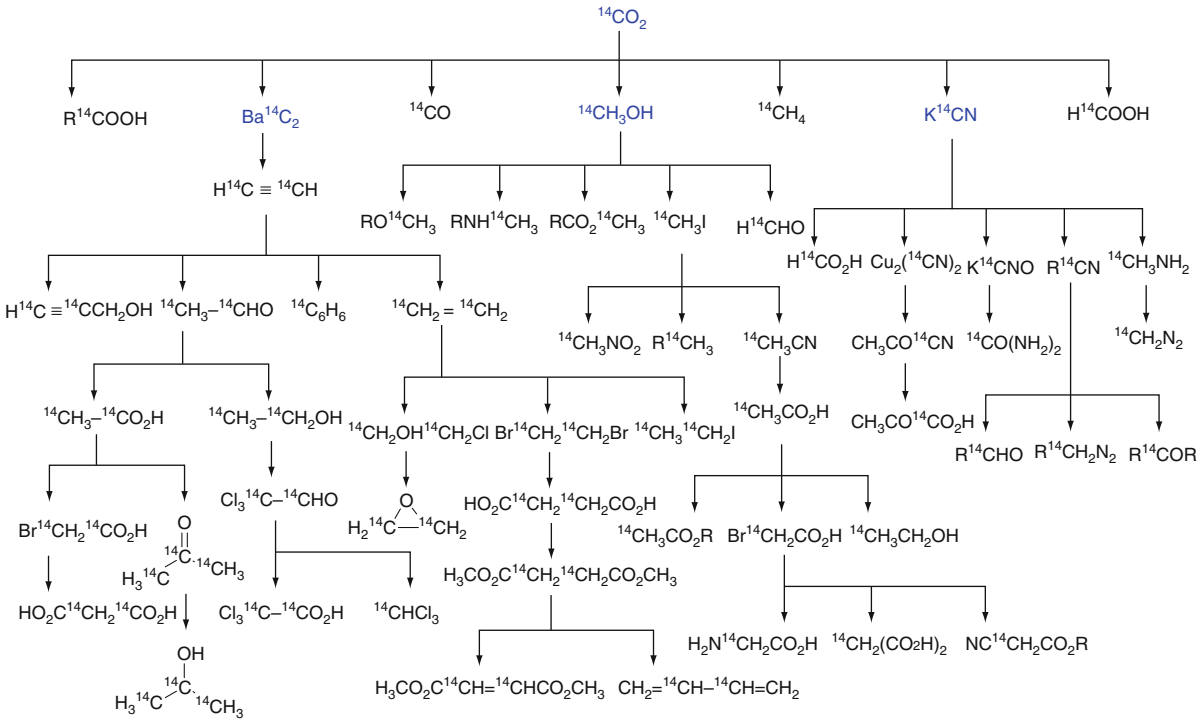
A ^{14}C labeled compound for which preparative methods are not well established should be synthesized as follows: first, the pathway is elaborated with nonradioactive material on the desired scale until a reliable process has been developed and the operator is adequately familiarized with the chemistry. Product purity should be checked by the usual chemical and physical methods. Then, the experiment is carried out at the tracer level to establish the nature of impurities and by products (e.g., volatility), to determine the yield of the desired product and to check for radio accountability. Finally, the procedures developed are duplicated with limited amounts of the ^{14}C labeled precursor.

B.11.2.4 Methods for ^{14}C-Syntheses

Reactor production of carbon 14 is achieved by neutron bombardment of solid beryllium nitride or solid aluminum nitride over a very long time and subsequent transformation of all ^{14}C compounds formed to $BaCO_3$ (Wilson 1966). That means, the preparation of labeled compounds is limited to $^{14}CO_2$ as the only practical starting material. Certain key intermediates can be used for the preparation of a great number of labeled compounds, for example, [^{14}C]potassium cyanide, [^{14}C]barium carbide, [^{14}C]methanol (see *Scheme B.11 2*). In principle both, chemical and biological methods can be applied for converting [^{14}C]barium carbonate and its simple derivatives into more complex labeled compounds, of which chemical synthesis is the most generally used.

B.11.2.4.1 Chemical Methods

Many good standard procedures for the chemical preparation of the more common ^{14}C labeled compounds have been summarized in recent literature reviews (McCarthy 2000) and in books or in book chapters (Muccino 1983; Murray and Williams 1958; Heys et al. 2009) (see also *Scheme B.11 2*). Today many specialized supply companies provide a variety of these basic ^{14}C labeled precursors or even more complex molecules on a custom synthesis basis. Usually the structural complexity of modern new drug development candidates requires multistep labeling



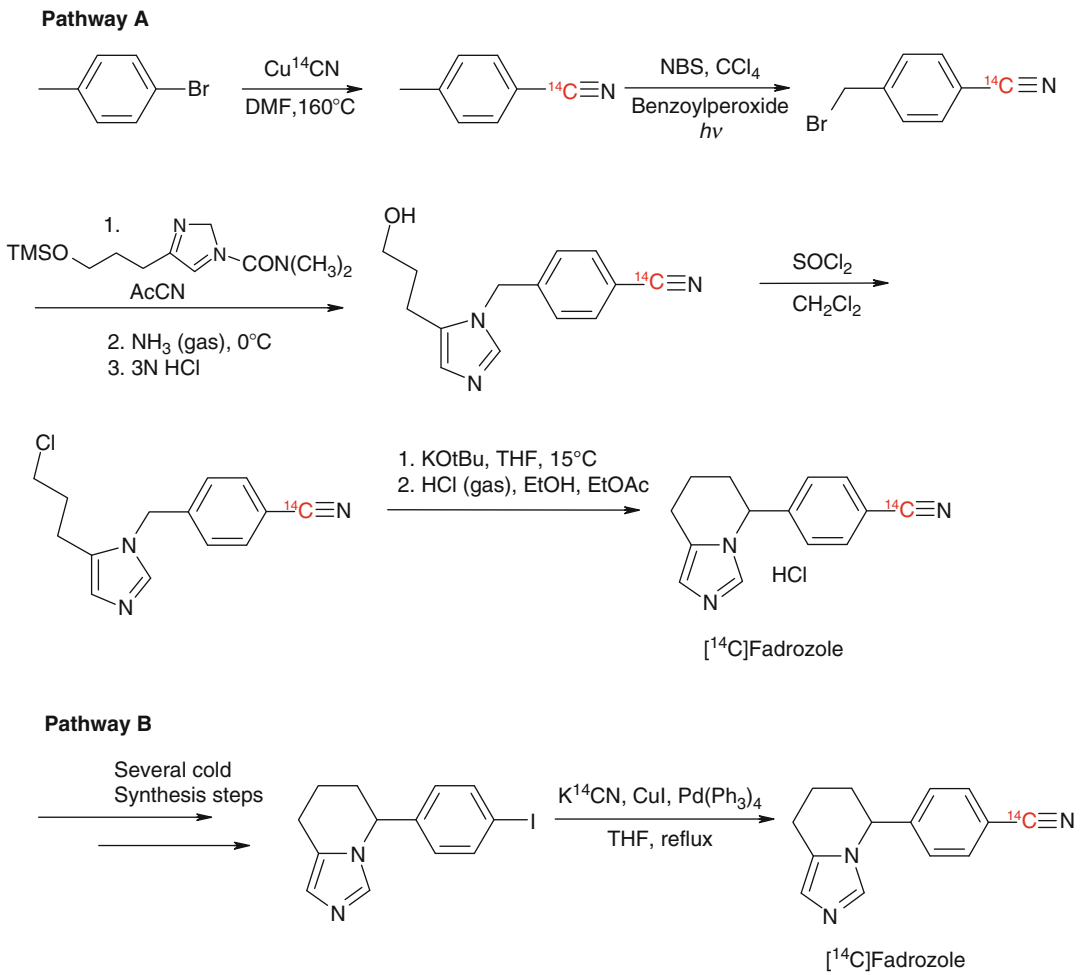
Scheme B.11-2

Synthetic routes to ^{14}C-labeled compounds (Raaen et al. 1968)

syntheses. Therefore, purchasing or outsourcing of basic labeled organic compounds to be used as starting materials for an in house synthesis of labeled drug development candidates is a common strategy in the pharmaceutical industry. If applicable, a late stage introduction of the ^{14}C label into an unlabeled advanced intermediate can be highly efficient (see also Sect. B.11.2.3, criteria (a)). For example, the Grignard reaction (Knochel 2005) of *organo* magnesium halides with $^{14}CO_2$, or a transition metal catalyzed cyanation (Allen et al. 1992; Sundermeier et al. 2003) with metal cyanide and subsequent saponification are convenient methods to introduce labeled carboxyl functionalities (Cao et al. 2007). Other small labeled building blocks frequently used for ^{14}C incorporation into organic molecules are, for example, [^{14}C]formaldehyde, [^{14}C]methyl iodide, [^{14}C]thiocyanate, and ^{14}C labeled acetic acid derivatives (McCarthy 2000).

A very good example of the advantage of a late stage labeling strategy is the synthesis of ^{14}C labeled Fadrozole. Initially the synthesis was performed according to pathway A (see *Scheme B.11 3*) starting with a cyanation of 4 bromotoluene and subsequent synthetic construction of the molecule (Markus et al. 1997). However, by developing a late stage ^{14}C cyanation of an unlabeled precursor (pathway B) the number of radioactive steps could be reduced from eight to only one (Allentoff et al. 2000).

Often reducing the number of radioactive steps is a major objective of route development activities because the amount of radioactivity employed, the radioactive waste produced and thus the costs of a synthesis can be



Scheme B.11-3
Synthesis of [^{14}C]Fadrozole

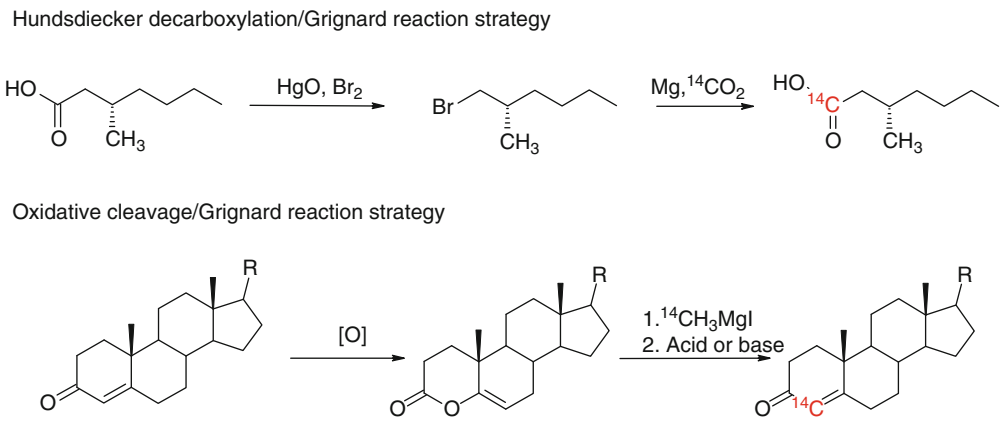
also reduced dramatically. Besides the recognition of avail able labeled reagents, of course accessibility of unlabeled precursors with reasonable synthetic efforts, has to be considered as well when planning a ^{14}C labeling synthesis (see also Sect. B.10.2.3, criteria (e)). One synthetic strategy applied for precursor synthesis is a degradation reaction either by Hundsdiecker decarboxylation (Kurosowa et al. 1997) or by oxidative cleavage (Shu and Heys 1994) as shown in *Scheme B.11 4*. Subsequent labeling affords the ^{14}C labeled version of the previous starting material using this protocol.

Other synthetic strategies for late stage labeling invo lve, for example, a directed metalation or halogen metal exchange for aromatic substitution and ^{14}C introduction as demonstrated, for example, in the synthesis of [$^{14}C_2$] WIN 63394 (Burgos et al. 1996) (see also *Scheme B.11 5*).

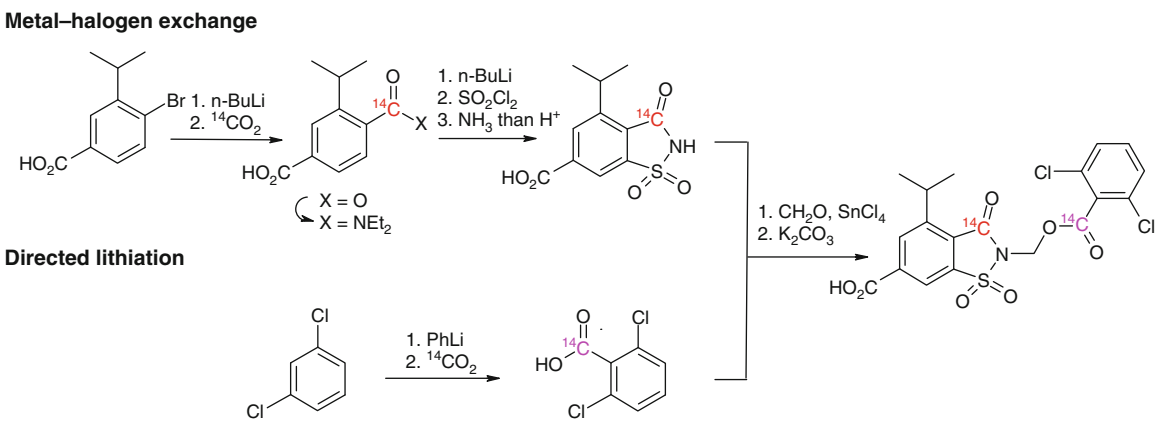
After successful introduction of the ^{14}C label, all fur ther chemical transformations can be performed applying classical or modern organic chemistry approaches includ ing for example asymmetric synthesis (Voges 2002) or cross coupling reactions (Derdau et al. 2003) as described for unlabeled compounds.

B.11.2.4.2 Biochemical Methods

Besides chemical methods, biochemical synthesis offers an option to obtain labeled natural products and organic compounds not always accessible by conventional synthe sis (Evans 1981; Benakis 1994). Oligopeptides, proteins, antibodies as well as a large number of pharmaceuti cally relevant compounds, for example, antibiotics are



Scheme B.11-4
Degradation/labeling strategy



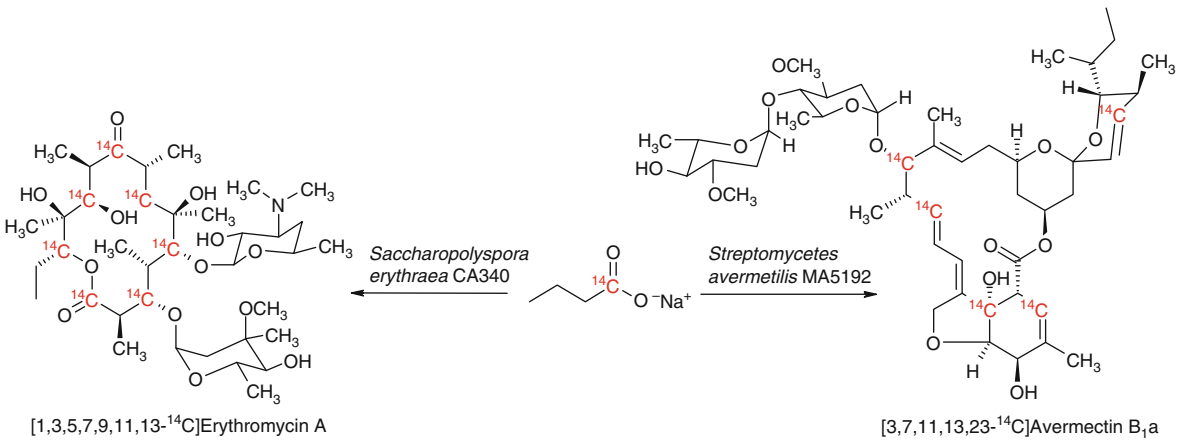
Scheme B.11-5
Metal halogen exchange and directed metalation strategy for ^{14}C-labelling of WIN-63394

synthesized by fermentation with the aid of yeast, bacteria, or fungi. Corresponding ^{14}C labeled compounds can be prepared if relevant ^{14}C labeled starting materials, for example, ^{14}C labeled amino acids are employed in the fermentation process. Yields are as important as in purely chemical syntheses, but unfortunately only a few bio chemical processes provide a single ^{14}C labeled product in high yield (Wallace et al. 1994). Decarboxylation and production of large quantities of $^{14}CO_2$ might be another drawback that needs to be considered when planning fermentation for labeling purposes.

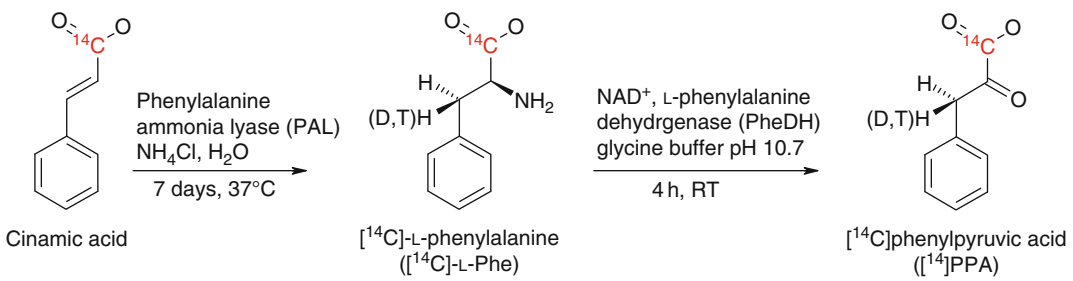
For example, [1,3,5,7,9,11,13 ^{14}C]erythromycin A was produced in liquid fermentation broths of *Saccharo polyspora erythraea* CA340 in shake flasks after the ad ministration of [1 ^{14}C]sodium propionate. The labeled erythromycin A was separated by extraction of the fermen tation broth and purified on Sephadex (see *Scheme B.11 6*) (Walker et al. 1996). A similar fermentation process with MA5192 (*Streptomycetes avermitilis*) and [1 ^{14}C] sodium propionate as precursor was also used for the synthesis of ^{14}C labeled avermectin B_1a (Ku et al. 1984). For both compounds, a total synthesis approach for ^{14}C label would have been very difficult, time and resource consuming, or even impossible.

On the other hand, enzymatic reactions are also applied for specific chemical transformations, for example, sapon ification, oxidation, and hydroxylation as part of multistep conventional chemical synthesis pathways for the prepa ration of ^{14}C labeled drug development candidates and corresponding ^{14}C labeled relevant metabolites (Allen et al. 2006, 2007). As an example for biochemical labeling, the enzymatic synthesis of ^{14}C labeled phenylpyruvic acid ([^{14}C]PPA) is shown in *Scheme B.11 7*.

The addition of ammonia to [^{14}C]cinnamic acid cat alyzed by the enzyme PAL (phenylalanine ammonia lyase) carried out in ammonia buffer at pH 9.8 led to the forma tion of [^{14}C] L phenylalanine ([^{14}C] L Phe). Subsequently this intermediate was converted to [^{14}C]PPA employing the enzyme PheDH (phenylalanine dehydrogenase) in the presence of NAD^+. As usual for enzymatic processes,



Scheme B.11-6
Biosynthetic labeling of erythromycin A and avermectin B_1a



Scheme B.11-7
Biochemical ^{14}C-labeling of phenylpyruvic acid

the yield of this reaction strongly depends on the incubation time, concentration of L Phe, buffer, pH, and enzyme quantity. The similar way in which the first step was carried out in fully deuterated or tritiated ammonia buffer gave [(3*S*) ^{2}H] L Phe and [(3*S*) ^{3}H] L Phe respectively, which offered the opportunity to get access to the corresponding deuterium and tritium labeled PPA as well (Skowera and Kanska 2008).

Another biochemical method for preparing labeled compounds is the photosynthesis, which may involve the assimilation $^{14}CO_2$ by algae such as *Chlorella vulgaris* (Godward 1960), cyanobacteria such as *Anacystis nidulans* (Tovey et al. 1974), or plants such as tobacco (*Nicotiana* sp.) or *Canna indica* (Putman and Hassid 1952) or by detached or full sized plants grown in a $^{14}CO_2$ atmosphere in sealed greenhouses or plastic bags (Benakis et al. 1986). This technique called "isotope farming" can provide high specific activities and good yields of ^{14}C labeled glucose, starch, nucleosides, amino acids, and lipids.

B.11.2.5 Stability of ^{14}C-Labeled Compounds

Compounds labeled with carbon 14 decay to nitrogen 14 with the emission of a β particle. Since the energy of these β rays by far exceeds bond energies of organic molecules, structural damage can occur. If the radiation energy is absorbed by the compound itself, the excited molecule may break up and/or react with other molecules. The activated decomposition fragments may also react in a sort of chain reaction with other molecules producing impurities (Bayly and Weigel 1960; Rochlin 1965). Typical reactions resulting from irradiation are dehydrogenation, oxidation, decarboxylation, deamination, condensation, and polymerization; in many cases through radical reactions (Sheppard 1972). In some cases, the shelf life is reduced from years to weeks or even days.

The rate of decomposition depends on storage conditions, specific activity, and chemical structure. Though it is not yet possible to foresee exactly the behavior of each compound, a thorough stability study may result in storage conditions that provide reasonable shelf life of the compound and thus reduce purification efforts and secure rapid availability of stock material. The following storage rules have proved to be successful and can be applied to reduce decomposition (Evans 1976; Bayly and Evans 1966) optimize storage conditions as regards chemical stability, store at the lowest practical temperature, dilute the specific activity, avoid high amounts of activity, store labeled compounds in solution, add radical scavengers or other stabilizers (Fredenhagen 2002) and avoid unnecessary re opening of vials and warming/cooling cycles.

In spite of all potential precautionary measures, the shelf time of labeled compounds is always limited and requires repurification of the material more or less frequently (Bayly and Evans 1968).

B.11.2.6 Purification

The development of a suitable purification method for ^{14}C labeled compounds can be crucial because typically a radiochemical purity of >98% is necessary for the planned studies (for specific applications up to 99.8% are required). Additionally, the limited shelf life of ^{14}C labeled compounds may require repurifying the ^{14}C labeled compound from time to time. Since synthesis impurities and degradation products can be structurally completely different, thus in some cases it might be necessary to develop different purification methods as well. Typically, ^{14}C labeled compounds are purified by column chromatography, semi preparative reversed phase HPLC or crystallization (Evans 1981).

B.11.2.7 Dilution

After purification, the ^{14}C labeled drug development candidate often needs to be diluted to obtain the specific activity required for the planned studies. To this end, the highly radioactive compound is homogeneously mixed with unlabeled material of the same compound by dissolving both in a suitable solvent. Subsequently, the solvent is removed by evaporation, the product crystallized, precipitated, or lyophilized to afford the diluted ^{14}C labeled drug development candidate ready for administration or further formulation to the drug product.

B.11.2.8 Analysis

After repurification and dilution, intensive analytical release testing including test items and specifications appropriate for the intended use are performed to guarantee consistent product quality (Dueker et al. 1998; Filer 1989). Typically, the identity of the compound is confirmed by NMR, IR, and/or LC MS, the radiochemical, chemical, and stereochemical purities are checked by analytical HPLC or GC and the specific activity is measured by liquid scintillation counters (LSC) and/or LC MS. In addition, specific applications or specific compound related

properties may require supplementary analytical tests, ion chromatography, water content determination, polymorphism, or particle size investigations.

B.11.3 General Aspects to be Considered for the Synthesis of Tritium-Labeled Compounds

B.11.3.1 Synthetic and Technical Considerations for ^{3}H-Labeling

Tritium labeled compounds are much less frequently applied for human ADME studies (see also Sect. B.11.1) because an essential problem is the integrity of the carbon tritium bond and the specificity of the labeling. On the other side, an advantage is the extremely high specific activity of 28.8 Ci/mmol of tritium, which is approximately 500 times higher than achievable with a single carbon 14 label (62.4 mCi/mmol) (Evans 1974). This property makes tritium irreplaceable for labeling of large molecules, particularly biomolecules such as peptides, proteins, oligonucleotides, and antibodies as well as for early labeling strategies to support discovery purposes.

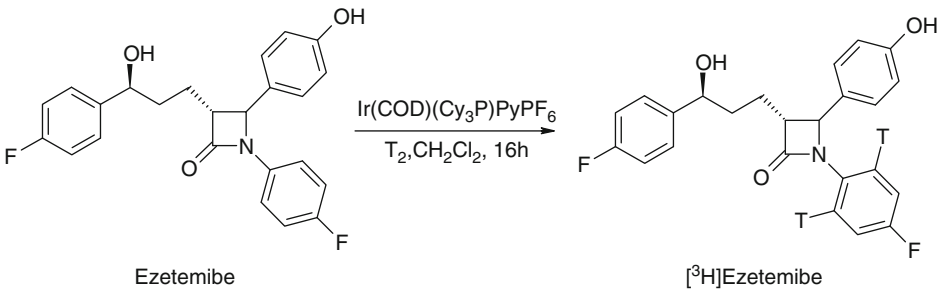
Tritium labeling is often much simpler than ^{14}C synthesis and labeling can often be accomplished very late in the overall synthesis. The typical sources for the tritium label are tritium gas or specific tritiated reagents and sometimes tritium water. Modern stainless steel manifolds allow a safe handling and storage of tritium gas (Benakis 1994). However, as for ^{14}C synthesis, a number of specialized supply companies offer custom tritiation services. In principle, similar points as mentioned under Sect. B.11.2.4 should be considered for the planning of a ^{3}H synthesis. Compared to ^{14}C synthesis the scale of tritiations is even further reduced. Often only a few milligrams of material have to be handled, which requires specific preparation techniques, operator training, and elaboration work. The shelf life of ^{3}H labeled compounds is usually even further reduced but for purification, dilution, and analysis of similar aspects as already mentioned for ^{14}C labeled materials have to be considered as well (see also Sects. B.11.2.6 B.11.2.8).

B.11.3.2 Chemical Methods for ^{3}H-Labeling

Comprehensive literature reviews summarizing synthesis techniques developed for tritium incorporation have been published recently (Muccino 1983; Murray and Williams 1958; Saljoughian and Williams 2000) and therefore only the main principles will be briefly explained in the following. There are two basic approaches for introducing tritium into organic molecules: exchange labeling (Atzrodt et al. 2007; Heys 2007; Lockley 2007) and synthetic tritiation methods (Evans 1981; Saljoughian 2002). In general, exchange labeling yields lower tritium abundance, often with the isotope being widely distributed over the molecule. On the other hand, exchange labeling can be highly cost and time efficient if it can be carried out directly on the target molecule.

An excellent example for the successful application of exchange labeling is the synthesis of tritiated Ezetemibe (Hesk et al. 2002). The tritium introduction was accomplished by a catalytic exchange with tritium gas starting directly from unlabeled Ezetemibe without any previous or subsequent reaction steps (see *Scheme B.11 8*).

In case of synthetic tritiations, the tritium is directly inserted into specific positions in the molecule resulting in high tritium abundances and specific activities. Basically, four different chemical methods can be used to introduce tritium into the target molecule: (1) reduction of reducible functions with tritiated reagents, (2) exchange of halogen by tritium, (3) hydrogenations of double or triple bonds



Ezetemibe [^{3}H]Ezetemibe

Scheme B.11-8
Synthesis of [^{3}H]Ezetemibe by catalytic exchange labeling

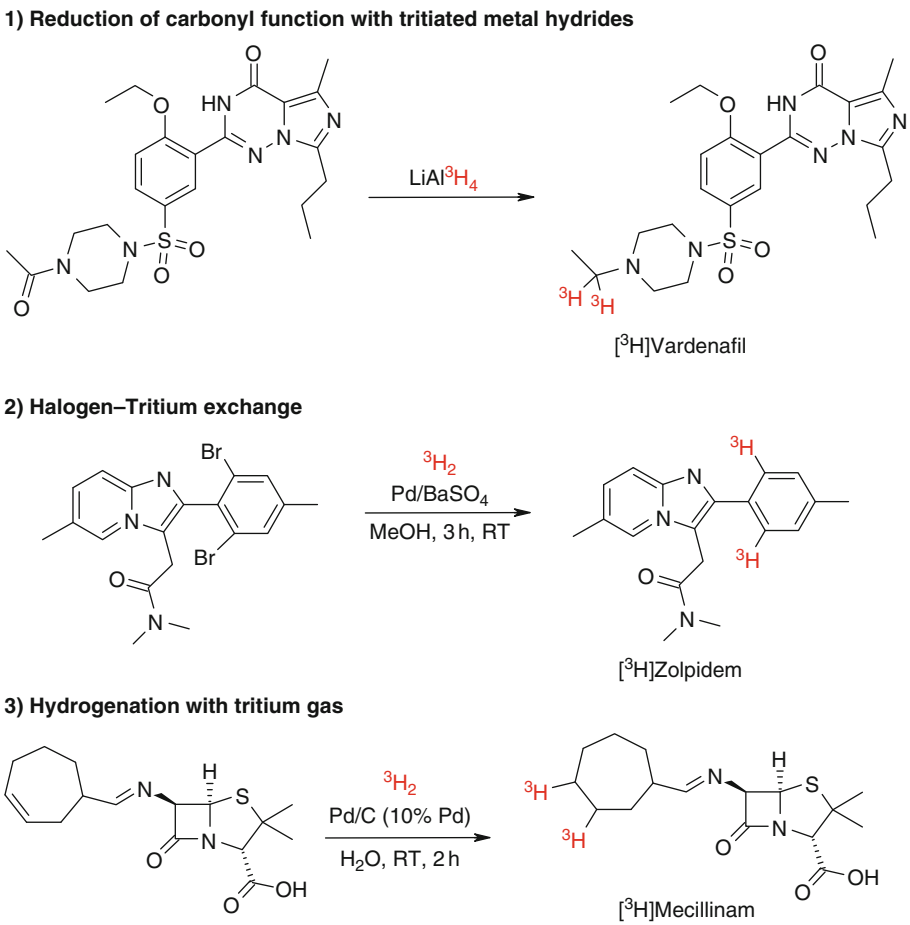
with tritium gas, and (4) application of tritiated small pre cursors such as methyl iodide in the labeling synthesis. Examples for the first three labeling strategies are depicted in *Scheme B.11 9*.

[^{3}H]Vardenafil was synthesized by reduction of a spe cial synthesized amide precursor with freshly prepared lithium aluminum tritide (Pleiss 2003). For the synthe sis of [^{3}H]Zolpidem at high specific activity, a suitable dihalogenated precursor was synthesized and then the tritium incorporated by a catalytic dehalogenation reac tion in the presence of tritium gas (Allen et al. 1986). The tritiation step for the synthesis of [^{3}H]Mecillinam was performed by treating the corresponding dehydro mecillinam prepared before a six step synthesis with tritium gas in the presence of 10% Pd/C as the catalyst (Frederiksen and Sörensen 2003). In all cases, specific unlabeled precursor molecules had to be synthesized and therefore synthetic tritiations are often limited by the chemistry required both before and during the labeling process.

B.11.4 Regulatory Requirements for Application of Radiolabeled Active Pharmaceutical Ingredients to Humans

B.11.4.1 General Study Requirements (Dain et al. 1994)

Administering radioactivity to human beings is a general ethical question due to the well established carcinogenic and/or teratogenic potential of radioactive compounds.



Scheme B.11-9

Examples for synthetic tritiations: (1) synthesis of [^{3}H]Vardenafil, (2) synthesis of [^{3}H]Zolpidem, and (3) synthesis of [^{3}H]Mecillinam

The radiation exposures should be "as low as reasonably achievable" (ALARA concept) and even with small doses of radioactivity given the residual risk should be minimized. Therefore, the concept of clinical studies administering radioactive drugs to humans has to be approved by a special ethical committee. Additionally, submission and approval of the Investigational New Drug Application (IND) for the US or if the study is carried out in the EU the Investigational Medicinal Product Dossier (IMPD) including detailed information on the synthesis, medication, and analytical release as well as a supportive stability study is required. The interpretation of regulatory requirements for the manufacturing process and the study design is subject to local national authorities and strongly depends on location and specifics of the study site and the country of the manufacturing facilities.

B.11.4.2 Impurities

Based on the ICH Guidelines Q3A *Impurities in New Drug Substances* and Q6A *Specifications in New Drug Substance* the limit for an unspecified impurity in drug substances for phase I/IIa/IIb is set at 0.2%. This threshold is also valid for the diluted radioactive drug substance (rDS). For the highly radioactive drug substance (hrDS), the usual internal release criteria require a radiochemical purity of at least 98% or even higher. This material (hrDS) usually undergoes at least a tenfold dilution with GMP (good manufacturing practice) produced cold material to afford the rDS and hence no radioactive impurity greater than 0.2% will be present.

B.11.4.3 Regulatory Requirements

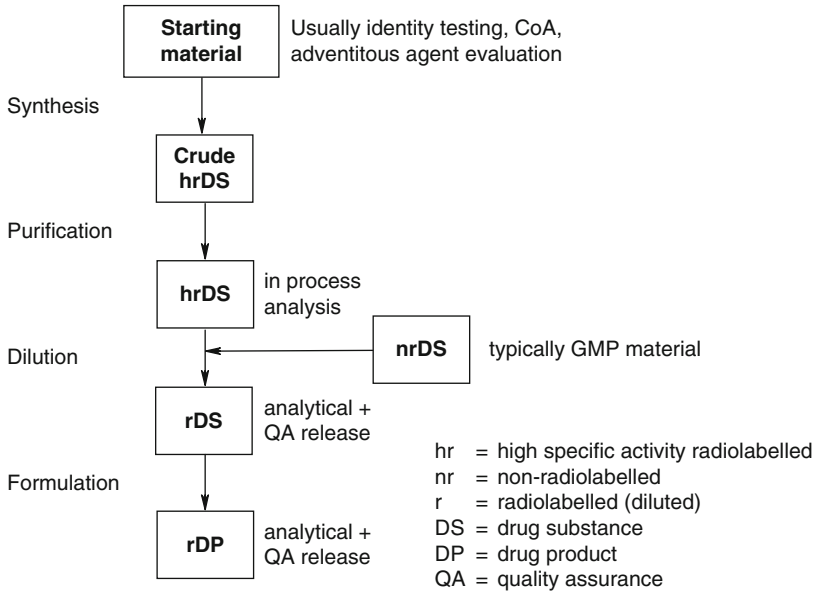
The GMP guide ICH Q7A *Good Manufacturing Practice for Active Pharmaceutical Ingredients* (APIs) does not apply to manufacturing/control aspects specific to radiolabeled compounds. However, Chap. 19 contains guidance for the manufacture of APIs used in clinical trials (APIs for investigational use during early phases of development) in general. For EU countries, the EU GMP Guideline Part II *Basic Requirements for Active Substances used as Starting Materials* including Chap. 19 *APIs for Use in Clinical Trials* is recommended although not required by community legislation. During the manufacturing process the hrDS is diluted with unlabeled API (manufactured according to GMP requirements) to achieve the specific radioactivity required for the planned study. Although typically in the final drug substance only very small radioactive amounts are incorporated, it could be considered as a radioactive API (rAPI). Therefore, the manufacture of radiolabeled APIs is covered by national drug laws and ordinances by some authorities whereas in other countries this has not come within the scope of regulatory GMP inspections and does not require certification. These inconsistent interpretations may result in different levels of GMP formally requested for the synthesis of radiolabeled APIs by national health authorities.

Consequently, different creative approaches combining quality principles with radiation safety aspects and other challenges inherent in radiosynthesis have been developed by pharmaceutical companies depending on national regulatory requirements (Lloyd et al. 2003; Fontana et al. 2000). Full GMP compliance for the whole synthesis or parts of it (e.g., only purification and dilution including in some cases full environmental control), different GMP or GLP like (Hong et al. 2008) or non GMP classified processes (Bonacorsi et al. 2007) have been applied. Generally, the local authorities are allowed to inspect the manufacturing facilities and to review compliance with appropriate regulations.

B.11.4.4 General Quality-Related Measures that Should be Applied for the Synthesis of Radioactive APIs

Irrespective of whether GMP is formally required or not, basically the synthesis of ^{14}C labeled drug development candidates to be administered to humans should follow higher quality standards compared to those for in vitro or animal study applications. Thereby the stringency of quality standards should increase as the process proceeds from early steps to final synthesis steps, purification, and dilution. Compared to orally administered compounds the synthesis of radiolabeled drug substances, which will be formulated for parenteral application should be subjected to more stringent control. Trace runs may be performed to confirm ("validate") the process. Usually all synthesis, manufacturing, analytical, and release activities are reviewed by an independent quality assurance organization to ensure compliance of all process steps with regulatory and internal company quality requirements. SOPs (Standard Operating Procedures) are established to define details of all quality related operations and processes.

Although, in principle, the labeled material could be any proportion of the administered drug substance (rDS), it is normally small in practice, typically forming less than 5% of rDS.



Scheme B.11-10

Typical process steps for the synthesis and manufacture of radiolabeled drug product

Assuming that the hrDS forms less than 10% of the rDS, even in a worst case scenario with a single 2% impurity in hrDS, after dilution this would end up in only 0.2% impurity of the final rDS (see also *Scheme B.11 10*). Therefore, it might be appropriate to focus increased quality requirements on the purification and dilution steps only.

Adventitious agents evaluation and a complete review of the synthesis concerning BSE/TSE (bovine spongiform encephalopathy/transmissible spongiform encephalopathies) and viral safety is requested by several authorities.

In addition raw materials, intermediates, solvents, and reagents as well as materials that will come into direct contact with the radiolabeled drug (dry reagents, charcoal, etc.) often have to be evaluated by testing or received with supplier's certificates of analysis (CoA) and subject of at least identity testing.

Retrievable and traceable recording of all process and testing related information (including signatures) in legible documents should be in place. In addition, several affiliations require written production instructions and records as well as a full quality assurance review of the synthesis documentation.

All equipment of the radiosynthesis laboratories that is critical to product quality (balance, preparative HPLC, pH meter) should be at least calibrated and maintained at appropriate intervals according to written procedures.

Glassware and other equipment that will come into direct contact with the radiolabeled compound for clinical use should be new or cleaned according to standardized procedures. Stirrer bars, syringes, needles, and glass pipettes should be new.

The manufacturing facilities including fume hoods and HPLC cabinets should be dedicated and cleaned. Appropriate measures must be taken to prevent product contamination or cross contamination. For parenteral application, a microbiological environmental monitoring of the work surfaces might be required in several countries.

Staff involved in the synthesis of radiolabeled materials for clinical use should be aware of the regulatory requirements and receive initial and continuing training, including hygiene instructions. For each employer specific duties and responsibilities should be recorded in written job descriptions.

B.11.5 Conclusion and Outlook

Suitable quality measures should be in place in order to assure that the manufacture, control, and release of radiolabeled compounds administered to human volunteers will satisfy quality requirements and ensures both patient safety and reliability of study results. In the last couple of years, the regulatory stringency for the synthesis of radiolabeled compounds for clinical use has been increased and certainly this will continue in the future.

However, on the other hand much progress has been made to develop new sensitive enabling techniques such as high sensitivity LSC and accelerated mass spectroscopy (AMS) (Vogel 2000; Vogel et al. 2007) allowing minimization of the radioactive dose. Especially AMS provides the option to decrease the radioactive dose by a factor of 1000 from around µ50 Ci to about 50 nCi (Garner 2000). The very high sensitivity of AMS permits the evaluation of micro dosing approaches including subtherapeutic doses (Lappin and Garner 2003). Smaller clinical doses and/or fewer radioactivity administrations to humans may also change the regulatory view on the synthesis of ^{14}C labeled com pounds. Today the downside of AMS is that all samples need to be converted into solid graphite, which is an expensive process and only a few laboratories are offering this service. Consequently, AMS is not yet used as a standard analytical technique. At the moment pharma ceutical companies designing ADME studies applying AMS only when it appears to be absolutely necessary, for example, in case of high potency drugs, large natural products, or having a very long biological half life (Lappin et al. 2006) but this will probably increase in the future when AMS becomes more accessible.

In the light of the recent FDA guidance *Safety Testing of Drug Metabolites*, stable isotopically labeled (^{13}C, ^{15}N, ^{2}H) analogues could be used more frequently to obtain quan titative and qualitative information on drug metabolism in early human ADME studies (e.g., First in Man) even without specific studies administering radiolabeled drug (Mutlib 2008). Modern LC/MS technologies and hyphen ation of liquid chromatography with chemical reaction interface mass spectrometry (CRIMS) (Jorabchi et al. 2005) presents an opportunity to perform quantitative measurement of metabolites even in the absence of authen tic standards or radiolabeled compounds (Abramson et al. 1996). However, despite its huge potential, this technology has not been fully developed and explored for routine human ADME studies.

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B.12 Drug–Drug Interaction Studies

Wolfgang Schmider · Xavier Boulenc

B.12.1 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) and to assess the necessity of a dose adjustment from the planned/established clinical dose for patients.

Furthermore, pharmacokinetics of a drug can also be altered by concomitantly administered drugs that have the potential to interact (US FDA 1999, 2006). 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).

The protocols were developed with consideration of the current good clinical practices and the studies were 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 (International Conference on Harmonization E6 1996; International Conference on Harmonization E8 1997). These principles have their origins in The Declaration of Helsinki (1996).

Design and conduct of the clinical studies presented in the examples below, all were in conformance with these principles. 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.

In this chapter, three examples of drug drug interaction studies are presented.

1. An inhibition study providing information on the potential pharmacokinetic interaction of CYP3A inhibitors with the investigational drug (investigational drug as "victim").
2. An induction study providing information on the effect of the investigational drug on CYP1A2 and CYP3A4 mediated metabolism (investigational drug as "perpetrator").
3. An induction study providing information on the potential effect of the investigational drug on the pharmacokinetics of the oral contraceptive EE and on its pharmacodynamic (contraceptive) effect (investigational drug as "perpetrator").

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B.12.2 Profiling the Effect of an Enzyme Inhibitor on Drug Pharmacokinetics

PURPOSE AND RATIONALE

The most frequent reason for drug drug interactions resulting in intolerable high drug concentrations and thereby causing safety issues is inhibition of cytochrome P450 (CYP) enzymes. The following aspects underlie the planning of an in vivo study aimed to investigate the effect of enzyme inhibition on the pharmacokinetics of the investigational drug.

Metabolic drug drug interaction studies should explore whether drugs in the marketplace are likely to affect the metabolic elimination of the investigational drug. In case the enzymes involved in the metabolic elimination of the investigational drug are polymorphic (e.g., CYP2D6), one has to bear in mind that unwanted high drug concentrations may also occur in absence of an inhibitor in poor metabolizers with regard to this enzyme. For studies investigating drug drug interactions at polymorphic enzymes, it is essential to exclude poor metabolizers from the study.

A specific objective of metabolic drug drug interaction studies is to determine whether the interaction is sufficiently large to necessitate a dosage adjustment of the drug itself or the drugs (inhibitors of the respective enzyme) it might be used with, or whether the interaction would require additional therapeutic monitoring. Potential drug drug interactions should be assessed relatively early in the drug development so that clinical implications or interactions can be assessed as fully as possible in later clinical studies.

Studies can usually be open label (unblinded), unless pharmacodynamic endpoints (e.g., adverse events that are subject to bias) are part of the assessment of the interaction.

The route of administration chosen for a metabolic drug drug interaction study is important. For the investigational drug, the route of administration should generally be the one planned for in product labeling.

In order to characterize the contribution of a metabolic pathway to the elimination of a drug and the respective effect of an enzyme inhibitor, exposure measures such as AUC and C_{max}, and pharmacokinetic parameters such as half life are recommended. In case a drug drug interaction is clearly present, the study design should be appropriate to obtain information allowing to draw conclusions on the clinical relevance. The sponsor of the study should be able to provide specific recommendations regarding dose or dose regimen adjustment, precautions, warnings, or contraindications of either the new drug or approved drugs.

PROCEDURE

The design of a study providing information on the potential pharmacokinetic interaction of CYP3A inhibitors with the investigational drug is presented in the *Protocol Outline*. For the purposes of simplicity, the description is limited to the collection, handling, and interpretation of data pertinent to the assessment of the interaction, although other parameters were also studied.

In the present case, in vitro studies had shown that the investigational drug is metabolized by CYP3A. Ketoconazole is a well known potent CYP3A inhibitor recommended as reference inhibitor for in vivo tests investigating the potential effect of CYP3A inhibition on drugs that are substrates for this enzyme (US FDA 1999, 2006). The study protocol described below is designed to achieve maximal inhibitory effect.

B.12.2.1 Protocol Outline

Interaction study to investigate a potential effect of ketoconazole on the investigational drug pharmacokinetic parameters in young, healthy, male subjects.

B.12.2.1.1 Objectives

Primary: The primary objective of the study was to assess the effect of repeated oral doses of ketoconazole (400 mg/day) on the pharmacokinetic profile of a single dose of the investigational drug (20 mg).

Secondary: The secondary objective of the study was to assess the clinical and biological safety and tolerability of the investigational drug given alone versus the investigational drug coadministered with ketoconazole.

B.12.2.1.2 Study Design

The study had a single center, open label, non randomized, two period, single sequence design.

In period 1 (day 1), subjects received a single dose of drug (20 mg) under fasted conditions, and PK samples were collected for 22 days.

Period 2 started with the administration of ketoconazole (400 mg) once daily for 5 days under fed conditions, which is sufficiently long to reach steady state conditions. On day 6, 20 mg of drug were coadministered with ketoconazole under fasted conditions. Thereafter, administration of ketoconazole was continued for 9 days under fed conditions in order to maintain steady state conditions.

In total, the wash out period between the two administrations of the investigational drug was at least 28 days.

B.12.2.1.3 Number of Subjects

Based on historical data on the drug, a true within subject standard deviation of 0.30 was assumed for AUC and C_{max}. With 18 subjects, the relative ratio (drug + ketoconazole vs. drug alone) of AUC and C_{max} means was estimated with maximum imprecision of 18.9% with 90% assurance. If the observed ratio is 2.5, then the 90% CI was no wider than 2.03 3.08, with 90% assurance, which was regarded to be acceptable. In order to ensure to obtain completed PK profiles of at least 18 subjects for the analysis, 22 subjects were included in the study.

B.12.2.1.4 Inclusion Criteria

The following inclusion criteria had to be met in order to standardize the study population: Male healthy subjects between 18 and 45 years of age. Body weight between 50 and 90 kg, with a BMI between 18 and 28 kg/m^2.

B.12.2.1.5 Treatments

Single oral administrations of 20 mg doses of drug on day 1 of period 1 and on day 6 of period 2, and repeated administrations of 400 mg doses of ketoconazole for 15 days in period 2.

B.12.2.1.6 Pharmacokinetic Data

Plasma concentrations of the investigational drug before and at predetermined times post dose in periods 1 and 2 were measured.

EVALUATION

Pharmacokinetic parameters of the investigational drug and ketoconazole were summarized by descriptive statistics.

The effect of ketoconazole on log transformed drug parameters (C_{max}, AUC_{last}, and AUC) was assessed with a linear mixed effects model. Estimates with 90% CIs for the difference in means between the two treatments (drug + ketoconazole vs. drug alone) were computed within the mixed model framework, and then converted to the ratio scale by antilogarithmic transformation. For $t_{1/2z}$, the magnitude of the effect of ketoconazole on drug pharmacokinetics was assessed with the p value within the mixed model framework.

CRITICAL ASSESSMENT OF THE METHOD

In the study design presented, the wash out period represented 3 4 half lives of the drug resulting in detectable amounts of drug in some pre dose samples of period 2. However, pre dose drug concentrations were clearly below 5% of C_{max}, thus having no relevant impact on the study outcome.

Generally, the period of blood sampling should be adapted to the expected half lives of the investigational drug in presence or absence of inhibitor. Due to the long half life of the investigational drug even in absence of an inhibitor, the AUC of one subject could not be analyzed because of an incomplete pharmacokinetic profile. In presence of ketoconazole, half life of the investigational drug was even longer resulting in four incomplete pharmacokinetic profiles (*Table B.12 1*).

Drug drug interaction study can use a randomized crossover (e.g., drug followed by drug + inhibitor, drug + inhibitor followed by drug), a one sequence crossover (e.g., either drug first and drug + inhibitor second always or the reverse), or a parallel design (drug in one group of subjects and drug + inhibitor in another). The one sequence crossover design presented in the example avoided the interindividual variability of the parallel design and a very long wash out period following coadministration of drug and ketoconazole.

Table B.12-1

Mean ± SD (CV%) [geometric mean] of drug PK parameters

PK parameter	Drug alone (N = 22)	Drug + ketoconazole (N = 21)
C_{max} (ng/mL)	105 ± 36.8	131 ± 47.4
	(35) [98.9]	(36) [123]
t_{max} (h)	1.50	3.00
	(1.00, 3.00)	(2.00, 4.07)
AUC_{last} (h × ng/mL)	2830 ± 1290	7220 ± 2820
	(46) [2490]	(39) [6620]
AUC[a] (h × ng/mL)	3130 ± 1440	8020 ± 3490
	(46) [2760]	(43) [7190]
$t_{1/2z}$ (h)	155 ± 85.1	190 ± 114
	(55) [132]	(60) [164]

Tabulated values are mean ± SD (CV%) [geometric mean] except for t_{max} where values are median (min, max)

[a] N = 21 (drug alone) or 17 (drug + ketoconazole)

When both investigational drug and interacting drug are likely to be given chronically over an extended period of time, administration of the investigational drug to steady state with collection of blood samples over one or more dosing intervals could be followed by multiple dose admin istration on the interacting drug, again with collection of blood for measurement of both the investigational drug and the interacting drug.

For both the investigational drug and the interacting drug, testing should maximize the possibility of finding an interaction. For this reason, the maximum planned or approved dose and the shortest dosing interval of the interacting drug (as inhibitor) should be used. Doses smaller than those to be used clinically may be needed for the investigational drug on safety grounds and may be more sensitive to the effect of the interacting drug.

Results of drug drug interaction studies should be reported as 90% CIs about the geometric mean ratio of the observed pharmacokinetic measures with and without the interacting drug (Schuirmann 1987). CIs provide an estimate of the distribution of the observed systemic expo sure measure ratio of drug + inhibitor versus drug alone and convey a probability of the magnitude of the interac tion. In contrast, tests of significance are not appropriate because small, consistent systemic exposure differences can be statistically significant ($p < 0.05$) but not necessar ily clinically relevant.

MODIFICATIONS OF THE METHOD

The study described above was designed to investigate the contribution of a specific CYP enzyme to the metabolic elimination of an investigational drug and the effect of CYP enzyme inhibition on the pharmacokinetic profile of the investigational drug. That means, the investigational drug is the "victim."

Alternately, one may need to study the effects of the investigational drug on already approved drugs. That means, the investigational drug is the perpetrator. In this case, the study design described above can be applied with the investigational drug as inhibitor and an approved drug as probe substrate. The choice of an appropriate substrate depends on the CYP enzyme inhibited by the investiga tional drug. In testing inhibition, the substrate selected should generally be one whose pharmacokinetics is mark edly altered by coadministration of known specific inhib itors of the enzyme system (i.e., a very sensitive substrate should be chosen) to assess the impact of the interacting investigational drug. Selected examples aretheophylline for CYP1A2, warfarin for CYP2C9, desipramine for CYP2D6, and midazolam for CYP3A.

Table B.12-2

Treatment ratio estimates and 90% CIs for drug pharmacokinetic parameters

PK parameter	Treatment ratio[a]	
	Estimate	90% CI
C_{max}	1.24	(1.05, 1.46)
AUC_{last}	2.58	(2.32, 2.86)
AUC	2.72	(2.45, 3.02)

[a](Drug + ketoconazole)/(Drug alone) ratio

If this study is positive for inhibition, further studies with other substrates may be useful, representing a range of substrates based on the likelihood of coadministration. For example, possible substrates for further study of a CYP3A inhibiting investigational drug might include dehydropyridine, calcium channel blockers, triazoloben zodiazepines, or for a CYP2D6 inhibiting investigational drug might include metoprolol. If the initial study is negative with the most sensitive substrate, it can be pre sumed that less sensitive substrates will also be unaffected.

B.12.2.2 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.

B.12.2.2.1 Results: Pharmacokinetics

The data pertinent to the assessment of potential effects of CYP3A inhibitors on the pharmacokinetics of the investi gational drug from the study described above can be summarized as follows (*Tables B.12 1* and *B.12 2*).

- Coadministration of repeated 400 mg once daily oral doses of ketoconazole with a single 20 mg oral dose of drug resulted in an increase in drug mean C_{max} (1.24 fold), AUC_{last} (2.58 fold), and AUC (2.72 fold).
- A statistically significant increase in drug $t_{1/2z}$ was noted when ketoconazole was coadministered ($p = 0.011$).

Taking together the above mentioned data, a statistically significant effect of ketoconazole on the pharmacokinetics of the investigational drug was observed. Hence, CYP3A contributes to a significant extent to the metabolism of the drug. However, due to the tolerability of the investiga tional drug, the increase in drug exposure was clinically not

relevant, and dose adjustment in presence of coadministered CYP3A inhibitors is not necessary.

REFERENCES AND FURTHER READING

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B.12.3 Exploratory Profiling of Enzyme Induction on Drug Disposition

PURPOSE AND RATIONALE

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 investigational drug itself and of concomitant medications.

Preclinical profiling for enzyme induction is complicated by the observation that, contrary to as seen for enzyme inhibition, the enzyme induction potential of a drug in man is difficult to assess preclinically, especially in nonhuman systems. There are however numerous hints, which can indicate that the drug under study has some activating effect on drug metabolizing enzymes such as the cytochrome P450 isozymes (CYP) 1A2 or 3A4. These hints can include a drop in systemic exposure to investigational drug after multiple dosing (possible autoinduction), 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 signals are observed, especially positive hints in human in vitro enzyme induction studies, then the in vivo induction potential of the investigational drug is typically studied in suitable explorative clinical studies. Given the expectation that enzyme induction is more likely to cause a reduced efficacy of investigational drug itself and/or of concomitant medications than to cause safety issues, the profiling of enzyme induction during early clinical development 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 induction 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 the investigational drug on CYP1A2 and CYP3A4 mediated metabolism, is presented in the *Protocol Outline*. 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 CYP1A2 and CYP3A4 mediated metabolism, although other parameters were also studied.

B.12.3.1 Protocol Outline

Safety, tolerability, and effect of CYP1A2 and CYP3A4 mediated metabolism of single and repeated oral doses of 400 and 1,200 mg drug or placebo in overweight or obese, but otherwise healthy men.

B.12.3.1.1 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 and 1,200 mg drug and (ii) the effect of the drug on CYP1A2 and CYP3A4 mediated metabolism.
Secondary: The secondary objective of the study was to investigate the pharmacokinetics of the drug after single and repeated oral doses of 400 and 1,200 mg in overweight or obese, but otherwise healthy men.

B.12.3.1.2 Study Design

The study had a single center, single and repeated dose, single trial period, parallel group, and double blind design.

Subjects received single doses of 150 mg caffeine, once before starting treatment with drug (day 1) and then again together with drug dosing on day 10. In addition, subjects received a single daily dose of 400 or 1,200 mg drug or matching placebo on day 2 and then again on days 4 10.

On days 1 and 10, CYP1A2 activity was monitored using the plasma concentrations of caffeine and its demethylated metabolite paraxanthine, and CYP3A4 activity was monitored using the urinary excretion of free cortisol and 6 β hydroxy cortisol and the ratios thereof (Rost and Roots 1994; Fuhr et al. 1996; Streetman et al. 2000; Kovacs et al. 1998; Tran et al. 1999).

B.12.3.1.3 Number of Subjects

The inclusion of eight subjects per dose group (400, 1,200 mg drug and matching placebo) was considered to be in line with common industry practice for this type of explorative study.

B.12.3.1.4 Inclusion Criteria

The following inclusion criteria were met: Men aged between 18 and 55 years; with 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 nonsmokers.

B.12.3.1.5 Treatments

Single (day 2) and repeated (days 4 10) oral doses of 400 or 1,200 mg drug or matching placebo after fasting. Single doses of 150 mg caffeine, once on day 1 and then again on day 10.

B.12.3.1.6 Pharmacokinetic Data

Plasma concentrations of the investigational drug, caffeine, and paraxanthine, before and at predetermined times post dose were measured.

Concentrations of the investigational drug, 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 CYP1A2 and CYP3A4 mediated metabolism from the 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 t, where t = 24 h post dose (AUC_{0-t}), AUC after extrapolation to infinity ($AUC_{0-\infty}$), apparent terminal half life ($t_{1/2z}$), 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. Changes from baseline (day 10 day 1) in caffeine clearance and 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: amount excreted (Ae) 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.

It is important that subjects enrolled in caffeine interaction studies must refrain from coffee, tea, and all other xanthine containing beverages throughout the study period.

In the described study, the time dependence and reversibility of the enzyme induction was not studied. Also the enzyme substrate used as a marker for the CYP1A2 activity was caffeine, which although frequently encountered in the target population and commonly used as a marker for CYP1A2 activity, is not a drug with a narrow therapeutic index used by the target population. The enzyme substrate used as a marker for the CYP3A4 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 CYP3A4 induction. Also urinary 6 β hydroxy cortisol and free

cortisol do not differentiate between intestinal and liver CYP3A4 activities.

MODIFICATIONS OF THE METHOD

The design of studies profiling of enzyme induction is 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 above provides explorative profil ing of potential enzyme induction after dosing over 2 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 hints from preclinical studies and experience with other mem bers of this chemical class characteristics for this drug, (ii) the chance/ability to build in the planned investiga tion 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 have severely impacted the market value of the investigational drug and thus should be profiled as early as possible.

In practice, the explorative profiling of enzyme induc tion is even less elaborate than described above because (i) it requires the use of enzyme substrates that are less amenable to inclusion in tolerability studies, and (ii) risk benefit considerations do not justify studies with a range of dose levels.

Instead, specific confirmatory clinical studies employ ing drugs with a narrow therapeutic index used by the target population and/or frequent usage in the target pop ulation are typically implemented in the late clinical devel opment as part of the range of drug drug interaction studies used to support the drug label.

B.12.3.2 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.

B.12.3.2.1 Results: Pharmacokinetics

The data pertinent to the assessment of potential drug medi ated effects on CYP1A2 and CYP3A4 mediated metabolism from study described above, as given in *Tables B.12 3* and *B.12 4*, can be summarized as follows.

- The mean $AUC_{0-\infty}$ for caffeine on day 10 was de creased by approximately 67%, and approximately

Table B.12-3

Mean (SD) plasma caffeine and paraxanthine pharmacokinetic variables (ng × h/mL) by treatment

Analyte variable		Treatment group		
Day	*N*	Placebo	400 mg	1,200 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 B.12-4

Mean (SD) ratio of urinary 6-β-hydroxy-cortisol/free cortisol excreted by treatment

Analyte variable		Treatment group		
Day	*N*	Placebo	400 mg	1,200 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)

80% when compared to day 1 for subjects treated with 400 and 1,200 mg drug, 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 approximately threefold in subjects treated with 400 mg drug and fivefold in subjects treated with 1,200 mg.
- All the caffeine/paraxanthine results suggest that the investigational drug induced CYP1A2 in humans and that the extent of induction was dependent on the dose administered.
- Given that a natural approximately 20 fold variation in CYP1A2 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 excre tion was about 11 for all treatments on both days 1 and 10 suggesting that there was no noticeable effect on CYP3A4 activity.

REFERENCES AND FURTHER READING

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B.12.4 Profiling the Effect of an Enzyme Inducer on Pharmacokinetics and Pharmacodynamics of Oral Contraceptives

PURPOSE AND RATIONALE

When discussing interactions, it is important to differen tiate between "detectable" and "clinically relevant" inter actions. It is accepted that for compounds with wide therapeutic margins, pharmacokinetic drug interactions may have little clinical relevance. An interaction is consid ered clinically relevant when the efficacy and/or toxicity of a drug is changed to such an extent that a dosage adjust ment 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. Pharmaco dynamic interactions may be caused by a wide variety of mechanisms; hence, detailed guidance for pharmacody namic studies is limited and the study design must be chosen on a case by case basis.

Typically, drug drug interaction studies include some form of comparison of the bioavailability of some marker substrate, for the example of ethinylestradiol (EE) given in the following lines, when dosed with or without concomitant dosing with the investigational drug. EE is one active component of oral contraceptives, and thus a very common concomitant medication for the drug, the target population of which is largely younger women. From ear lier in vitro and animal in vivo studies, the drug was known to be potent inducer of both phase I and phase II metabo lizing enzymes, including those enzymes reportedly involved in the clearance of EE. Since a clinically relevant drug drug interaction between the investigational drug and oral contraceptives would impact the product label and probably also impact the market value of the drug, the study described in the following lines was performed.

PROCEDURE

The design of a typical drug drug interaction study is pre sented here. 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 poten tial pharmacokinetic and pharmacodynamic interactions, although other parameters were also studied.

B.12.4.1 Protocol Outline

Effects of repeated once daily drug doses on the safety, pharmacodynamics, and pharmacokinetics of EE after dos ing with monophasic oral contraceptives containing EE in healthy overweight or obese women.

B.12.4.1.1 Objectives

Primary: The primary objective of the study was to study the effects of repeated once daily drug doses on the phar macokinetics of EE after dosing with monophasic oral contraceptives containing EE in healthy overweight or obese women.

Secondary: The secondary objective of the study was (i) to assess whether the investigational drug affects the contra ceptive effect of the oral contraceptives containing EE 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 the drug.

B.12.4.1.2 Study Design

Single center, double blind, randomized crossover study in young healthy overweight or obese women. Subjects crossed over with respect to drug or placebo that was given double blinded. The study ran over two menstrual cycles.

Cycles 1 and 2 involved dosing with either drug or matching placebo on days 6 20, dosing with the subjects regular EE containing oral contraceptive on days 8 28, hos pitalization on days 19 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, that is, the first day after completing the previous cycle (menstruation gen erally 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 were advised to continue use of these measures for at least 28 days after completing cycle 2. Dropouts were not to be replaced.

B.12.4.1.3 Number of Subjects

Based on published variability in pharmacokinetic studies of EE in lean subjects, taking confidence intervals (CIs) of 80 125%, residual variance ranged from 10% to 33%. Based on these residual variance values, calculated samples sizes ranged from 6 to 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 EE in lean subjects, (ii) published sample sizes in other studies of this type of study ranged from 12 to 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% CIs for area under the curve (AUC) ratio's EE + drug and EE alone, 24 subjects com pleted the study as planned.

B.12.4.1.4 Inclusion Criteria

The following inclusion criteria were met. Women aged between 18 and 45 years; with body mass index (BMI) of 25.0 35.0 kg/m^2; who are willing to use prescribed barrier contraceptive methods; who apart from being over weight 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 contra ceptives); who are using monophasic contraceptives containing EE as the estrogen compound; who present normal gynecological histories and normal, regular men strual 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 nonsmoking or light smokers.

B.12.4.1.5 Treatments

Cycles 1 and 2: Oral administration of (i) the subjects' normal oral contraceptive once daily (mornings before breakfast) on days 8 28 of each cycle and (ii) drug or matching placebo, once daily (mornings before breakfast) on days 6 20 of each cycle (15 doses in total).

B.12.4.1.6 Pharmacokinetic/ Pharmacodynamic Data

Plasma concentrations of EE on day 20 in cycles 1 and 2.

Plasma concentrations of the investigational drug, 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 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_{0-24}, $t_{1/2}$) were calculated using a non compartmental analysis employing a linear/log trapezoidal method.

EE in plasma: Descriptive statistics and comparison of plasma EE concentrations in cycles 1 and 2. Analysis of variance on log transformed data, 90% CIs for AUC ratio of EE + drug and EE alone ($AUC_{EE + Drug}/AUC_{EE}$).

Analysis of variance was performed on the log transformed AUC_{0-24} of EE to estimate intra subject variability. The intra subject variability was subsequently used to estimate the 90% CI of the $AUC_{EE + Drug}/AUC_{EE}$ ratio.

Drug in plasma: Individual plasma concentrations were tabulated together with standard descriptive statistics for each variable.

Progesterone in serum: Descriptive statistics and comparison of serum progesterone concentrations in cycles 1 and 2. Descriptive comparison of the proportion of subjects who ovulated while receiving drug and contraceptive concomitantly and the proportion of subjects who ovulated while 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.

17 β Estradiol in serum: Descriptive statistics and comparison of serum 17 β estradiol concentrations in cycles 1 and 2. Individual serum concentrations of 17 β estradiol were tabulated.

B.12.4.2 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.

B.12.4.2.1 Results

The data pertinent to the assessment of potential pharmacokinetic and pharmacodynamic interactions from the study described above, as given in *Table B.12 5*, can be summarized as follows.

In this study, nine different brands of oral contraceptives were used. The dose of EE per pill most commonly taken was 30 µg (by 17 subjects) and ranged from 20 to 50 µg across all subjects 24 subjects who completed the study as planned.

Pharmacokinetics: When EE was administered in combination with the investigational drug, arithmetic mean $t_{1/2}$ and T_{max} values were comparable for both treatments. Geometric mean C_{max} and AUC_{0-24} values were approximately 20 30% lower, respectively, than when EE was administered with placebo (*Table B.12 5*). The resulting treatment ratio ($AUC_{0-24,\ EE + Drug}/AUC_{0-24,\ EE + placebo}$) was 0.73 with a 90% CI of 0.68 0.78. Since these values were below the predefined 90% CI of 0.8 1.25, a

Table B.12-5

Summary statistics of pharmacokinetic parameters for ethinylestradiol

Parameter	EE + placebo				EE + Drug			
	n	Arithmetic mean	CV (%)	Geometric mean	*n*	Arithmetic mean	CV (%)	Geometric mean
C_{max} (pg/mL)	24	98.3	39	91.1	24	79.4	43	72.5
AUC_{0-24} (pg × h/mL)	24	933	34	879	24	672	32	637
$t_{1/2z}$ (h)	24	22	27	21	24	21	28	20
T_{max} (h)	24	1.2	55		24	1.2	54	

pharmacokinetic interaction between the investigational drug and EE can be concluded. The mechanism for this statistically significant reduction in systemic exposure to EE is unknown; however, since the drug is known to induce CYP1A2 in man, and CYP1A2 and phase II enzymes in animals, the observed effect could reflect a metabolic interaction between drug and EE.

Pharmacodynamics: Serum concentrations of proges terone and 17 β estradiol are considered reliable indica tors for the occurrence of ovulation. Since progesterone and 17 β estradiol concentrations were comparable for both treatments, and progesterone serum concentrations did not exceed 1.4 ng/mL (as defined in this study, pro gesterone concentrations above 1.4 ng/mL on day 20 of a menstrual cycle indicated ovulation) for both treatments, it was concluded that drug administration did not affect the contraceptive effect of EE based oral contraceptives and that no ovulation occurred in any of the subjects.

CRITICAL ASSESSMENT OF THE METHOD

The study described above could have been powered to study the effect of the drug on pharmacodynamic effects of EE, 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 the investigational drug.

Furthermore, it was not studied whether or not the observed effects on the pharmacokinetics of EE were revers ible, and if so, the time course thereof.

The lack of prior knowledge of the time course of any drug mediated induction of phases I and II metaboliz ing enzymes complicated the interpretation of the data obtained from the example study. Based on previous clinical studies, a dosing regimen for the investigational drug was chosen to ensure that enzyme induction was maximal at the time shortly before ovulation. However, it was not known whether the observed enzyme induction would fade after prolonged exposure to the drug, or whether on rechallenge, the same magnitude of enzyme induction would be seen, or if the observed induction was reversible.

This example however followed the recommended basic profiling sequence, that is in vitro profiling, confir mation of the in vitro observation in animals, explorative profiling in man. 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 approa ches and definitive studies designed to support clear label ing statements.

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 (EMEA CPMP/EWP/560/95 1997; US FDA 1997, 1999, 2006). 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 har monization of study designs and marker substrates employed, and in the manner in which the data obtained is interpreted, for example, by the development of classi fication systems. In addition, while existing guidance mainly covers cytochrome P450 mediated drug interac tions, the importance of other mechanisms such as trans porters has been recognized, and should also be addressed.

MODIFICATIONS OF THE METHOD

The following general issues and approaches should be considered (for a more detailed discussion see EMEA CPMP/EWP/560/95 1997; US FDA 1997, 1999, 2006). In the following discussion, the term *substrate* (S, in our example EE) is used to indicate the drug studied to deter mine if another drug, which is termed the interacting drug (I, in our example the investigational drug), changes its exposure. 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 uniquely correct study design for studying drug drug interactions can be defined.

The following considerations may be useful when choosing a study design.

- Interpretation of findings from these studies will be aided by a good understanding of dose/concentration and concentration/response relationships for both desir able and undesirable drug effects. In certain instances, reliance on endpoints other than pharmacokinetic measures/parameters may be useful.
- The inhibiting/inducing drugs and the substrates should be dosed so that the exposure of both drugs is relevant to their clinical use.
- 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.

- When attainment of steady state is important, long half lives of the substrate, interacting drugs, and/or their metabolites should be considered.
- 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.
- Studies can usually be open label (unblinded), unless pharmacodynamic endpoints subject to bias are part of the assessment of the interaction.
- 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 coadministration 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 coadministration 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 pharmacokinetic/pharmacodynamic (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% CIs about the geometric mean ratio of the observed pharmacokinetic measures with (S + I) and without the interacting drug (S). CIs 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.

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B.13 In Vitro/In Vivo Correlation for Drug–Drug Interactions

Xavier Boulenc · Wolfgang Schmider · Olivier Barberan

B.13.1 Introduction

Over the last 15 years, drug drug interactions (DDIs) have become one of the emerging topics of the clinical drug development. For drugs with narrow therapeutic indices, the increase or decrease of plasma concentrations can lead to adverse effects or loss of efficacy, respectively. The increase of exposure caused by DDIs can be substantial (e.g., ketoconazole and midazolam with an interaction ratio of 16 fold for AUC is a well known example) in particular with CYP3A4 (› *Fig. B.13 1*). Indeed, health authorities released dedicated guidelines in the late 1990s, some of them recently updated (FDA Guidance for Indus try 2006). In this context, simulations or predictions of DDI, bridging the gap between in vitro outcomes and clinical situation are challenging for the pharmaceutical industry, fueled by recent growth of knowledge in molec ular biology, computer based simulation/predictions, and a better understanding of the inhibition and induction mechanisms.

From an industrial point of view, a drug can be con sidered as a perpetrator and/or a victim. Victims are those drugs whose clearance is affected by a perpetrator. This last category are those drugs (or other environmental factors) that inhibit or induce the proteins (i.e., enzymes, trans porters) responsible for clearing a victim drug. A pharma cokinetic drug interaction implies that a perpetrator causes a change in the clearance of the victim, in turn either decreasing or increasing concentrations of the vic tim drug in plasma and presumably at the site of action.

Most of the proteins (but certainly not all!) to which drugs may bind are commercially available or readily pro duced in house, their role in pharmacokinetics may be dissected and assessed. Such tests include in vitro inves tigations to characterize a drug as a victim or a perpetra tor toward drug metabolizing enzymes, transporters, or plasma proteins. Quantitative metrics describing the inter action of the drug with the protein, such as binding con stants, inhibition constants, induction potential are part of this assessment and allow qualifying a drug as a potential victim and/or perpetrator toward a given enzyme/protein. It is generally accepted that all DDIs are graded according to the concentrations of the interacting drugs (Ito et al. 1998). The magnitude of the interaction requires mathe matical models that have been proposed in the literature, based on several assumptions (Bachmann 2006).

DDI prediction has been conceptualized to assist in drug development decision. For that purpose, during the last 10 years, major improvements for DDI prediction have been proposed in particular for CYP450 based DDI, even if the former equations have never been con sidered as debatable (Rowland and Matin 1973). So far, DDI predictions have been focused on metabolic drug interactions. But there are many pharmacokinetic inter actions other than those occurring at enzymatic sites, such as those involving transporters or altered physiological functions. With many drugs highly bound to plasma and tissues proteins, and with activity residing in the unbound drug, it is noteworthy that plasma protein based DDI, through displacement, were considered as major concerns during the 1970s and 1980s. In practice, this concern is currently considered as unfounded in most cases (Beneth and Hoener 2002). During the last two decades, trans porters have been identified as one of the major factors determining the pharmacokinetic properties of drug; DDI predictions have been studied and are currently one of the upcoming fields of investigations. This chapter mainly deals with metabolizing enzyme drugs based DDI predictions.

For the major CYPs involved in drug metabolism (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) assessments of CYP metabolism, inhibition, and induction are determined as early as possible in research and develop ment stages. The mechanism of the CYP inhibition (re versible or mechanism based inhibition (MBI) also called time dependent inhibition (TDI)) is a crucial factor to determine and the result can potentially influence drug development strategies. This is why predicting, as early as possible, the magnitude of clinical DDIs caused by com pounds for which CYP inhibition and/or induction profile were determined is imperative to avoid potential dangerous DDIs between compounds and putative co medication.

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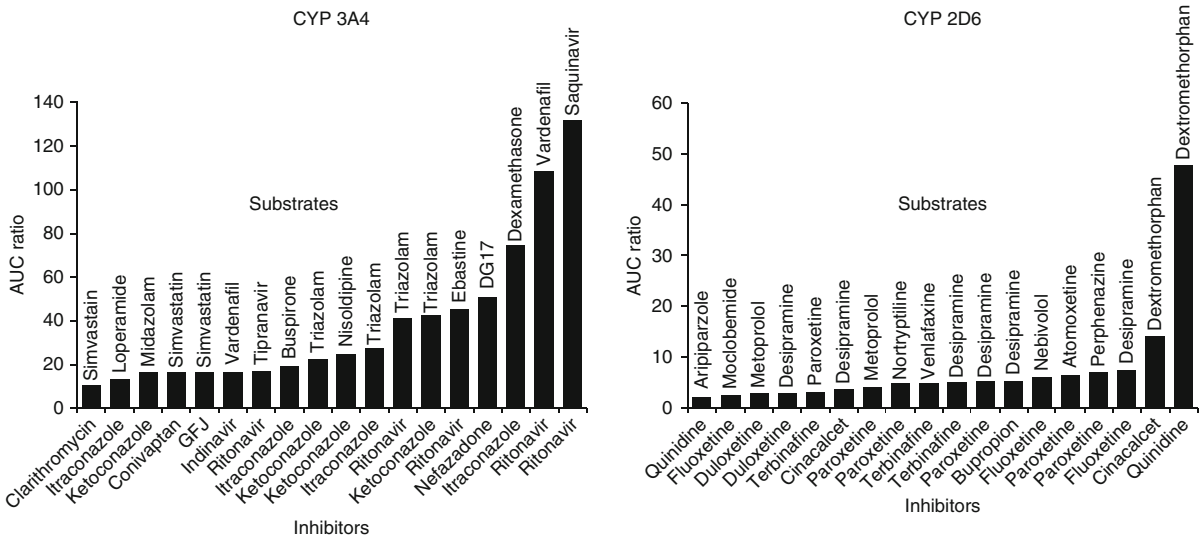

Figure B.13-1

Highest interaction ratios for the two major CYP isoforms CYP3A4 and CYP2D6. *Source*: AurSCOPE ADME/DDI® database (June 2008 release)

B.13.1.1 Methods and Assumptions

Most approaches to predict metabolic DDIs are based on in vitro in vivo extrapolation (IVIVE); however, the effects of inhibition and/or induction of drug metabolism on in vivo pharmacokinetics are highly variable and depend on the properties of the drug, the route of administration, etc. (Rowland and Matin 1973; Tucker 1992). Only main routes of administration, intravenous (bolus or infusion) and per os, were considered in this article.

B.13.1.1.1 Victim-Based Equations

Models describing the clearance decrease (inhibition), depending on the administration route, have been pro posed several years ago by Ito et al. (1998).

Intravenous Administration

The change in AUC of victim after intravenous bolus administration ($AUC_{(iv)}$) and during intravenous infusion (C_{ss}) can be expressed by the following equation, if the dose or infusion rate is constant (Ito et al. 1998)

$$\frac{AUC_{i_{(iv)}}}{AUC_{(iv)}} = \frac{C_{ss}i}{C_{ss}} = \frac{1}{f_h \times (Cl_h i/Cl_h) + 1 - f_h} \qquad (B.13.1)$$

where f_h represents the fraction of the hepatic clearance in the total clearance and Cl_h and $Cl_h i$ the hepatic clearance without and with perpetrator, respectively.

When Cl_h is rate limited by the hepatic blood flow rate (high clearance drug) and $Cl_h i$ is still rate limited, AUC ratio is equal to unit (Eq. (B.13.1)) indicating that there is no change in AUC and therefore no DDI.

Prediction of DDIs after intravenous administration of the victim can be described by Eq. (B.13.1) but remains poorly reported in the literature. Nevertheless, predictions involving high and low clearance drugs were addressed by Ito et al. (1998).

For high clearance drug ($E_h = 1$) Cl_h is rate limited by the hepatic blood flow rate if the hepatic clearance in presence of the perpetrator ($Cl_h i$) is still rate limited ($E'_h = 1$), AUC ratio is equal to unit indicating no change in AUC and therefore no DDI.

For low clearance drugs ($E_h<1$), Cl_h and $Cl_h i$ are described by the unbound intrinsic clearance of the victim without and with perpetrator. If the protein binding is not affected by the perpetrator, the AUC ratio can be estimated by Eq. (B.13.2), where f_h is the fraction of hepatic clearance (Cl_h) in total clearance (Cl_{tot}), $f_m(E_k)$ is the fraction of victim clearance mediated by the inhibited metabolic pathway k and Clu_{int_k} is the intrinsic metabolic clearance of substrate for the pathway k in the liver.

$$\frac{AUC_i}{AUC} = \frac{1}{f_h \times \sum_k^n {}_1 \frac{f_m(E)_{h,k}}{\text{Fold change } Clu_{int_k}} + 1 - f_h \times \sum_k^n {}_1 f_m(E)_{h,k}} \qquad (B.13.2)$$

High clearance drugs ($E_h = 1$), having Cl_h rate limited by the hepatic blood flow but where the hepatic clearance in

presence of the perpetrator is no more rate limited, are not addressed by Ito et al. (1998). This phenomenon occurs most of the time when perpetrator inhibition is so strong that the clearance of the victim becomes very small and consequently widely below the hepatic blood flow ($E'_h << 1$) and can lead to significant DDIs (AUC ratio >2) (Fig. B.13 2). For example, administration of keto conazole at 400 mg once a day or administration of an inactivator like erythromycin at 500 mg twice a day can fit into this category.

Per os Administration

Even if intravenous administration remains of importance, mainly for antineoplastic drugs or drugs given exclusively at hospitals, per os administration is the route of administra tion the most used for drugs on the market.

Most approaches to predict metabolic DDIs of drugs given by oral route are based on IVIVE and were de scribed for the first time by Rowland and Matin (1973). Equation (B.13.2) describes the average increase in the area under the plasma concentration time curve (AUC) of a victim drug following administration of one or more perpetrator drug(s), where $f_m(E_k)$ is the fraction of victim clearance mediated by the inhibited metabolic pathway k and $\mathrm{Clu}_{\mathrm{int}_k}$ is the intrinsic metabolic clearance of substrate for the pathway k in the liver.

The ratio of AUC estimated by using the Rowland equation takes into account only metabolism in the liver.

However, many enzymes are expressed in the human small intestine, not only cytochromes P450 enzymes (CYP3A4, CYP3A5, CYP1A1, CYP2C9, CYP2C19, CYP2D6, and CYP2J2) but also transporters (P glycoprotein, MRP1, and MRP2). CYP3A4 was determined as the most ex pressed in the small intestine, accounting for 80% of total P450s, followed by CYP2C9 (14%), CYP2C19 (2%), and CYP2J2 (1.4%) (Lin et al. 1999; Paine et al. 2006). The total amount of CYP3A expressed in the human small intestine (65.7 70.5 nmol) represents approximately 1% of the hepatic estimate (Paine et al. 1997). Furthermore, the contribution of intestine to the magnitude of DDI, may be significant, considering high levels of inhibitors in the gut lumen achieved during absorption.

Intestinal inhibition was incorporated in the DDI prediction model as the ratio of the intestinal wall avail ability in the presence and absence of the inhibitor (F'_g and F_g, respectively) by Rostami Hodjegan and Tucker (2004) and also by Obach et al. (2006) and Wang et al. (2004).

$$\frac{\mathrm{AUC}_i}{\mathrm{AUC}} \quad \frac{F'_g}{F_g} \times \frac{1}{f_h \times \sum_k^n {}_1 \frac{f_m(E_k)}{\text{Fold change Clu}_{\mathrm{int}_k}} + \left(1 \quad f_h \times \sum_k^n {}_1 f_m(E_k)\right)} \qquad (B.13.3)$$

This approach is applicable for both reversible and irre versible inhibition interactions and also induction (increase of clearance to be considered, in this case). The concept of change in clearance can be applied also for the determination of the ratio of the intestinal wall availability

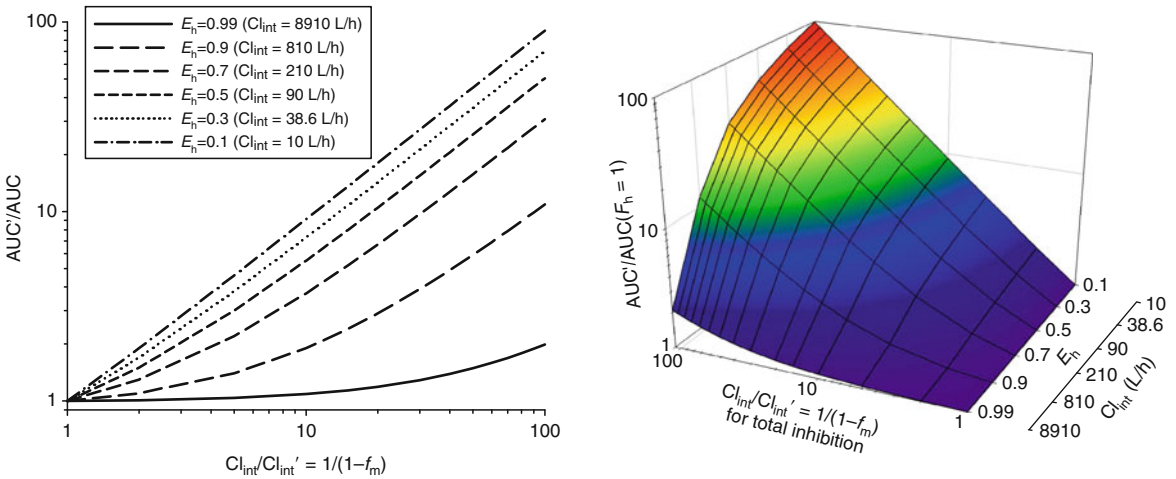

Figure B.13-2

Estimated AUC ratio after substrate administered by IV route using Eq. (B.13.1). Impact of E_h (without inhibitor) or the corresponding Cl_{int} and Cl_{int}/Cl'_{int} on AUC ratio. Simulations were conducted using the well-stirred model

in the presence and absence of perpetrator (see Eq. (B.13.4)).

$$\frac{F_g^{'}}{F_g} = \frac{1}{F_g + (1 - F_g) \times \frac{Cl'_{int,g}}{Cl_{int,g}}} \qquad (B.13.4)$$

The $F_g^{'}/F_g$, ratio can be estimated in three different ways (Galetin 2007; Galetin et al. 2008) as outlined below. The F_g control values can be determined by in vitro methods, considering the gut metabolic Cl_{int}, permeability and natural villous blood flow (see Yang et al. 2007a, b for details) and also in vivo after oral and IV administrations of the compound (Galetin et al. 2007). The details of these methods are beyond the scope of this article. However, in vivo determination, maximal $F_g^{'}/F_g$, and model predicted F_g'/F_g methods are summarized here after.

In vivo determination involves an intravenous and an oral administration in the presence of an inhibitor. This method is of less interest compared to the others because of the poor availability of data sets and the difficulty to obtain them.

A second in vivo method has been proposed with grapefruit juice (GFJ). A single administration of GFJ is able to selectively inhibit intestinal but not hepatic CYP3A4 through the fluranocoumarins that cause inhibition of the enzyme. Hence, the F_g of a CYP3A4 substrate can be determined comparing the AUC after oral administration with and without GFJ (Yang et al. 2007a, b).

The "worst case" scenario is used, that is, maximal inhibition of intestinal CYPs resulting in F_g' = 1, and therefore in a maximal F_g ratio equal to $1/F_g$. Maximal inhibition can be used as a pragmatic estimation of the F_g'/F_g (Galetin et al. 2007) mostly when perpetrators are potent competitive inhibitors or inactivators.

Model predicted F_g ratio is obtained from the decrease in the intestinal intrinsic clearance in the presence of an inhibitor using the in vitro obtained inhibitory constant (competitive or mechanism based) and the estimated inhibitor concentration in the intestinal wall during absorption phase (Obach et al. 2006; Rostami Hodjegan and Tucker 2004).

Considering per os administration, metabolism in the liver, and also in the gut must be determined for assessing DDIs in particular for CYP3A4 and UGT substrates.

Parallel Pathway: Fraction Metabolized ($f_m(E)$) However, the magnitude of a DDI is dependent not only on the characteristics of the perpetrator drug but also on the pharmacokinetic properties of the victim drug. These considerations are common to IVIVE of metabolic inhibitory drug interactions in general and will be briefly reviewed here.

Consideration of the fraction of the total clearance of the victim drug, mediated via the enzyme being inactivated ($f_m(E)$), is crucial when a risk assessment for interaction between a specific victim and perpetrator drug pair is evaluated.

The relationship between fold increase in AUC and the $f_m(E)$ of the victim drug is illustrated in *Fig. B.13 3* for various values of fold decrease in intrinsic clearance. Getting initial estimates of $f_m(E)$ of the victim drug is not always straightforward. Fractions metabolized of the victim drugs are estimated by using in vitro or in vivo approaches.

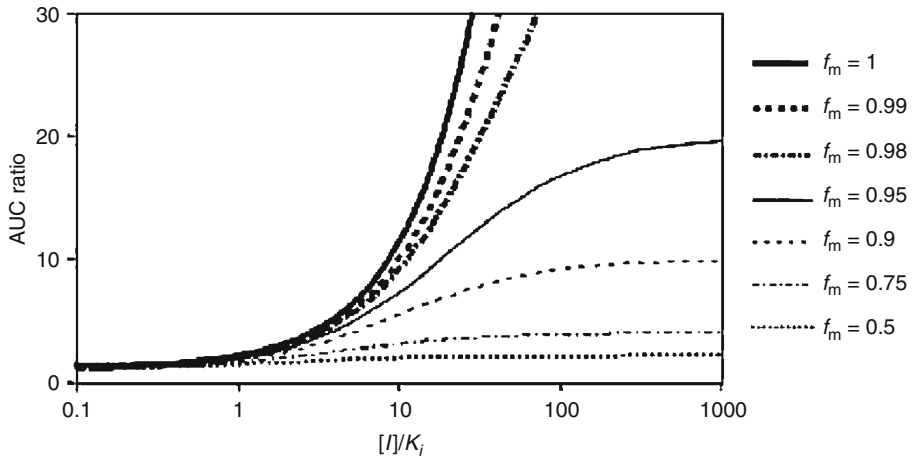

Figure B.13-3

Effect of parallel pathways of drug elimination on predicted AUC ratio assuming a competitive inhibition

In vitro approaches mainly involve reaction phenotyping of major metabolic pathways by using factors to scale to in vivo metabolic clearance from recombinant CYP experiments. The fraction metabolized for an enzyme ($f_m(E)$ vitro) is estimated by dividing the estimated clearance for this enzyme (different pathways) by the total in vitro clearance (Eq. (B.13.5)).

$$f_m(E_i)\text{vitro} = \frac{\sum_{k\ 1}^{p} \text{Cl}_{\text{int},u_k}(rE_i)\times \text{SF}_{ki}}{\sum_{j\ 1}^{n}\left(\sum_{k\ 1}^{p} \text{Cl}_{\text{int},u_k}(rE_j)\times \text{SF}_{kj}\right)} \quad (B.13.5)$$

where there are j enzymes with corresponding $\text{Cl}_{\text{int}_k}(rE_i)$ values calculated from enzyme kinetic parameters for different pathways k in each recombinant system and where SF_{kj} was a scaling factor corresponding to the pathway k and enzyme j.

Each Cl_{int} value is calculated from the general Michaelis Menten equation: $\text{Cl}_{\text{int}} = V_{\max}/K_m$, assuming $[S]$ concentration of substrate is much lower than K_m value. Otherwise, $[S]$ is not negligible and saturation occurs, depending on the $[S]$ value; the full following equation is to be considered for each $[S]$ value: $\text{Cl}_{\text{int}} = V_{\max}/(V_{\max} + [S])$.

When recombinant systems are used, a relative activity factor (RAF) was proposed as scaling factor to allow for differences in the activity of the enzymes per unit of microsomal protein compared with that in human liver microsomes (HLM) (Crespi 1995). Initially the RAF was defined relatively to $V_{\max}$ (Eq. (B.13.6)) but RAF based on intrinsic clearance ($\text{RAFCl}_{\text{int}}$) was also used and it was demonstrated that prediction accuracy of metabolic clearance in HLM was increased when $\text{RAFCl}_{\text{int}}$ was used instead of $\text{RAF}V_{\max}$ (Emoto and Iwasaki 2007). Furthermore, recombinant CYP microsomes co expressed with cytochrome b5 might be suitable for the prediction, at least in the case of recombinant microsomes from insect cells (Emoto et al. 2006). Nakajima et al. (2002) suggested that the expression level of cytochrome b5 recombinant CYP microsomes and NADPH CYP oxidoreductase used for the prediction was not crucial while cytochrome b5 and NADPH CYP oxidoreductase are expressed in recombinant systems. Co expression of cytochrome b5 might facilitate the scaling of data in recombinant CYP microsomes to the predicted value in HLM.

$$\text{RAF}_{kj} = \frac{V_{\max_{kj}}(\text{HLM})}{V_{\max_k}(rE_j)} \quad (B.13.6)$$

RAF has units of pmol of enzymes/mg of protein. Hence, the contribution of the activity of each enzymes involved in the metabolism of a NCE could be scaled to HLM. The RAF approach has been applied, for example, for the determination of the relative contribution of metabolic pathways to net mirtazapine biotransformation (Stormer et al. 2000). This parameter was originally suggested for CYPs but was also used for UGTs with a slight modification (Toide et al. 2004).

The RAF approach does not take into account interindividual variability since it is very difficult to differentiate experimental variability from the true interindividual variability in liver samples.

Alternatively, enzyme abundance could be used as scaling factor (Eq. (B.13.7)) to estimate fraction metabolized in vitro, by extrapolation of recombinant clearances to HLM clearances (Rodrigues 1999).

$$f_m(E_i)\text{vitro} = \frac{\sum_{k\ 1}^{p} \text{Cl}_{\text{int},u_k}(rE_i)\times E_{i\,\text{abundance(HLM)}}}{\sum_{j\ 1}^{n}\left(\sum_{k\ 1}^{p} \text{Cl}_{\text{int},u_k}(rE_j)\times E_{j\,\text{abundance(HLM)}}\right)} \quad (B.13.7)$$

where there are j CYPs with corresponding $\text{Cl}_{\text{int}_k}(rE_i)$ values calculated from enzyme kinetic parameters for different pathways k in each recombinant system and where enzyme abundance (E_j) was the amount of j enzyme per mg microsomal protein (pmol/mg of prot) in HLM.

However, abundances are needed to correct for the relative contribution of each enzyme to the total metabolism and most of the time this is not always straightforward. Shimada et al. (1994) remain the most cited source for abundance information, despite the fact that the study was based on liver samples from only 30 Caucasian and 30 Japanese subjects. A meta analysis of literature was done by Rowland Yeo et al. (2004) including more than 200 human liver samples for Caucasian subjects only.

Recently, abundance of enzymes in the liver (pmol/mg of protein) was determined from a literature analysis of 18 separate studies based on 315 livers using weighted mean (Barberan, unpublished results). Only data from adult Caucasians (more than 16 years old) were included, and sources were verified to exclude duplication of individual data in the analysis (*Table B.13 1*).

However, using enzyme abundances as scaling factor do not allow for any difference in activity per unit amount of CYP between the expression system and the liver. So Proctor et al. (2004) proposed incorporating a scaling factor (ISEF, intersystem extrapolation factor) in the abundance method to take into account different intrinsic activities in recombinant enzyme systems. ISEF is a dimensionless number used as a direct scaler to convert data obtained with a recombinant enzyme system to an HLM one.

$$\text{ISEF}_{kj} = \frac{V_{\max_{kj}}(\text{HLM})}{V_{\max_k}(rE_j)\times E_{j\ \text{abundance (HLM)}}} \quad (B.13.8)$$

Table B.13-1

Abundances of enzymes in the liver determined by literature analysis of 18 separate studies based on 315 livers

CYP	Abundance pmol/mg (HLM)
1A2	48.8
2A6	21.5
2B6	14.6
2C8	15.5
2C9	69.6
2C19	15.4
2C18	2.5
2D6	9.5
2E1	74.3
3A4	173
3A5	59.4

where E_j abundance (HLM) refers to the abundance of the jth enzyme in the liver and where V_{max} refers to metabolism of a probe substrate by an individual enzyme. The units of V_{max}(HLM), $V_{max}(rE)$, and abundance are pmol/min/mg protein, pmol/min/pmol enzyme, and pmol enzyme/mg protein, respectively.

As with RAF the ISEF may be defined with respect to V_{max} (Eq. (B.13.8)) but also to intrinsic clearance. ISEF is inserted in Eq. (B.13.9) as follows:

$$f_m(E_i)\text{vitro} = \frac{\sum_{k\ 1}^{p} \text{Cl}_{\text{int},u_k}(rE_i) \times \text{ISEF}_i \times E_{i\ \text{abundance(HLM)}}}{\sum_{j\ 1}^{n} \left(\sum_{k\ 1}^{p} \text{Cl}_{\text{int},u_k}(rE_j) \times \text{ISEF}_j \times E_{j\ \text{abundance(HLM)}}\right)} \quad (B.13.9)$$

where there are j CYPs with corresponding $\text{Cl}_{\text{int}_k}(rE_i)$ values calculated from enzyme kinetic parameters for different pathways k in each recombinant system and where enzyme abundance (E_j) was the amount of j enzyme per mg microsomal protein (pmol/mg prot)in HLM and ISEF_j was an intersystem extrapolation factor corresponding to enzyme j.

The second in vitro CYP reaction phenotyping method consists in using HLM incubated with or without chemical inhibitors or inhibitory antibodies. Use of appropriate inhibitor concentrations is essential and it is important to ensure that the extent of inhibition (e.g., percent of inhibition versus control) with the antibodies or the chemical inhibitor is comparable for each CYP in order to avoid any bias in the calculation leading to an over or under estimation of a given CYP isoform involvement in the clearance. In the case where a compound is metabolized by multiple CYP isoforms, a combinatorial approach with two or more inhibitors (or antibodies) may be considered (Zhang et al. 2007). $f_m(E)$ for a given CYP isoform is calculated similarly to what is done in vivo with Eq. (B.13.12), Cl ratio is obtained with in vitro clearances with or without inhibitor of the CYP isoform. For polymorphic CYP isoform, genotyped/phenotyped HLM can be used. Clearance comparison between poor and extensive HLM metabolizers enables $f_m(E)$ determination as done in vivo (see Eq. (B.13.11)). Instead of HLM, another more physiological in vitro material can be considered: human hepatocytes. The approach is the same, even if specific inhibitors are more difficult to select. The advantage of this last method over the others is the lack of scaling factors necessity, and the "more physiologic" feature of the material. Other tissue based systems are sometimes used (e.g., liver tissue slice) with a similar methodology. Nevertheless, the lack of specificity of the inhibitors/antibodies is a concern and complication may arise due to differing effects on enzyme kinetics of various substrate and inhibitor concentrations.

It is also worth noting that low turnover compounds (low Cl_{int}), metabolized by more than two CYPs are difficult to reaction phenotype. It is particularly true with HLM and hepatocytes methods as in this case clearance difference with or without inhibitor are difficult to assess.

A variety of in vitro systems can be used to evaluate the roles of different CYPs in the metabolic clearance of a victim, and each system has its own pros and cons. Therefore, it is often recommended to use more than one system and to integrate all of them to consider various aspects of the experimental design and the entire in vitro data set to make an assessment of CYP reaction phenotype.

Instead of using in vitro experiments, in vivo approaches were applied to determine $f_m(E)$ like phenotyping, chemical in vivo inhibition, nonlinear regression, or renal excretion.

The simplest case is that of polymorphic expression of the enzyme being inhibited with the existence of clearly definable extensive metabolizer (EM) and poor metabolizer (PM) populations, for example, CYPs 2D6, 2C9, or 2C19. The fraction metabolized by the polymorphic enzyme could be expressed by the following relationship (10.10) involving EM and PM clearance or AUC (Gibbs et al. 2006).

$$f_m(E) = \left(1 - \frac{\text{Cl(PM)}}{\text{Cl(EM)}}\right) \times \frac{1}{1 - \text{EF}} = \left(1 - \frac{\text{AUC(EM)}}{\text{AUC(PM)}}\right) \times \frac{1}{1 - \text{EF}} \quad (B.13.10)$$

where EF (enzyme function) is the ratio of polymorphic enzyme function in a PM relative to an EM subject. As described above, EF values greater than 0 and less than 1 could represent the degree of impairment in enzyme function resulting from the polymorphism.

For the specific case where CYP2D6 is the polymorphic enzyme of interest and EF = 0 meaning that the polymorphic enzyme pathway is absent in the PMs and that only one enzyme is subject to inhibition, Eq. (B.13.10) can be simplified to

$$f_m(E) = 1 - \frac{Cl(PM)}{Cl(EM)} = 1 - \frac{AUC(EM)}{AUC(PM)} \qquad (B.13.11)$$

In such cases, knowledge of the pharmacokinetics in EM versus PM subjects will help to define $f_m(E)$. For example, this approach was extensively and successfully used to estimate f_m(CYP2D6) (Ito et al. 2005) for a set of nine CYP2D6 victims (Venkatakrishnan and Obach 2005).

Alternatively, initial estimates of $f_m(E)$ may be obtained from the results of clinical DDI studies with selective potent inhibitors of enzymes that produce essentially complete inhibition of the enzyme of interest (in vivo chemical inhibition method). A total inhibition ($[I]/K_i >> 1$ (competitive inhibition) or $\frac{k_{inact} \times [I_u]_{H,j}}{k_{deg,H} \times ([I_u]_{H,j} + KI_{u_j})}$ >>1 (for MBI) produce a maximum AUC ratio possible that is solely dependent on $f_m(E)$ (B.13.12)

$$f_m(E) = 1 - \frac{1}{AUC_{ratio}} = 1 - Cl_{ratio} \qquad (B.13.12)$$

A total inhibition is assumed when, for example, ketoconazole is administered at 400 mg/day for CYP3A or when paroxetine is administered at 30 mg/day for CYP2D6. This method was used by Ohno et al. (2007, 2008) to estimate fraction metabolized of CYP3A4 victims by using ketoconazole or itraconazole as selective competitive inhibitors and diltiazem as mechanism based inactivator. A similar study on CYP3A4 was published by Shou et al. (2008) but with an extended list of inhibitors (troleandomycin, ritonavir, mibefradil, fluconazole, itraconazole, clarithromycin, saquinavir, erythromycin, and GFJ). An estimate of 0.93 for the f_m(CYP3A) value for the CYP3A probe substrate midazolam has been derived based on an observed mean 16 fold increase in total exposure following administration of 400 mg/day ketoconazole (Obach et al. 2006).

When a significant number of clinical interaction data exists for each victim (multiple inhibitors) a more sophisticated method, involving nonlinear least squares regression, was proposed. Clinical interactions were fitted to Eq. (B.13.3) for competitive inhibitors (Brown et al. 2005; Ito et al. 2005) and total hepatic input concentration ($[I]_{in}$) was used as the inhibitor concentration for all the predictions. An estimate of 0.94 for the f_m(CYP3A4) has been derived based on the AUC ratio observed in vivo and $[I]/K_i$ ratio for 10 DDIs involving midazolam as the victim drug.

For CYP3A4 substrate, the isoform involvement is classically investigated with a standard study design (i.e., 400 mg ketoconazole QD administration). Using a set of substrate co administered in clinic in these same conditions, observed AUC ratios and $f_m(E)$ values estimated in vitro (human hepatocytes) were fitted in order to estimate the inhibitory index and the corresponding $[I]/K_i$ of ketoconazole in these clinical conditions (*Fig. B.13 4*). In this approach, the uncertainty of each $f_m(E)$ value, determined in vitro, of the substrates (11 compounds) used in the model, are assumed to compensate each other in order to allow an accurate prediction of ketoconazole $[I]/K_i$ in these clinical conditions. With this estimated $[I]/K_i$ (=50 based on our model) of ketoconazole, it makes easier the CYP3A4 $f_m(E)$ determination of an investigational compound tested in the same clinical conditions. A number of other approaches were employed to approximate $f_m(E)$. Initial values were obtained from estimates of total metabolism calculated indirectly from urinary recovery of unchanged drug (renal excretion method) mainly for CYP3A4 (Brown et al. 2005). A good alternative for

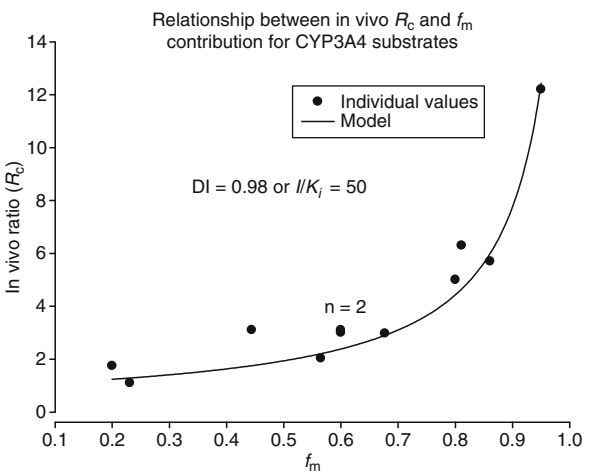

Figure B.13-4

Relationship between in vivo observed AUC ratio after 400 mg QD ketoconazole coadministration in the same conditions for 11 CYP3A4 drug substrates. The model used is $R_c = 1/[(f_m(1 \quad DI) + (1 \quad f_m)]$; with $DI = [I]/(K_i + [I])$. Fitting the model allow to determine a DI of 0.98, corresponding to a $[I]/K_i$ in vivo of 50

polymorphic enzymes is "phenocopying," that is from the difference between the urinary recovery of metabolites in both the presence and absence of a selective inhibitor (Back and Orme 1989) and was employed for the deter mination of $f_m(E)$ for CYP2C9 substrate tolbutamide using sulfaphenazole as inhibitor.

A number of approaches exist to obtain $f_m(E)$ values and were described above. The importance of considering $f_m(E)$ to predict accurate DDIs was emphasized by many authors not only for mechanism based or competitive inhibition (Brown et al. 2005; Ito et al. 2005; Obach et al. 2005; Ohno et al. 2008) but also for induction (Ohno et al. 2008). For example, assuming a competitive inhibition and only one pathway inhibited, determination of abso lute value of $f_m(E)$ is of great importance whether the particular enzyme under examination contributes more than 50% of the total clearance. Despite the potency ($[I]/K_i$) of the inhibitor, a maximal AUC ratio of 2 is obtained when $f_m(E)$ is equal to 0.5. With a value of $[I]/K_i$ over 10 a small increase in $f_m(E)$ will impact AUC ratio dramatically. This phenomenon enhances exponen tially in conjunction with the increase of $f_m(E)$ (*Fig. B.13 3*). Consequently, accurate determination of $f_m(E)$ will be mandatory for exact prediction of DDI.

Whether the usage of $f_m(E)$ is not debatable this is not the case of methods employed to determine the value for a particular substrate. The general tendency when *Figs. B.13 5* and *B.13 6* are observed is a significant variability of $f_m(E)$ values not only for CYP2D6 but also for CYP3A. For example, Felodipine, a substrate of CYP3A4, has a maximum and a minimum value of 0.99 and 0.81, leading to a maximum AUC ratio (assuming a total competitive inhibition $[I]/K_i >> 1$) of 100 and 5.26, respectively.

Gut level: Metabolism of drugs occurred not only in the liver but also in the gut and most of the time only CYP3A4 was considered (Galetin et al. 2006; Einolf 2007; Fahmi et al. 2008, 2009), because it was determined as the most expressed in the small intestine, accounting for 80% of total CYPs (Lin et al. 1999; Paine et al. 2006). Nevertheless, many other CYPs are expressed in the human small intes tine (CYP3A5, CYP1A1, CYP2C9, CYP2C19, CYP2D6, and CYP2J2) and are also involved, with a less extent, in the metabolism of drugs. So considered only CYP3A4 can lead to less accurate prediction than expected and to critical issues if metabolism of the drug in the gut is mainly due to others CYPs than the CYP3A4.

Consequently, when more than one cytochrome P450 is involved in the intestinal metabolism, a fraction of victim clearance mediated by the inhibited or induced

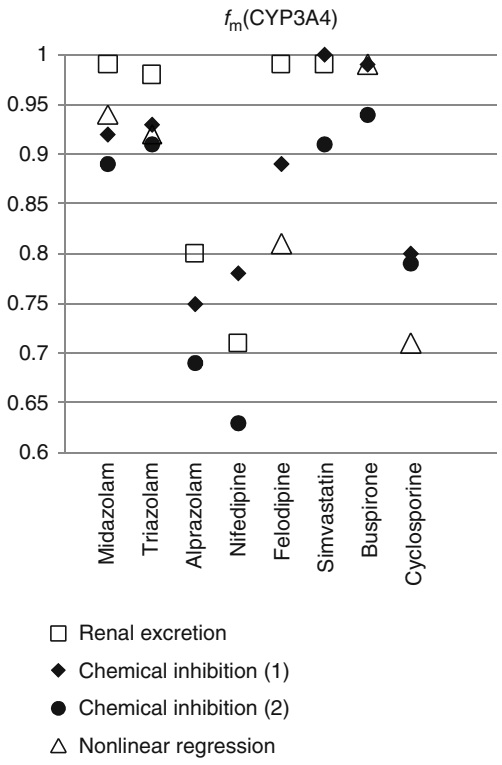

Figure B.13-5

f_m(CYP3A) values were collected from literature analysis using the AurSCOPE ADME/DDI® database (December 2008 release) based on renal excretion, chemical inhibition, and nonlinear regression

metabolic pathway must be involved in the prediction model, as it was done in the liver (*Eq. (B.13.13)*).

$$\frac{Cl'_{int,g}}{Cl_{int,g}} = \sum_{k\ 1}^{n} \frac{f_m(E)_{k,g}}{\text{Fold change Clu}_{int_{k,g}}} = 1 - \sum_{k\ 1}^{n} f_m(E)_{k,g} \qquad (B.13.13)$$

where $f_m(E)_{k,g}$ is the fraction metabolized by enzyme k in the gut wall.

The net intrinsic metabolic clearance in the gut (Cl_{int,u_g}) is contributed by all the intestinal enzymes in volved in the metabolism of the compound. Assuming that intrinsic metabolic clearance in the gut and in the liver are the same when expressed per pmol of enzyme (Yang et al. 2004), the contribution of a particular enzyme to the net intrinsic metabolic clearance in the gut may be estimated from the product of the intrinsic unbound clearance per unit of enzyme multiplied by a scaling factor

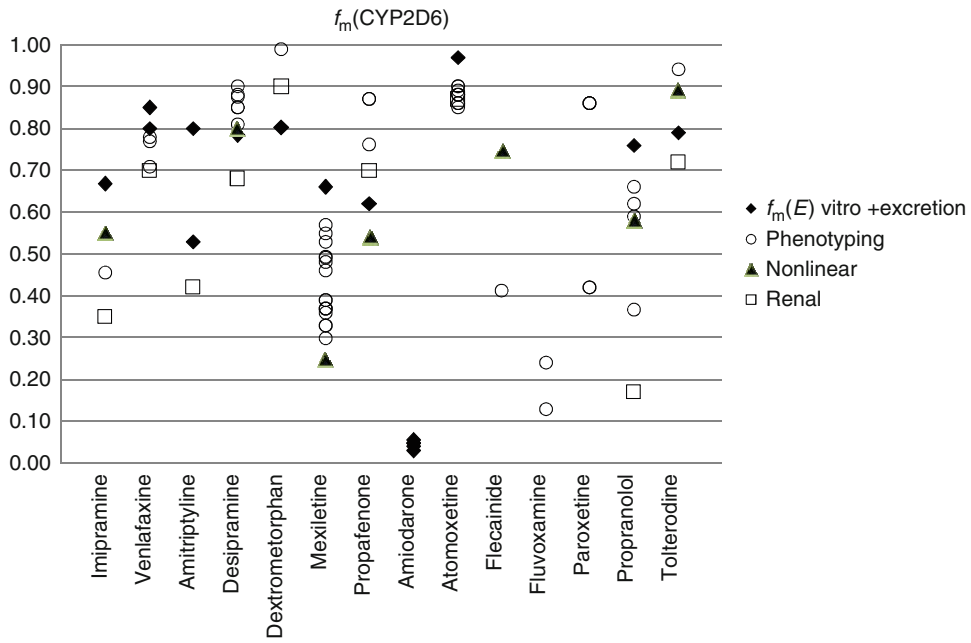

Figure B.13-6

f_m(CYP2D6) values were collected from literature analysis using the AurSCOPE ADME/DDI database (December 2008 release) based on renal excretion, nonlinear regression, phenotyping, and in vitro determination methods

(10.15). This scaling factor could be the same than those used in the liver (ISEF, RAF, or abundance).

$$f_m(E_i)_g = \frac{\sum_{k\ 1}^{p} \mathrm{Cl}_{\mathrm{int},u_k}(rE_i) \times \mathrm{SF}_{ki}}{\sum_{j\ 1}^{n}\left(\sum_{k\ 1}^{p} \mathrm{Cl}_{\mathrm{int},u_k}(rE_j) \times \mathrm{SF}_{kj}\right)} \quad (B.13.14)$$

General considerations: The in vivo situation is some times more tricky as total clearance and $f_m(E)$ values can vary, reflected by a nonlinear pharmacokinetic. Herewith are presented the three clinical major situations for which $f_m(E)$ values of the enzymes involved in a given compound can vary.

1. *Saturation of one metabolic pathway*: One metabolic pathway can be saturated when increasing dose whereby the overall clearance is decreased and all the $f_m(E)$ values are rearranged ($f_m(E)$ of the not saturated pathways are increased) (*Fig. B.13 7*).
2. *Induction of one metabolic pathway*: In the other way round, the relative involvement of a given isoform can increase in case of induction; in this case the overall clearance is increased and all other $f_m(E)$ values are also rearranged (decrease).
3. *Genotyping*: It is also worth noting that genetic poly morphism involving CYP genes, has of course a tre mendous impact on the $f_m(E)$ polymorphic enzyme but also on the nonpolymorphic ones. In subjects who lack the major metabolic clearance pathway (poor metabolizer), remaining pathway(s) become impor tant (*Fig. B.13 8*). Authorities recommend addressing these pathway(s) in these specific populations (FDA Guidance for Industry 2006).

B.13.1.1.2 Perpetrator-Based Equations

Equations proposed for perpetrators depend on the mech anism by which they have an impact on the clearance of the victim. Basically, three interaction mechanisms have been described: reversible inhibition, TDI, and induction.

Competitive Inhibition

In the presence of competitive inhibition, the fold change in intrinsic hepatic clearance inhibited by enzyme k can be applied to the liver or the gut and will be determined by the unbound concentration of perpetrator(s) (in the liver or in the gut) and its associated unbound equilibrium dissociation constant ($K_i u$) as follows (Eq. (B.13.15)), assuming perpetrators are acting through the same mech anism of inhibition:

$$\text{Fold change } \mathrm{Clu}_{\mathrm{int}_{h/g,k}} = 1 + \sum_{j\ 1}^{p}\left(\frac{[I_u]_{h/g,j}}{K_i u_j}\right) \quad (B.13.15)$$

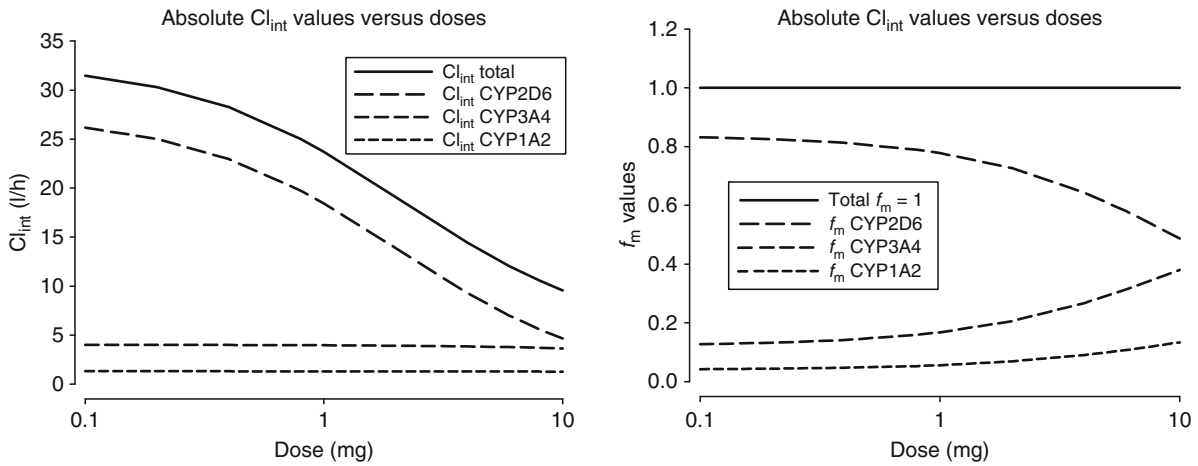

Figure B.13-7

Absolute Cl_{int} (*left*) and corresponding $f_m(E)$ values (*right*) for a compound metabolized by CYP1A2, 2D6, and 3A4 with a saturation of CYP2D6. Data were simulated with the following V_{max}, (pmol/min/pmol of CYP) and K_m (μM) parameter values: CYP2D6 (K_m = 0.2; V_{max} = 5.6); 1A2 (K_m = 32; V_{max} = 43); 3A4 (K_m = 10; V_{max} = 40). $f_m(E)$ is calculated with Eq. (B.13.6); each Cl_{int} values are calculated for each substrate concentration with $Cl_{int} = V_{max}/(K_m + [S])$ (see parallel pathway, $f_m(E)$ calculation in this chapter). For clearance calculation, concentration was assumed to be equal to the portal concentration with a fraction absorbed F_{abs} = 1 and gut bioavailability F_g = 1

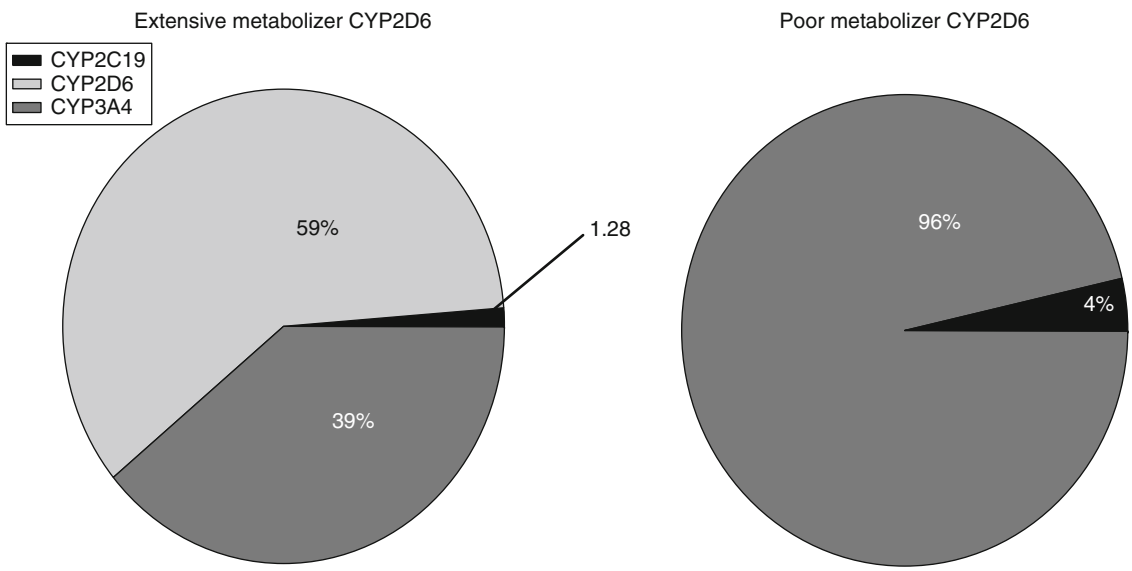

Figure B.13-8

CYP3A4, 2D6, and 2C19 isoform relative contributions in intrinsic clearance of CYP2D6 EM and PM subjects for a substrate metabolized by these three enzymes

where $[I_u]_{h/g,j}$ is the unbound concentration of perpetrator j at the enzyme site (liver or gut) and $K_i u_j$ the unbound inhibition constant for perpetrator j obtained from in vitro studies after accounting for nonspecific binding (see $fu_{(mic)}$ determination in this chapter). Whereas the determination of the perpetrator concentration in the liver or in the gut will be detailed in a dedicated chapter, the way of measuring and determining inhibitory constant (K_i, IC_{50}) is discussed below.

The human liver microsomal assay (HLM assay) is accepted as the "gold standard" for in vitro DDI assessment as it is felt to be closest to the native enzyme environment regardless the batch variability, potential transporter involvement, and cell penetration complications associated with human hepatocytes. The use of heterologous expression systems is not without complications either. The stoichiometry between NADPH cytochrome P450 reductase, cytochrome b5, and individual CYP enzymes is sometimes different in heterologous expression systems than in microsomes. The HLM assay was used by many authors but extensively by the group of Walsky for assessing in vitro DDI involving CYP2B6 and CYP2C8 (Walsky and Obach 2004; Walsky et al. 2005, 2006).

A short description of the methods used to measure enzyme activity, with an emphasis on the key points to manage will be described in this chapter. A complete view of these methods could be found in specialized reviews (Fowler and Zhang 2008; Rodrigues 2002) or in regulatory guidance (FDA Guidance for Industry 2006).

In practice, however, CYP inhibition measurement is almost always performed by analyzing inhibition of substrate metabolism. Determination of in vitro parameters remains a crucial step in the extrapolation process not only because most of the approaches used for predicting DDI interaction are based on IVIVE, but also because in vitro parameters are used extensively in the extrapolation process. A wrong determination of in vitro parameters will conduct most of the time to a wrong prediction whatever the method used (MSM, MDM, $[I]/K_i$), but sometimes, and this is more critical for future development of product, to a wrong prediction that could be misunderstood because realistic enough to be confound with a true one.

For all these reasons, inhibitory constants must be determined as precise as possible by using standardized methods. Unfortunately, using only these standardized methods is not enough to avoid discrepancies between the determinations of the same inhibitory constant, so effects contributing to differences in data between assay types are discussed below.

When determination of inhibitory constants is performed, the substrate, of course, is metabolized but inhibitors metabolism could occur during incubation and if the proportion of this metabolism is significant could conduct to, obviously, a discrepancy between measured IC_{50}s. Incubation duration and enzyme concentration are crucial elements to take into account for minimizing inhibitors metabolism. A recent study showed that inhibitors metabolism may occur in a significant amount when using 0.5 mg/mL HLM and 20 min incubation time (Di et al. 2007). Consequently, to minimize inhibitor metabolism, protein concentration and incubation time must be kept as low as possible; normally this is the case in modern inhibition experiment where HLM concentration of 0.05 0.2 mg/mL and an incubation time of 3 5 min are used.

Of course, when dealing with inhibitors metabolism, inhibitor metabolites must be taken into account with the possibility that metabolites more inhibitory than the parent substance were generated in significant quantities. This aspect is well documented when inhibition is time dependent (see Sect. B.13.1.1.2.2) but this is not the case for competitive inhibition.

In theory, the same IC_{50} value should be generated for inhibition of the same enzyme when performed under the same conditions (protein concentration, solvent concentration, buffer, and enzyme source) if different substrates are used at the K_m concentration. However, this is often not the case in practice. There are differences in inhibition activities of test compounds, dependent upon the substrate chosen for some CYPs, especially CYP3A4 but also CYP2C9.

Due to the substrate differential response observed for CYP3A4, the recommended approach to CYP3A4 DDI analysis is the use of multiprobes (Tucker et al. 2001; Bjornsson et al. 2003) where the lowest inhibition constant (K_i) used indicates the "worst case scenario" for a potential interaction. An analysis of 26 in vivo interaction studies between four different CYP3A4 substrates and three competitive inhibitors namely ketoconazole, itraconazole, and fluconazole were done by Galetin et al. (2005) and based on the percentage of AUC ratio predictions that were within the twofold standard, midazolam as an in vitro substrate for CYP3A4 predicted 77% of the inhibitory DDIs quantitatively accurately, testosterone as an in vitro substrate predicted 71% of the DDIs accurately, nifedipine as an in vitro substrate predicted 69% of the DDIs accurately, and quinidine as an in vitro CYP3A4 substrate predicted 81% of the DDIs quantitatively accurately. It was concluded by Galetin et al. (2005) that

midazolam and quinidine provided the best assessment of DDIs involving CYP3A4.

Nevertheless, the preferred substrates proposed by the FDA in the last draft for CYP3A4 were midazolam and testosterone, quinidine was proposed neither as preferred nor as acceptable substrate (*Table B.13 2*).

An additional complicating issue in the assessment of CYP3A inhibition potential is the occurrence of homo/heterotropic cooperativity (allosteric effect) in vitro (Galetin et al. 2003). These "atypical" phenomena attributed to the existence of multiple binding sites have been associated with CYP3A. Recent publications indicate similar behavior for CYP2C9 (Egnell et al. 2003; Hutzler et al. 2003; Foti and Wahlstrom 2008) and UDP glucuronosyltransferase enzymes (Uchaipichat et al. 2004). Nevertheless, the in vivo consequences of these allosteric mechanisms are still unclear, even if recently authors proposed to explain some in vivo results observed with flavonoids in particular, by such phenomena (Henshall et al. 2008).

Time-Dependent Inhibition

The inactivation of CYPs by reactive products that form heme or protein adducts or a metabolic inhibitory com plex (MIC) is referred to as mechanism based inhibition (MBI) or time dependent inhibition (TDI). MBI is char acterized as an irreversible or quasi irreversible inactiva tion of the CYP, requiring synthesis of new enzyme for recovery of activity. TDI is generally involved in more complex DDI than reversible competitive inhibition as it can result in a more profound and prolonged effect than the therapeutic dose or exposure might suggest. For CYP3A4, the number of TDI interactions accounted

Table B.13-2

Preferred and acceptable chemical substrates for in vitro experiments provided by FDA Guidance for Industry (2006)

CYP	Substrate preferred	K_m (μM)	Substrate acceptable	K_m (μM)
1A2	Phenacetin-*O*-deethylation	1.7 152	7-Ethoxyresorufin-*O*-deethylation	0.18 0.21
			Theophylline-*N*-demethylation	280 1230
			Caffeine-3-*N*-demethylation	220 1565
			Tacrine 1-hydroxylation	2.8, 16
2A6	Coumarin-7-hydroxylation	0.30 2.3		
	Nicotine C-oxidation	13 162		
2B6	Efavirenz hydroxylase	17 23	Propofol hydroxylation	3.7 94
	Bupropion-hydroxylation	67 168	S-mephenytoin-*N*-demethylation	1910
2C8	Taxol 6-hydroxylation	5.4 19	Amodiaquine *N*-deethylation	2.4
			Rosiglitazone para-hydroxylation	4.3 7.7
2C9	Tolbutamide methyl-hydroxylation	67 838	Flurbiprofen 4′-hydroxylation	6 42
	S-warfarin 7-hydroxylation	1.5 4.5	Phenytoin-4-hydroxylation	11.5 117
	Diclofenac 4′-hydroxylation	3.4 52		
2C19	S-mephenytoin 4′-hydroxylation	13 35	Omeprazole 5-hydroxylation	17 26
			Fluoxetine *O*-dealkylation	3.7 104
2D6	(±)-Bufuralol 1′-hydroxylation	9 15	Debrisoquine 4-hydroxylation	5.6
	Dextromethorphan *O*-demethylation	0.44 8.5		
2E1	Chlorzoxazone 6-hydroxylation	39 157	p-Nitrophenol 3-hydroxylation	3.3
			Lauric acid 11-hydroxylation	130
			Aniline 4-hydroxylation	6.3 24
3A4/5	Midazolam 1-hydroxylation	1 14	Erythromycin *N*-demethylation	33 88
	Testosterone 6 b-hydroxylation	52 94	Dextromethorphan *N*-demethylation	133 710
			Triazolam 4-hydroxylation	234
			Terfenadine C-hydroxylation	15
			Nifedipine oxidation	5.1 47

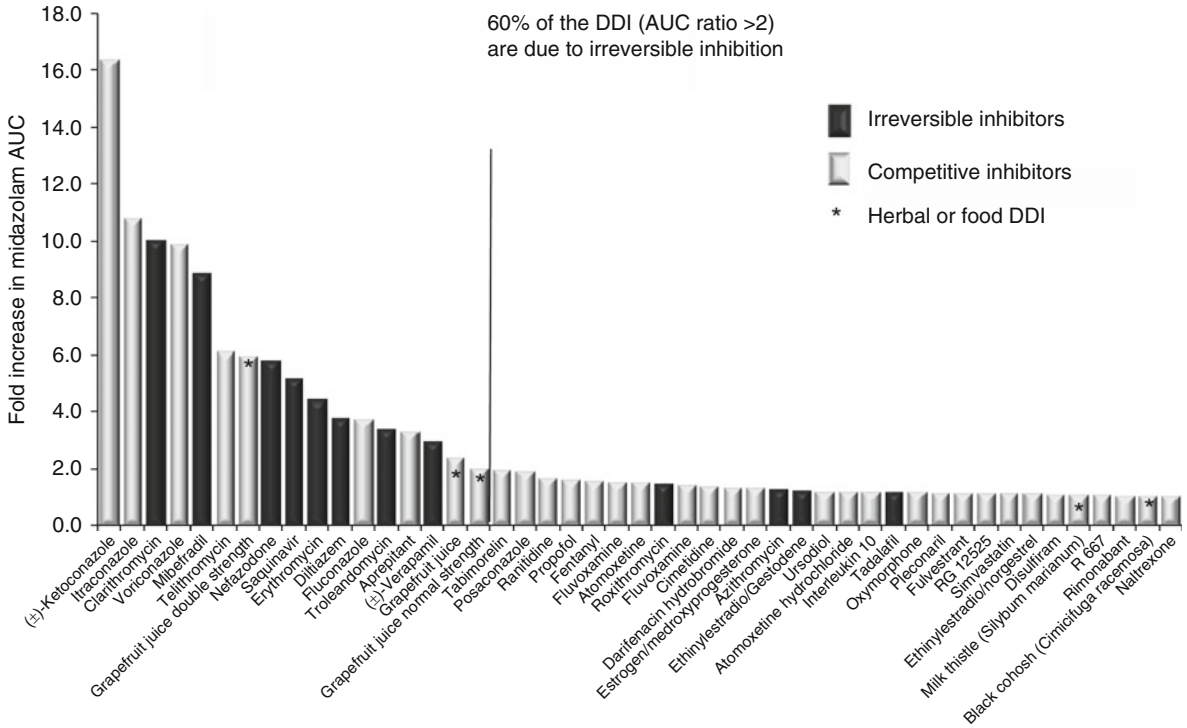

Figure B.13-9

Observed in vivo DDI between midazolam as victim and potential CYP3A4 perpetrators. *Source*: AurSCOPE ADME/DDI database (December 2008 release)

for around 60% of all the significant interaction (AUC ratio > 2) (*Fig. B.13 9*).

In the presence of inactivation, the fold change in intrinsic hepatic clearance will also be determined by the pseudo first order apparent inactivation rate (k_{obs}). This apparent inactivation rate is dependent upon $[I_u]_{h/g}$ (unbound inhibitor concentration in the liver or the gut), K_{Iu} (inhibitor concentration at which half maximal inactivation rate is achieved) and the true first order inac tivation rate constant (k_{inact}).

$$\text{Fold change } \mathrm{Clu}_{\mathrm{int\,h/g},k} = 1 + \sum_{j\ 1}^{p} \left(\frac{k_{\mathrm{inact}} \times [I_u]_{h/g,j}}{k_{\mathrm{deg,h/g}} \times ([I_u]_{h/g,j} + \mathrm{K_I} u_j)} \right) \qquad (B.13.16)$$

where $k_{deg,h/g}$ is the natural degradation rate constant for the enzyme in the liver or in the gut.

Such prediction clearly depends on reliable determi nations of enzyme turnover (k_{deg}) and inhibitory con stants (k_{inact} and K_I) involved in calculations.

Mechanisms proposed to be involved in the degradation of native and inactivated CYPs involve lysosomal, ubiquitin independent/dependent, and 20S/26S proteasomal systems (Correia and Liao 2007) and is commonly accepted that the kinetic change in enzyme level is a zero order rate for enzyme synthesis and a first order rate for en zyme degradation. At steady state, enzyme synthesis rate (K_{syn}) rate equal degradation rate ($k_{deg,h} \times E_{ss}$) (10.18), but when this balance is disturbed by external events the enzyme level will change to reach a new steady state (*Fig. B.13 10*)

$$K_{syn} = k_{deg,H} \times E_{ss} \qquad (B.13.17)$$

Furthermore, the lack of information on in vivo turnover rates (k_{deg}) of several human CYP isoforms represents an important source of uncertainty in the IVIVE process. Determination of rate constant of degradation in human remains a challenge even if different types of experiments (from in vitro to in vivo) have been used. A determination in the liver was done for all the major CYP isoforms using the techniques described in *Table B.13 3*. Nevertheless a great variability was measured depending on the methods but also on the sources of hepatocytes, enzymes for in vitro experiment, and on the patient population for the in vivo experiment. For example, the estimate of average CYP3A4 half life in the liver ranges from 26 to 140 h and was based

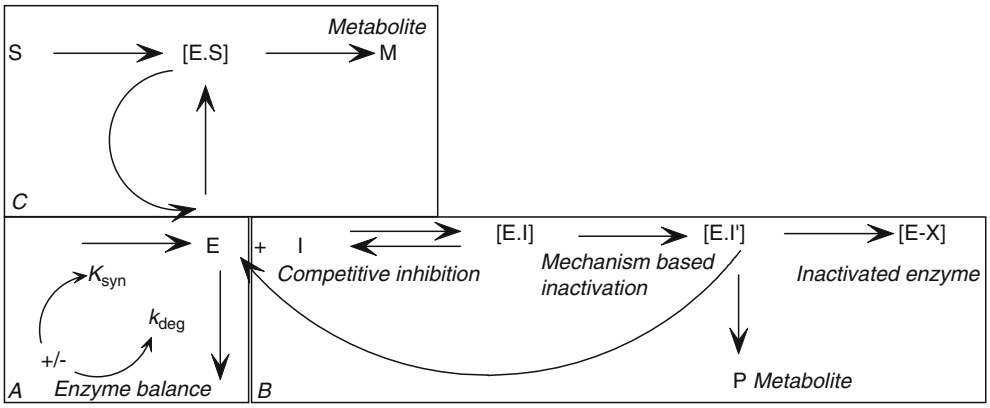

Figure B.13-10

Schematic illustration of in vivo enzyme turnover (A), the kinetic scheme depicting mechanism-based inactivation (B), the interaction between an MBI and a substrate, competition with a probe substrate (C)

Table B.13-3

Methods for determining the turnover of hepatic CYPs

In vitro methods	Description	Reference
Radiolabeling of enzyme (''Puke-Chase'' method)	Incorporation of [3H]-leucine into the enzyme by preincubation, replacement with unlabeled leucine in the culture medium, and measurement of radioactivity in specific enzyme	Elshourbagy et al. (1981), Newman et al. (1982)
Degradation of enzyme in cultured hepatocytes or liver slices	In vitro studies have demonstrated that CYP apoprotein and enzyme activities decline in parallel over time in cultured human hepatocytes and liver slices. Assuming that these changes are the result solely of endogenous enzyme degradation, turnover may be estimated based on the time profile of enzyme level	Renwick et al. (2000), Wright and Paine (1992), Lake et al. (1996), Paine (1990), Guillouzo et al. (1985)
Induction of CYP enzymes in hepatocytes	Enzyme turnover half-life may be estimated from the increase in enzyme level or activity from the basal to the maximally induced state in cultured hepatocytes. Incubation durations of 48 or 72 h, is recommended by US FDA guidelines	Lasker (2002), LeCluyse (2001), Li et al. (1997), FDA Guidance for Industry (2006)
In vivo methods		
Recovery of enzyme activity after enzyme induction	This method consists in the administration of repeated doses of an enzyme inducer, preferably until a new steady-state level of enzyme is reached. The inducer is then discontinued and return of enzyme activity to the basal level is followed using a specific probe substrate	Abramson (1986)
Recovery of enzyme activity after mechanism-based inhibition (MBI)	An inactivator requires metabolic activation by the target enzyme to form a reactive intermediate that binds irreversibly or quasi-irreversibly to the enzyme, resulting in loss of enzymatic activity until recovery is achieved through synthesis of new enzyme	Silverman (1988, 1995)
Pharmacokinetic modeling of auto-induction	Chronic exposure of drugs results in increased expression of the CYP enzyme(s) involved in their own metabolism. Modeling of the plasma concentration time data of an auto-inducer after multiple doses allows characterization the half-life of the induced enzyme	Levy (1986)

on 13 determinations using in vivo and in vitro methods. Clearly, all in vitro methods will have some experimental deficiencies in reflecting in vivo reality (Yang et al. 2008).

Because of the kinetic profile of enzyme turnover, enzyme synthesis is a zero order (K_{syn}) and enzyme de gradation is a first order rate (k_{deg}), the same increase in the final level produced either by the same (absolute value) fold increase in enzyme synthesis rate or fold decrease in the enzyme degradation rate constant, the time profile up to the final enzyme level will be quite different. A decrease of the enzyme degradation rate constant will prolong the turnover half life while an increase of the rate of synthesis will not. Therefore, when DDI predictions involved mech anism based inactivators or inducers the in vivo impact should be carefully managed and analyzed and the use of extremum values should be avoided. *Table B.13 4* repre sents the common mean values of half lives of degradation used for DDI prediction coming from literature analysis.

The common determination of MBI parameters (k_{inact} and K_I), also called conventional experimental protocol (CEP) was proposed by Silverman (1988) and involved two different experimental protocols with a common part that consists in a preincubation of the enzyme and cofac tors with different inhibitor concentrations for varying incubation times.

- Followed by a dilution of the reaction mixture and further incubation of a probe substrate to assess the capability of the inactivator to block the active enzyme.
- Or without dilution but after a direct addition of a large amount of substrate probe to the preincubation mixture.

There are advantages and drawbacks for both of these methods.

- The first one, needs a large amount of enzymes in the preincubation step to provide enough enzyme activity. Therefore, this may increase the need of taking into account the fraction unbound in microsomes during the calculation of DDIs. The dilution volume is also a critical step in the determination of in vitro inhibi tory constants because the dilution is used to "quench" the inactivation reaction. A low volume of dilution will let the inactivation proceed during the determination of the remaining enzyme activity with substrate probe (Silverman 1988).
- With the second one, the inactivation reaction is not stopped by the dilution step, so further MBI competi tion may occur during the incubation step with probe substrate.

Table B.13-4

Common mean hepatic values of half-lives of degradation used for DDI prediction coming from literature analysis

Enzymes	k_{deg_h} (min^{-1})	Reference
CYP1A2	0.000296	Venkatakrishnan and Obach (2007), Obach et al. (2007)
CYP2B6	0.000361	Venkatakrishnan and Obach (2007)
	0.00026	Obach et al. (2007)
CYP2C19	0.000444	Venkatakrishnan and Obach (2007)
	0.00026	Obach et al. (2007)
CYP2D6	0.000226	Venkatakrishnan and Obach (2007), Einolf (2007), Obach et al. (2007)
	0.00024	Grime et al. (2009)
CYP3A4	0.000321	Venkatakrishnan and Obach (2007), Einolf (2007), Fahmi et al. (2008)
	0.00016	Fowler and Zhang (2008), Galetin et al. (2006)
	0.00016 0.00050	Grime et al. (2009)
	0.000769 0.00128	Wang et al. (2004)
CYP2C9	0.00026	Obach et al. (2007)

These two methods need an incubation time as short as possible compared to the preincubation one in order to minimize the interaction between inhibitor and substrate (Ito et al. 1998).

The calculation of k_{inact} and K_I use the natural logarithm of remaining enzyme activity plotted against the preincubation time according to Eq. (B.13.18), the slopes of the initial log linear phases represent the observed inactivation rate constants (k_{obs})

$$k_{obs} = \frac{d[E]_t}{dt} / [E]_t = \frac{k_{inact} \times [I]}{K_I + [I]} \qquad (B.13.18)$$

Two methods are generally used to obtain k_{inact} and K_I values; one is a linear method (Kitz and Wilson 1962) and the other a nonlinear regression method.

A modification of the conventional protocol (CEP) was proposed recently by Yang et al. (2007a, b). Named mechanistically based experimental protocol (MEP) because using CEP for characterizing MBI, it may introduce substantial bias in estimating parameter values (Yang et al. 2005). This may act upon the ability to predict the in vivo consequences of MBI from in vitro data.

The MEP involves three steps and relies on the simultaneous fitting of three differential equations to estimate the relevant CYP inhibition constants more precisely than traditional methods.

Inhibitory constants (k_{inact}, K_I, and K_i) are determined in the MEP and optimized by using a genetic algorithm. MEP is considered by Yang et al. (2007a, b) to be superior to the CEP with regard to accuracy, precision, and efficiency. Its application may allow better prediction of the in vivo implications of MBI.

Two other methods, generating IC_{50} values rather than k_{inact}, were used for identifying inactivators that consist in measurement of decreases in IC_{50} occurring with a preincubation with liver microsomes and NADPH (Obach et al. 2007; Atkinson et al. 2005). The IC_{50} measured after such a preincubation was highly correlated with the k_{inact}/K_I ratio measured after a full characterization of inactivation. These methods were analyzed and compared (Grime et al. 2009) to establish potential usage in terms of IVIVE.

Induction

In the presence of induction, the fold change in intrinsic clearance will also be determined by inducer concentrations at the enzyme site $[I]_{h,g}$ (in the liver or in the gut), the maximum fold induction, and the concentration of inducer associated with half maximum induction (EC_{50}), but the way of combining these three elements could be different. Until now, two predictive models exist for the calculation of the fold change in intrinsic clearance based on an empirical calibration factor and applied in the liver and in the gut (Fahmi et al. 2008) or on a Hill equation applied only in the liver (Shou et al. 2008) (see Eqs. (B.13.19) and (B.13.20)):

$$\text{Fold change } Clu_{int_{h/g,k}} = \frac{1}{1 + \sum_{j\ 1}^{p} \left(\frac{d \times E_{max} \times [I_u]_{h/g,j}}{[I_u]_{h/g,j} + EC_{50,j}} \right)} \qquad (B.13.19)$$

The d parameter in Eq. (B.13.19) represents an empirical calibration factor for the purposes of in vitro to in vivo induction scaling. As such, its value was estimated through correlation of predicted and observed AUC ratios.

$$\text{Fold change } Clu_{int_k} = \frac{1}{1 + \sum_{j\ 1}^{p} \left(\frac{E_{max_j} \times [I]^n_{H,j}}{([I]^n_{H,j} + EC^n_{50,j})} \right)} \qquad (B.13.20)$$

The n parameter in Eq. (B.13.20) represents the Hill coefficient.

In many cases, E_{max} and EC_{50} are not readily obtained from concentration response curves measured by in vitro induction assays because of limitations imposed by drug solubility, cell permeability, or toxicity. In these instances, the slope of the induction response curve (equivalent to E_{max}/EC_{50}) at a more experimentally feasible low concentration range of the inducer can be used for the prediction. This method (B.13.21) is, however, only applicable if in vivo concentrations of an inducer are low ($[I]_{H,j} << EC_{50,j}$).

$$\text{Fold change } Clu_{int_k} = \frac{1}{1 + \sum_{j\ 1}^{p} (\text{Slope}_j \times [I]^n_{H,j})} \qquad (B.13.21)$$

An alternative approach was investigated by Ohno et al. (2008). Such a method was developed on the basis of the principle that the extent of alterations in the area under the plasma concentration time curve (AUC) is predicted on the basis of in vivo information from minimal clinical studies without using in vitro data. These authors propose to determine the apparent CYP3A4 contribution to the total oral clearance of the substrates from inhibitory DDI studies using a potent CYP3A4 such as itraconazole or ketoconazole. For the inducers, for a given dose and treatment duration, apparent clearance increase was calculated based on the reduction of the AUC for standard CYP3A4 substrates such as midazolam or simvastatin. For drug candidates, CYP3A4 involvement in the clearance can be determined from inhibitory clinical study as described or from in vitro investigations (f_m assessment, see "Parallel

Pathway: Fraction Metabolized ($f_m(E)$)" in this chapter) to predict quantitative AUC decrease prediction after coadministration with a given inducer.

B.13.1.1.3 Integrated General Model for Oral and IV Administration of Victim Drug: Use of Fumic for Corrected In Vitro Parameters Determination

This combined model is based on calculating the net effect of competitive inhibition, inactivation, and induction in both the intestine and liver based on results presented above.

Per os

$$\frac{\mathrm{AUC}_i}{\mathrm{AUC}} = \frac{1}{F_g + (1-F_g)\times\left(\sum_k^n{}_1 \frac{f_m(E)_{g,k}}{C_{g,k}\times M_{g,k}\times T_{g,k}} + 1 - \sum f_m(E)_{g,k}\right)} \times \frac{1}{f_h \times \sum_k^n{}_1 \frac{f_m(E)_{h,k}}{C_{h,k}\times M_{h,k}\times T_{h,k}} + 1 - f_h \times \sum f_m(E)_{h,k}} \quad (B.13.22)$$

IV

$$\frac{\mathrm{AUC}_i}{\mathrm{AUC}} = \frac{1}{f_h \times \sum_k^n{}_1 \frac{f_m(E)_{h,k}}{C_{h,k}\times M_{h,k}\times T_{h,k}} + 1 - f_h \times \sum_k^n{}_1 f_m(E)_{h,k}} \quad (B.13.23)$$

where $C_{h,k}$ and $C_{g,k}$ are the terms for reversible inhibition in the liver and in the gut, respectively, associated to enzyme k and assuming inhibition by multiple perpetrators.

$$C_{h,k} = 1 + \sum_j^p{}_1 \frac{[I_u]_{h,j}}{K_i, u_{kj}} \quad (B.13.24)$$

$$C_{g,k} = 1 + \sum_j^p{}_1 \frac{[I_u]_{g,j}}{K_i, u_{kj}} \quad (B.13.25)$$

where $M_{h,k}$ and $M_{g,k}$ are the terms for TDI in the liver and in the gut, respectively, associated to enzyme k and assuming inhibition by multiple perpetrators

$$M_{h,k} = 1 + \sum_j^p{}_1 \frac{k_{\mathrm{inact}\,j} \times [I_u]_{h,j}}{k_{\mathrm{deg}_{h,j}} \times ([I_u]_{h,j} + K_I, u_{kj})} \quad (B.13.26)$$

$$M_{g,k} = 1 + \sum_j^p{}_1 \frac{k_{\mathrm{inact}\,j} \times [I_u]_{g,j}}{k_{\mathrm{deg}_{g,j}} \times ([I_u]_{g,j} + K_I, u_{kj})} \quad (B.13.27)$$

where $T_{h,k}$ and $T_{g,k}$ are the terms for induction in the liver and in the gut, respectively, associated to enzyme k and assuming induction by multiple perpetrators

$$T_{h,k} = 1 + \sum_j^p{}_1 \frac{E_{\max_{k,j}} \times [I_u]_{h,j}}{[I_u]_{h,j} \times \mathrm{EC}_{50\,k,j}} \quad (B.13.28)$$

$$T_{g,k} = 1 + \sum_j^p{}_1 \frac{E_{\max_{k,j}} \times [I_u]_{g,j}}{[I_u]_{g,j} \times \mathrm{EC}_{50\,k,j}} \quad (B.13.29)$$

Microsomal Nonspecific Binding

Microsomal binding of drug substrates and/or perpetra tors is increasingly recognized as a potential source of artifact arising in the course of in vitro studies of drug metabolism. Nonspecific binding of substrate to micro somal protein is an important aspect of the extrapolation of in vivo drug clearance from data obtained with the liver or recombinant expressed microsomal systems. These, in turn, may produce inaccurate predictions when in vitro data are used to estimate in vivo pharmacokinetics (Obach 1996, 1997; Obach et al. 1997; Venkatakrishnan et al. 2000, 2001; Kalvass et al. 2001). Non specific binding to micro somal systems may also likewise influence estimation of potency of metabolic inhibitors (Gibbs et al. 1999), re sulting in an overestimation of K_i (Margolis and Obach 2003; Brown et al. 2006; Tran et al. 2002). Consequently, in vivo hepatic clearance and the extent of inhibitory drug interactions were often underpredicted.

Investigators have tried to use relative low micro somal protein concentration to avoid the nonspecific binding (Jones and Houston 2004). However, relative high concentrations (1 2 mg/mL) were still needed when studying phase II metabolic reactions in particular (Soars et al. 2002) and in vitro assessment of the TDI potential (Ghanbari et al. 2006). As such, it is essential to correct the metabolic kinetic parameters ($\mathrm{Cl}_{\mathrm{int}}$, K_m, and K_i) by the unbound fraction to microsomes ($\mathrm{fu}_{(\mathrm{mic})}$) in order to ensure accurate pharmacokinetic estimation of potential drug candidates. Experimentally, $\mathrm{fu}_{(\mathrm{mic})}$ is assessed by in vitro methods (dialysis, ultra filtration, ultra centrifu gation) (Jones and Houston 2004). In addition QSAR approches were developed.

For drugs corresponding $\mathrm{fu}_{(\mathrm{mic})}$ values were estimated using the Austin et al. (2002) method or the Hallifax and Houston (2006) one (see Eqs. (B.13.30) and (B.13.31), respectively).

$$\mathrm{fu}_{(\mathrm{mic})} = \frac{1}{1 + C \times 10^{0.56\times\log P/D\ \ 1.41}} \quad (B.13.30)$$

$$\mathrm{fu}_{(\mathrm{mic})} = \frac{1}{1 + C \times 10^{0.072\times\log P/D^2 + 0.06\times\log P/D\ \ 1.126}} \quad (B.13.31)$$

C is the microsomal protein concentration (g/L), log P/D is the log P for basic compounds ($pK_a > 7.4$), and log $D7.4$ for neutral or acidic compounds ($pK_a > 7.4$).

B.13.1.2 Basic Equations and Authorities Requirements

The first attempts of DDI prediction considered simple equation separating perpetrator and victim.

For inhibition, the reversible inhibition risk, for exam ple, is evaluated, after oral administration of the victim drug, considering the simple Eq. (B.13.32):

$$R_c = \frac{Cl_{int}}{Cl_{int,i}} = \frac{AUC_i}{AUC} = 1 + \frac{[I]}{K_i} \qquad (B.13.32)$$

with R_c = AUC ratio of substrate with and without inhib itor coadministration.

Some attempts have been made to predict clinical interactions with such simple equation (Blanchard et al. 2004; Ito et al. 2004). Nevertheless, this relationship can not be considered for prediction as it relies on a major assumption: victim drug is eliminated by way of a single metabolic pathway, which is not true in most of cases (Ito et al. 2005). (See Parallel pathway B.13.1.1.1 in this chapter) However, this approach is used for ranking inhibitor but cannot predict a specific DDI as substrate $f_m(E)$ is not taken into account. The pragmatic dimen sionless ratio $[I]/K_i$ reflects the strength of inhibition of the compound for a given in vivo concentration. This is a useful predictive parameter, recommended by health authorities, for a given compound in order to qualify the inhibition risk toward CYP isoforms: higher the ratio is, higher the risk is. In other words, addressing in clinic, the inhibition of the isoform corresponding to the highest ratio allows pharmaceutical industry to characterize the highest inhibition risk for a given clinical condition (dos age, administration route, dosing interval, etc.). Should no inhibition observe (substrate probe AUC increase <2) with the highest potentially inhibited isoform, then no further clinical study will be warranted (*Fig. B.13 11*). If this first clinical study is positive, the next most poten tially inhibited isoform must be investigated, and so on until no interaction is observed. In the other way round, assessing this simple ratio is also useful for ranking com pounds for a given isoform, in particular at discovery level before entering in development in order to select the compound reflecting the lowest risk of inhibition.

In addition to this relative approach, this simple equa tion can be used for an absolute risk assessment. A value higher than 0.1 of $[I]/K_i$ is considered as positive,

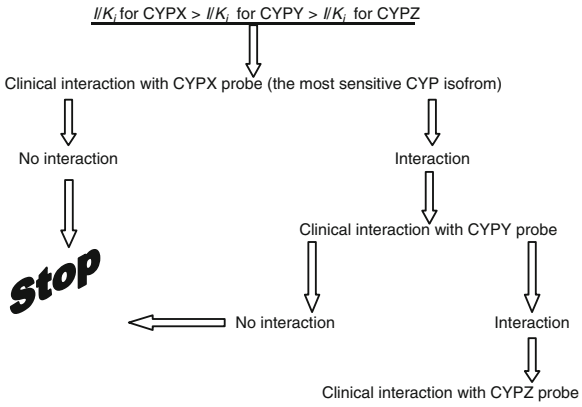

Figure B.13-11

Rank order strategy for clinical studies to be conducted. The compound is tested as perpetrator for three CYP isoforms with increasing risk of inhibition *X* > *Y* > *Z*

reflecting a potential risk of inhibition in the FDA Guid ance for Industry (2006) as well as in the scientific com munity (Bjornsson et al. 2003). A first absolute risk assessment can be done with this ratio, considering the threshold value of 0.1 as recommended in the authorities guideline.

Regarding MBI, a corresponding equation to Eq. (B.13.33) has been proposed (Venkatakrishnan and Obach 2007):

$$R_c = \frac{Cl_{int}}{Cl_{int,i}} = \frac{AUC_i}{AUC} = 1 + \frac{k_{inact}}{k_{deg}} \times \frac{1}{1 + \frac{1}{([I]/K_I)}} \qquad (B.13.33)$$

Nevertheless, this equation, with two dimensionless ratios ($[I]/K_I$ and k_{inact}/k_{deg}) is not widely used and recommended in the current authority's guideline for risk assessment as done for reversible inhibition. This is because of the level of uncertainty linked to these param eters and in particular the natural enzyme turnover k_{deg} (see Sect. B.13.1.1.2.2).

For CYP3A4, for which the highest number of mech anism based inhibitors has been described, this parameter is still debatable in the literature (Yang et al. 2008). Nev ertheless, once k_{deg} selected, risk assessment can be pro posed positioning the inhibitor on an abacus, knowing k_{inact} and $[I]/K_I$ (*Fig. B.13 12*).

Whatever the mechanism inhibition, it is generally recommended to consider unbound in vivo inhibitor con centration as well as unbound K_i and K_I for reversible and nonreversible inhibition, respectively. (This hypothesis will be more detailed in Sect. B.13.1.3.1.)

Unbound K_i and K_I are assessed experimentally, after determination of $fu_{(mic)}$ representing the unbound

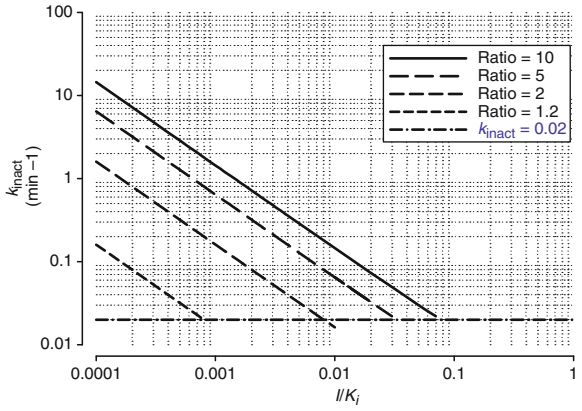

Figure B.13-12

Abacus for MBI risk assessment, assuming $f_m(E) = 1$ and $k_{deg} = 0.00016\ min^{-1}$. Ratio represents the AUC ratio with and without coadministration of inhibitor. The threshold $k_{inact} = 0.02\ min^{-1}$ corresponds to the accepted lowest limit detectable using the classical current in vitro test

fraction of the inhibitor concentration in vitro after incubation with hepatic microsomes or estimated (Hallifax and Houston 2006; Austin et al. 2002) (see Sect. B.13.1.1.3.1 for more details).

For substrate compound, interaction ratio can simply be estimated using the following equation:

$$R_c = \frac{Cl_{int}}{Cl_{int,i}} = \frac{AUC_i}{AUC} = \frac{1}{1 - f_m} \qquad (B.13.34)$$

with AUC_i: substrate exposure with inhibitor; AUC: substrate exposure without inhibitor, where $f_m(E)$ is the relative contribution of a given CYP isoform in the overall clearance or in the metabolic clearance if the compound is eliminated through metabolism only.

This basic equation assumes inhibition is total (very high $[I]/K_i$ for reversible inhibition) and does not take into account the intestinal first pass that might be important for CYP3A4 substrate. Authorities (FDA Guidance for Industry 2006) recommend to investigate and identify enzyme involved in a metabolic pathway (CYP phenotyping) representing more than 25% of the overall clearance ($f_m(E) = 0.25$). This value is calculated taking account all other clearance parts (renal, biliary, etc...) in addition to the metabolic one. Therefore, it is necessary to have the complete picture of the elimination pathways of the investigational compound. Generally, data obtained after administration of the radiolabeled compound to human provide the definitive pieces of information on the routes of drug clearance. $f_m(E)$ value for a given CYP isoform is not easy to determine. This so called reaction phenotyping or isozyme mapping, involves the use of different in vitro materials: recombinant protein, liver subcellular fractions, hepatocytes in primary culture, enzyme selective chemical inhibitors, and antibodies (Zhang et al. 2007) (see "Parallel Pathway: Fraction Metabolized ($f_m(E)$)" B.13.1.1.1 in this chapter). In most of cases, several in vitro models are used. In particular, recombinant system is an attractive and recent tool as it allows to investigate and identify straightforward the isoform responsible of a metabolic reaction with the corresponding Michaelis Menten parameters (K_m, V_{max}), in opposite to the relative lack of specificity of inhibitors used in HLM or hepatocytes. However, when choosing this system, it is mandatory to bridge the gap between in vitro and in vivo situation, in terms of CYP relative abundances and intrinsic enzymatic activity, wherefore specific factors (relative activity factors, ISEFs) must be considered. In vitro and in vivo data can be mixed for determining the relative involvement of a given CYP isoform versus the overall clearance (see "Parallel Pathway: Fraction Metabolized ($f_m(E)$)" B.13.1.1.1 in this chapter).

B.13.1.3 Mechanistic Static Model (MSM)

The MSM used the general mathematical model (Eqs. (B.13.22) and (B.13.23)) and all the items described in Sect. B.13.1.1 but it is based upon the assumptions of a "well stirred" model for hepatic blood clearance and a linear pharmacokinetic of the victim. It is assumed that the victim is only metabolized in the liver (and intestine for per os administration) and also that perpetrator and victim were given at the same time.

It ignores transient plasma binding displacement of the victim during the absorption phase and time variant perpetrator concentration leading to a different extent of inhibition of substrate metabolism during "first" and subsequent pass through the liver.

This model also assumes that the fraction of victim absorbed (F_{abs}), hepatic blood flow rate (Q_h), the gut blood flow rate (Q_g), and the fraction of drug unbound in the blood do not change in the presence of the perpetrator.

The general mathematical model developed for predicting DDIs' either after intravenous or per os administration requires an estimation of the perpetrator concentration at the enzyme site.

B.13.1.3.1 Perpetrator Concentration at the Enzyme Site

$[I]$ has been defined as the concentration of inhibitor at the enzyme site, which in practice, is unmeasureable.

One of the key challenges, however, is the estimation of most appropriate value of $[I]$. The estimation of $[I]$ should be based on in vivo concentrations of a given perpetrator that change over time, raising the question of whether if the systemic plasma concentration or the hepatic inlet concentration is the most relevant concentration (*Table B.13 5*).

In addition, the impact of plasma protein binding remains controversial even if the use of total plasma systemic concentration has been advocated in the past as a means of predicting reversible inhibition DDI; however, this is contrary to the well established "free drug hypothesis" of drug action in DDI predictions. Do we take into account for the estimation of $[I]$ the total plasma concentration or the unbound plasma concentration?

In most of the publications dealing with the impact of the inhibitor concentration on accuracy of DDI prediction (*Table B.13 6*) correction to account for the free drug fraction was shown to be important and determined as the concentrations leading to the most accurate predictions not only for mechanism based inhibitors but also for reversible inhibitor mechanisms. Nevertheless, it is essential to note that total concentration was also determined by other authors (Brown et al. 2006; Houston and Galetin 2005) as the best concentration leading to the more accurate DDI predictions.

Consideration of the systemic plasma concentration as opposed to the hepatic inlet concentration is also crucial.

Most of the publications predicting DDIs involving reversible competitive inhibitors have determined that the hepatic inlet concentration ($[I]_{in,avg,u}$ or $[I]_{in,max,u}$) (*Table B.13 6*) provided the most accurate DDIs predictions. Whereas, prediction of DDIs involving MBI or induction mechanisms are more accurate when systemic plasma concentration ($C_{max,u}$) was introduced in the calculations. This result was also supported by our unpublished results where it was found that using unbound systemic plasma concentrations ($C_{max,u}$ or $C_{avg,u}$) for the prediction of DDIs between midazolam victim and eight inactivators of CYP3A4, reduced significantly overprediction due to the use of the hepatic inlet concentrations ($[I]_{in,max,u}$ or $[I]_{in,avg,u}$) and increased the accuracy (*Fig. B.13 13*).

Table B.13-5

Consolidates some of the methods for computing (and selecting) values for [*I*] found in the literature for DDI prediction

Choice of [*I*]	Estimation of [*I*]
Maximum steady-state plasma concentration	$[I]_{max} = C_{max}$
Unbound concentration	$[I]_{max,u} = fu \times C_{max}$
Average steady-state plasma concentration	$[I]_{avg} = C_{avg}$
Unbound concentration	$[I]_{avg,u} = fu \times C_{avg}$
Maximum hepatic inlet plasma concentration	$[I]_{in,max}$ $C_{max} + \frac{F_{abs} \times k_{abs} \times D}{Q_h}$
Unbound concentration	$[I]_{in,max,u} = fu \times [I]_{in,max}$
Average hepatic inlet plasma concentration	$[I]_{in,avg}$ $C_{avg} + \frac{F_{abs} \times k_{abs} \times D}{Q_h}$
Unbound concentration	$[I]_{in,avg,u} = fu \times [I]_{in,avg}$

Table B.13-6

Literature analysis of perpetrator concentrations used for predicting DDIs using MSM or $[I]/K_i$ approach

Model	Perpetrators	CYPs involved	Best [*I*]	Reference
MSM	Competitive MBI	3A4	$[I]_{in,max,u}$ (competitive)	Fahmi et al. (2009)
	Inducer		$[I]_{max,u}$ (MBI and inducer)	
MSM	Competitive MBI	3A4, 2D6, 1A2, 2C9, 2C19	$[I]_{in,max,u}$	Einolf (2007)
MSM	Competitive	3A4, 2D6, 2C9, 2C19, 1A2	$[I]_{in,avg,u}$	McGinnity et al. (2008)
MSM	MBI	3A4, 1A2, 2D6, 2C9, 2C19	$[I]_{max,u}$	Obach et al. (2007)
MSM	Competitive	2C9, 2D6, 3A4	$[I]_{avg}$	Brown et al. (2006)
MSM	Competitive	3A4, 1A2,2D6, 2C9, 2C19	$[I]_{in,max,u}$	Obach et al. (2006)
MSM	Competitive	3A4	$[I]_{avg}$	Houston and Galetin (2005)
$[I]/K_i$	Competitive	2C9, 2D6, 3A4	$[I]_{in,avg}$	Ito et al. (2004)
$[I]/K_i$	Competitive	3A4, 1A2, 2D6, 2C9, 2C19	$[I]_{max,u}$	Blanchard et al. (2004)
$[I]/K_i$	Competitive	3A4, 1A2, 2D6, 2C9, 2C19	$[I]_{in,max,u}$	Ito et al. (2002)
MSM	Competitive	2C9	$[I]_{in,max,u}$	Kanamitsu et al. (2000)

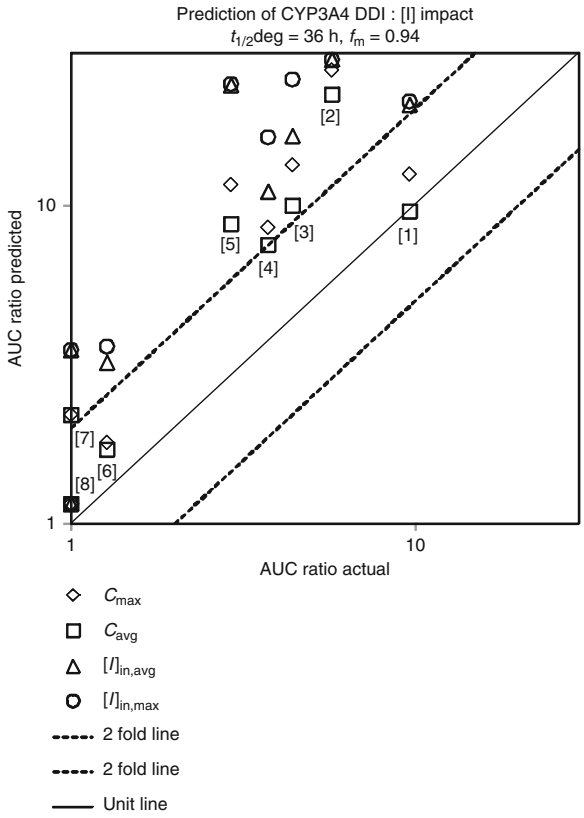

Figure B.13-13

Relationship between the observed midazolam AUC ratio in vivo and AUC ratio predicted for 8 CYP3A4 inactivators (1: clarithromycin, 2: saquinavir, 3: erythromycin, 4: verapamil, 5: diltiazem, 6: fluoxetine, 7: azithromycin, 8: ethynylestradiol). The plot represents predictions using the average or maximum systemic unbound drug plasma concentration (square or lozenge dots, respectively) or the average or maximal inlet hepatic (triangle or circle dots, respectively).The solid line represents line of unity, whereas dashed lines represent the twofold limit in prediction accuracy

It was found that the reversible inhibition portion performed the best when the unbound portal vein concentration was used for concentration in the liver, whereas for irreversible inactivation and induction, the unbound systemic concentration was best.

Even if these results appeared inconsistent from physiological point of view they can be rationalized.

For reversible inhibition, much of the interaction occurs during absorption and the hepatic first pass after, the concentrations of perpetrator decrease below values required to exhibit reversible interaction. Consequently, the use of the hepatic inlet concentration for predicting DDIs makes sense when reversible inhibitors are involved.

Conversely for inactivation and induction, the use of systemic concentrations makes sense in that the DDIs caused by inactivators and inducers continue to occur after first pass exposure of the intestine and the liver is over.

Even if most of the results can be rationalized, some are contradictory. For example, total systemic plasma concentration (C_{avg}) was determined as the concentration leading to the most accurate prediction for reversible competitive inhibitors (Brown et al. 2006; Houston and Galetin 2005). These considerations may underline some limitations of the mathematical model.

Systemic or hepatic inlet concentrations can be entered into the mathematical model as total or unbound exposure. So, it is common practice with such approaches to empirically select surrogate measures of exposure in the liver and intestine that provide the best correlation of predicted and observed DDIs reported in the literature.

Although practically useful, such empirical oversimplifications surely limit predictive accuracy within the data set and it will be reckless to apply the method outside the data set.

Furthermore, it would be inappropriate to conclude from publication of Houston and Galetin (2005) that CYP3A4 interactions are fundamentally driven by total systemic perpetrator concentration and could lead to an over interpretation of the mathematical model.

Rate constant of absorption (k_{abs}) values are used for [I] in vivo determination and if necessary estimated, assuming a first order absorption rate, according to Eq. (B.13.35)

$$T_{max} = \frac{\ln(k_{abs}/k_{el})}{k_{abs} - k_{el}} \qquad (B.13.35)$$

where T_{max} (h) is the time to reach the maximum concentration and k_{el} (h^{-1}) the elimination rate constant coming from the same experiment.

When k_{abs} values are not available; a default value of 0.1 min^{-1} is assumed corresponding to gastric emptying.

Because perpetrator and victim were given at the same time the concentration in the intestinal wall during absorption phase ($[I_u]_g$) will be described by the following equation:

$$[I_u]_{j,g} = \frac{F_{abs} \times k_{abs} \times D}{Q_g} \qquad (B.13.36)$$

Two commercial softwares using the MSM approach have been developed and are currently available on the market, Simcyp population based Simulator® is developed by

Simcyp Ltd, Sheffield, UK (http://www.simcyp.com), and DDI Predict 2009 Edition® by Aureus Pharma, Paris, France (http://www.aureus pharma.com).

- Even if the MDM approach (see Sect. B.13.1.4) is the cornerstone of the Simcyp population based Simulator® software, it includes also a MSM approach.

This MSM approach encompasses all the aspects of metabolism, inhibition, and induction of a drug and takes also advantage of the interindividual variability used extensively in the MDM approach. Compounds of interest (e.g., in house and marketed compounds) features, considered as inhibitor or substrate, are used as input data in Simcyp software. For marketed compounds, parameters must be sought after in the literature or generated in house. However, some marketed compound parameters are available in the Simcyp library.

- *DDI Predict 2009 Edition*® provides a graphical report containing all potential DDIs between a drug candidate and a large panel of marketed or withdrawn drugs.

The predictions are supported by calculation of the change of the AUC ratio based on plasma concentration of drug candidates in the presence or absence of enzyme (CYPs, UGTs) inhibitors. The new edition extends the prediction of DDIs in cases where multiple metabolic pathways are involved and provides also new functionalities including prediction of fraction metabolized ($f_m(E)$) based on scaling factors (RAF, ISEF, abundance), gut metabolism, and others.

To calculate interactions, *DDI Predict 2009 Edition*® is using a large library of drugs (more than 1,500) containing more than 7,000 and 8,000 inhibition and PK data points, respectively. The use of a large library avoids time wasting and provides prediction of DDIs for a large panel of potential co medications.

B.13.1.4 Mechanistic Dynamic Model (MDM)

MDM refers intrinsically to physiologically based pharmacokinetic (PBPK) models (Edginton et al. 2008; Schmitt and Wilmann 2004). The objective of PBPK is to describe mathematically all physical and physiological processes, which determine the pharmacokinetics of a drug. In other words, this type of modeling intends to describe most of the mechanisms involved in the absorption, distribution, metabolism, and elimination of the compound. In opposite, classical modeling PK starts from an in vivo concentration time curve. In this case, the experimental data set is used to fit the data into a two or three compartments model. Using such model, pharmacokinetic of population (PKPOP) approach allows to identify covariates (e.g., co administered drug) explaining a part of the variability. By contrast, PBPK models are derived from the anatomical and physiological structure of the organism studied. They establish virtual population by building up models integrating physicochemical and metabolic, distribution, in vitro parameters of the drug classically generated during discovery and development process, using cellular or subcellular models, like in vitro metabolic parameters, (K_i, K_m, V_{max},...), and the related population parameters like, physiological parameters: tissue structure, tissue composition, tissue volume, and associated blood flow (*Fig. B.13 14*). Recently, the use of both mixing these parameters PBPK, the possibility exists, making some reasonable assumptions to describe how the drug interacts with the system in specific conditions. For a CYP450 victim, this condition can be with or without coadministration with a perpetrator, allowing predicting the magnitude of the interaction in a given population. The use of predictive population modeling allows for a priori assessment of potential effect of physiological properties and/or the presence of a co administered drug on the pharmacokinetic parameters of the victim. This effect might be different in a subgroup of population because of a mixing effect of the perpetrator and the physiological parameters (Jamei et al. 2009). As an example, if a victim is eliminated through metabolic route via CYP3A4 isoform and renal excretion, the magnitude of the exposure increase in renal impaired subjects co administered with a CYP3A4 inhibitor can be investigated, allowing to identify a subgroup of subjects among a given population that are likely to be overexposed.

In the beginning of development, use of predictive population PBPK models allows for the a priori prediction and interindividual variability without the need for actual in vivo data. However, in order to address DDI using PBPK, requests two models, one for the victim and one for the perpetrator, which might be time consuming and not reasonable in discovery stage for a set of candidates. Once human actual PK data have been produced after first in man trial, refinement of the model is mandatory and can aid in designing clinical DDI in healthy volunteers or specific populations. PBPK allows to address several questions, during drug development, related to the DDI playing with doses, dose regimen, dose staggering and populations to predict pharmacokinetic profiles in given clinical conditions.

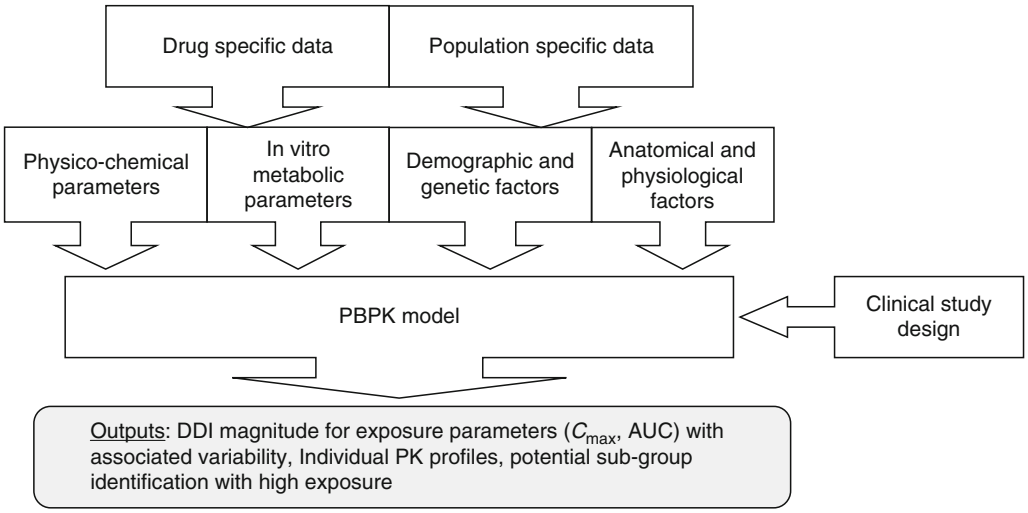

Figure B.13-14

PBPK diagram: drug and population specific parameters, used as input data

Several commercial softwares have been developed and are currently available on the market like PK Sim®, Gastro Plus®, Simcyp® (nonexhaustive list) (Dong et al. 2008). All these tools include metabolism and metaboli cally based DDI simulations in addition to the other ADME simulated properties, even if DDI and metabolism were the initial focus of Simcyp® only and remains the "core" of this software (Rostami Hodjegan and Tucker 2007; Jamei et al. 2009).

Detailed review of the use of PBPK as well as it use during drug development is beyond the scope of this article. The readers are invited to refer good recent reviews on that topic (Schmitt and Wilmann 2004; De Buck and Mackie 2007; Edginton et al. 2008; Dong et al. 2008; Espié et al. 2009).

B.13.1.5 MSM Versus MDM

Some authors compared MSM and MDM approaches with observed clinical results, for a set of interaction ratios, considering two mechanisms reversible, time based inhibitions (Einolf 2007), and induction (Fahmi et al. 2009). MDM was investigated with a PBPK embed ded in the Simcyp® software. From a qualitative point of view, of 100 clinical trials, the two approaches performed well at identifying an interaction (greater or equal to two fold) versus noninteracting drug with a percent around 80% (Einolf 2007). From a quantitative point of view, 67 and 75% were correctly predicted (within twofold of actual value) for MSM and MDM, respectively (Einolf 2007). Among trial interactions, with an interaction equal or higher than twofold (n = 59), the percentages are 53 and 64% for MSM and MDM, with a tendency of overprediction for MSM (with 39%) and underprediction for MDM (with 24%). Statistical analysis showed a pretty close value of geometric mean square error. Notably, for MBI this difference might not be due to the models per se, but more likely linked to the k_{deg} considered for modeling, which was different for MSM and MDM, as the magnitude of the prediction is highly dependent upon the value of this parameter. More recently, Fahmi et al (2009) used a mathematical model that simultaneously incorporates reversible inhibition, TDI, and induction of CYP3A4 iso form (see General Integrated Model in this chapter). This model, used as MSM, was compared with PBPK of Simcyp® (MDM), with 50 in vivo clinical trials. Actually, only five drugs exhibited all three interaction mechanism, among 30 compounds used in the study; for all others, at least one mechanism pertained to an interaction ratio of unity in the full mathematical model. MSM and MDM predicted correctly (interaction versus no interaction, defined as a twofold change in exposure) in 44 and 45 of the total 50 trials with a geometric mean square error of 1.74 and 1.47, respectively. Of the trials that had a clinical DDI effect higher or equal to 2, the increase in AUC was predicted within 50% of the actual value in 21 and 24 of the trials for MSM and MDM, respectively. Overall, the authors concluded that the static and dynamic models yielded comparable performance in predicting in vivo DDIs from in vitro data.

These two analyses, comparing MSM and MDM sug gest similar accuracy prediction. However, most MSM provide point estimates of the average DDI, with the as sumption of one precipitant concentration and the same in CYP450 enzyme levels across the population, whereby the risk to individuals is not evaluated. Nevertheless, a MSM approach incorporating the variability of CYP450 enzyme levels is conceivable, and is addressed with the "steady state" option in Simcyp® software and is planned to be incorporated in DDI Predict® from Aureus Pharma. But this represents only one source of variability that might be coped with MSM, whereas MDM is supposed to take into account all the sources with a time based approach of predicted concentration allowing simulating pharmacoki netic profiles, with the important interaction C_{max} ratio of the victim, of both perpetrator and victim. Moreover, a computer simulated program addressing MDM can have advantage to simulate DDI in specific population and considering trial design details (e.g., dose staggering). This method can also cope with nonlinearity of victim in particular as concentration of the substrate and K_m values are considered with the full Michaelis Menten equation, whereas MSM assumes clearance is constant.

A comparison between MSM and MDM approaches is provided in *Fig. B.13 15*. Basically, MSM provide a not time consuming but average estimation of metabolically based DDI magnitude whereas MDM is intended to pro vide subject based one (i.e., intersubject variability, full PK profile). Depending on the question, the development step and the needed accuracy of the answer, MSM or MDM approach must be selected adequately. MDM approach requires detailed information on metabolic path ways and interaction that are available relatively late in drug development. For these reasons, it is sometimes hazardous to use such approach too early in development with over abundance of detailed outputs pertained to variability, population comparison, nonlinearity, etc., if the input data are not reliable enough. Basically, the dynamic model is to be expanded along drug development and accurately used for prediction when validated with actual clinical data.

Type of data	Items	MSM	MDM (PBPK)
Input	Physico-chemical properties of drugs not needed	Yes	No
	In vitro metabolic data of the drugs not needed	No	No
	Distribution data (e.g. f_{up}) not needed	No	No
	No time-consuming	Yes	*No
	Hypothesis for the most relevant [*I*] not needed	No	Yes
Output	Magnitude of interaction clearance ratio	Yes	Yes
	Investigation of more than one perpetrator	Yes	Yes
	Combination of more than one mechanism (e.g. MBI + reversible inhibition)	Yes	Yes
	Inter-subject variability addressed	**Yes	Yes
	PK profile determination, tissue distribution addressed	No	Yes
	Interaction ratios determination for other PK parameters than clearance (e.g. C_{max}) investigated	No	Yes
	Co-variate impact on interaction (e.g. effect of hepatic impairment on interaction magnitude) addressed	No	Yes
	Nonlinear PK with time and/or dose of substrate addressed	No	Yes

* Two PBPK models needed
** Not all variabilities but some of them can be taken into account (e.g. intersubject enzyme abundance variability)

Figure B.13-15
MSM and MDM comparison for DDI investigations

B.13.2 Conclusion

For pharmaceutical industry, building a drug interac tion plan requires in vitro outcomes and mathematical equations to predict the likelihood of significant or high risk DDI clinical outcomes. However, a complete under standing of the relationship between in vitro findings and clinical outcomes of metabolic based DDI is still emerg ing. For inhibition, representing the most frequent meta bolic based DDI mechanism, in particular, the last decade has shown how the mechanism of inhibition (mechanism based versus Michaelis Menten kinetic) must be accu rately addressed in order to improve reliability of the prediction model, using the appropriate corresponding equations (Obach et al. 2006). Increasing understanding of the potential clinical drug interaction magnitude caused by compounds as soon as possible in the develop ment is imperative for druggability to avoid facing not manageable clinical DDI in the late development steps. The Pharmaceutical Research Manufacturers (PhRMA) has developed some years ago, and published minimal best practice guidelines for predicting DDIs for drug development purpose (Bjornsson et al. 2003). Behind the proposed mathematical equations, two concepts have been recently proposed for DDI prediction (Einholf 2007): MSM and MDM. Basically, the former is referring to an "average approach" without taking into account the intersubject variability inside a population. The time var iant change in the concentrations of the perpetrator and victim are also not considered. MDM refers to PBPK parameters model. Advantages and pitfalls of these two approaches have been detailed in this chapter.

Although excellent quantitative concordance of pre dicted and actual clinical results have been described, in some situations, predictions by both MSM and MDM lead to over or underestimations (Blanchard et al. 2004; Ito et al. 2004). This lack of accuracy is generally attributed to some unknown mechanisms (e.g., MBI instead of reversible inhibition, parallel elimination pathway, inhibi tion due to a metabolite and not the unchanged drug, inhibition, or induction of an important cellular transport mechanism). Some of them have been successfully pro posed to improve predictions. For example, the unexpected inhibition (based on in vitro data to the unchanged com pound) of cerivastatin clearance mediated by CYP2C8, by gemfibrozil has been clearly explained by Shitara et al. (2004) who demonstrated that 1 *O* β glucuronide of gemfibrozil is a strong CYP2C8 inhibitor through an MBI, and not the aglycone. Gemfibrozil example is mean ingful on that respect as the same authors showed as well that the glucuronide inhibits OATP1B mediated uptake of cerivastatin (Shitara et al. 2004). Gemfibrozil exemplifies the necessity to get a complete picture of the in vitro results (metabolism and transporters based in this case) in order to explain clinical results. Obviously, gemfibrozil clinical outputs have been explained a posteriori, pointing out the lack of knowledge of such mechanisms, a priori in complex situation for most of cases, reflecting that in some cases DDI in clinic is caused at different levels. Addressing all the possible mechanisms that might be part of the DDI picture for a given compound is for sure, the major challenge for the upcoming years in order to improve the liability and the accuracy of the DDI predictions.

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B.14 Specific Studies for Formulation Development

Roland Wesch

PURPOSE AND RATIONALE

The bioavailability of a drug depends on the properties of the drug product, a combination of drug and formulation properties. The support for formulation development by means of clinical PK studies is multifaceted and, in fact, covers all routes of administration, intravascular routes as well as extravascular ones, like oral, intramuscular, or subcutaneous routes, and in most instances vaginal, dermal, ocular, topic, rectal, nasal, or pulmonary administration. The drugability (disease tailored exposure profiles mediated by optimized delivery systems) of pharmacologically active substances will remain one of the major challenges in drug development, especially if poorly absorbable, poorly soluble compounds are considered. Rare exceptions include some drugs belonging to BCS class I (highly soluble, highly permeable) that rapidly dissolve from Immediate Release solid oral drug products.

Formulation development requires close coordination of various functions, for example, galenics, analytics, process development, preclinical, and clinical pharmacology. Clinical PK is just one, however, an important component in this framework.

Details about how to deal with changes in components or composition of drug products are described in published regulatory guidances (Guidance for Industry Waiver of in vivo bioavailability and bioequivalence studies for immediate release solid oral dosage forms containing certain active moieties/active ingredients based on the biopharmaceutics classification system (BCS). U.S. Department of Health and Human Services et al. 1999; SUPAC IR 1995, 1997), while no formal guidance exists that in particular covers all types of formulation interactions. Details about clinically relevant (drug drug) interactions and assessment of equivalence of formulations can be found in Guidance for Industry Statistical Approaches to Establishing Bioequivalence (2001), Steinijans and Hauschke (1997), CPMP (2002), and CPMP (1998).

In order to optimize drug exposure, the development of modified release formulations is an option. In vitro tests (stability testing, dissolution) are routinely used as a first step in formulation development in order to build an absorption model for the prediction of in vivo exposure via the in vitro/in vivo correlation. The assessment of efficacy in disease models and exposure (PK) in animals will be applied to lead candidates only, before going to man.

Reasons for change of formulation include, but are not limited to, poor bioavailability of solid oral formulations, limitations in drug load for oral or parenteral formulations, profound food effect, too early/too late onset of action (absorption, distribution), too short/too long duration of action (metabolism, elimination), or high intra and inter individual variability.

PROCEDURE

The design of an exploratory formulation development study with SAR001 is presented below in Part A.

In the project with SAR001, the formulation development study explored the relative bioavailability of three prototype nanocrystal (NC) formulations (tablet, granules, lyophilisate) versus a soft gelatine capsule (SGC) formulation that was used in early clinical phases. This study should help to determine selection and development of alternative formulations to be used in Phase III studies, as the current SGC had *limitations in the unit strength* possibly not suitable for long term efficacy trials. The food effect was also investigated, as in an animal pilot study, the *magnitude of food effect* was more important for NC dispersion when compared to SGC.

In another project (HMR456) modified release (MR) formulations were developed in order to overcome the *short elimination half life,* and thus, a *short duration of action* of the Immediate Release formulation. The study design and the main PK results are presented elsewhere in this textbook (food effect chapter). In Part B, we will discuss the value of the deconvolution tool, which was applied to the PK results of the study.

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B.14.1 PART A

B.14.1.1 Protocol Outline

Relative bioavailability of three prototype nanocrystal formulations of SAR001 in comparison to a soft gelatin capsule formulation of SAR001 under fasting and fed conditions to healthy subjects.

B.14.1.1.1 Primary Objective

To assess the relative bioavailability of three prototype nanocrystal formulations of SAR001, in comparison to the soft gelatin capsule formulation, by assessing plasma concentration of SAR001 under fasting and fed conditions.

B.14.1.1.2 Study Design

Open, randomized, two group, four treatment, four period, four sequence crossover study using two parallel groups under fasting or fed conditions (Groups I and II, respectively). All single oral drug administration periods within groups were separated by a washout of 7 days.

B.14.1.1.3 Inclusion Criteria

Healthy male subjects, aged between 18 and 45 years, with a Body Mass Index (BMI) between 18 and 28 kg/m^2 inclusive, and liver function tests and creatine kinase values within reference ranges.

B.14.1.1.4 Treatments

Treatment A: Single dose of 80 mg SAR001 in soft gelatin capsule (SGC).
Treatment B: Single dose of 80 mg SAR001 in uncoated tablet.
Treatment C: Single dose of 80 mg SAR001 in granules for oral suspension.
Treatment D: Single dose of 80 mg SAR001 as lyophilisate for oral suspension.

All treatments were administered under two food conditions: fasting (overnight fasting + 4 h postdose, Group I) and fed (high fat high calorie breakfast starting 30 min and ending 5 min predose, Group II).

B.14.1.1.5 Pharmacokinetic Data

Concentration of SAR001 in plasma before and at predefined times after dosing.

EVALUATION

Only part of the evaluation will be presented here.

Standard descriptive statistics were calculated for each parameter and each treatment.

To determine the relative bioavailability of any pair of formulations, 90% confidence intervals (CI) of formulations ratio for AUC_{0-168} and C_{max} were displayed under each food regimen.

For C_{max}, AUC_{0-72}, and AUC_{0-168}, formulation effect was assessed using a linear mixed effects model separately for each food regimen on log transformed parameters. Estimates with 90% confidence intervals of pairwise formulations ratio of geometric means were computed within the linear mixed effects model framework.

Similarly, food effect was assessed for each formulation using a linear mixed effects model on log transformed parameters. Estimates and 90% confidence intervals of food regimens ratio of geometric means were computed within the linear mixed effects model framework.

To determine the food effect on each of the four formulations, 90% CI of fed/fasted ratio for AUC_{0-168} and C_{max} were displayed for each formulation separately.

CRITICAL ASSESSMENT OF THE METHOD

Due to the exploratory pilot character of such a formulation development study, a sample size calculation in its proper sense is not routinely performed. Often, a number of 12 study completers per cohort is considered sufficient for this purpose. This quite small cohort size "encourages" to implement high complexity with multiple objectives within a single trial. If solid formulations are to be compared, the same unit strength for each formulation is recommended in order to avoid an intrinsic source of variability, for example, if two tablets with 40 mg each are compared to a single capsule with 80 mg drug load. The number of dose units should be kept low, again to avoid a source of variability. Standardization is questionable if up to ten units or even more have to be swallowed with a limited amount of non carbonated water (e.g., 240 mL).

Preceding studies should help to define the necessary washout period in order to avoid carryover effects. If the predose concentration is higher than 5% of C_{max} in a given study period, the predose value should be subtracted from all postdose concentrations as corrective action.

Concerning food, not only composition (constituents, calories) but also start and end of food intake in relation to drug administration should be standardized.

In this example, the study subjects were stratified for food condition, which results in four high fat high calorie meals for members of Group II, and in four 14 h lasting fasting periods for members of Group I. In order to avoid late dropouts in crossover studies that apply within comparisons of both food conditions, it may be advisable to define the compliance to high fat high calorie breakfast as an inclusion criterion for the study.

In all studies with parallel groups, care should be taken that baseline demographics for example, gender ratios, body weight, BMI, age are similar between groups. A possibility to overcome this request is the implementation of the same reference treatment for each group. An example for this approach can be found in the food effect chapter.

MODIFICATIONS OF THE METHOD

If in late clinical development the need for a formulation switch becomes evident, and a formulation comparison is necessary for bridging purposes, the exploratory character of a formulation development study gets lost and a bioequivalence study has to be conducted.

B.14.1.2 Part A

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the pharmacokinetic results obtained from the study described above under "PROCEDURE" is presented below (*Tables B.14 1 B.14 3*; *Figs. B.14 1* and *B.14 2*).

B.14.1.2.1 Results – Pharmacokinetics

In fasting or in fed condition, whatever the formulation, t_{max} was about the same, with median values ranging from 2 to 4 h post administration.

In fasting condition, SAR001 exposure was lower with nanocrystal formulations compared to soft gelatin capsules (SGC). In comparison to SGC, variability of pharmacokinetic parameters was similar for the lyophilisate, slightly higher for the tablets and higher for the granules.

In fed condition, SAR001 exposure was close to that observed with SGC for granules and lower than that observed with SGC for tablets. Variability of pharmacokinetic parameters was about the same, whatever the formulation.

Food effect (relative bioavailability) based on AUC_{0-72} was about twice as high with nanocrystal formulations compared to soft gelatin capsules.

B.14.2 Part B

PROCEDURE

The immediate release PK properties of the active metabolite HMR123 of drug HMR456 were not sufficient to support a twice a day dosing (terminal elimination half life too short, time above PD concentration threshold too short). Therefore modified release (MR) formulations were developed. The use of a deconvolution tool will be discussed in this section.

The design of the exploratory bioavailability study on modified release drug products is presented in brief below. Detailed information is given in the food effect chapter. For the assumptions of the hypothetical deconvolution tool, in vitro/in vivo dissolution data from a predecessor study with the same compound was used.

B.14.2.1 Protocol Outline

Comparison of pharmacokinetics and safety of Modified Release formulations of 600 mg HMR456 with that of an immediate release formulation.

B.14.2.1.1 Primary Objective

To compare the PK characteristics of modified release (MR) formulations of HMR456 with the PK of an immediate release (IR) formulation of HMR456.

B.14.2.1.2 Secondary Objective

To assess the influence of food on the PK of MR formulations of HMR456.

Hypothetical in vivo dissolution was performed in addition to the objectives mentioned in the study protocol. Application of the method, results, and interpretation will be discussed here.

B.14.2.1.3 Study Design

Single center, open label, single dose, four period crossover study design with two parallel treatment groups.

Single oral doses of 600 mg HMR456 were given under fasting and under non fasting conditions.

Table B.14-1

Pharmacokinetic population: numbers by treatment/condition

Condition/treatment	Soft gelatin capsules	Granules for oral suspension	Lyophilisate for oral suspension	Uncoated tablets
Fasting	13	13	13	13
Fed	13	14	13	12

Table B.14-2

Relative bioavailability estimates and 90% confidence intervals between formulations

Food condition	Parameter	Formulation comparison	Ratio estimate and 90% CI
Fasted	C_{max} (ng/mL)	Granule vs. capsule	0.30 (0.20, 0.45)
		Lyophilisate vs. capsule	0.29 (0.19, 0.43)
		Tablet vs. capsule	0.17 (0.11, 0.25)
	AUC_{0-72} (ng·h/mL)	Granule vs. capsule	0.47 (0.34, 0.64)
		Lyophilisate vs. capsule	0.44 (0.32, 0.60)
		Tablet vs. capsule	0.29 (0.21, 0.39)
	AUC_{0-168} (ng·h/mL)	Granule vs. capsule	0.50 (0.39, 0.63)
		Lyophilisate vs. capsule	0.52 (0.41, 0.67)
		Tablet vs. capsule	0.37 (0.29, 0.49)
Fed	C_{max} (ng/mL)	Granule vs. capsule	0.67 (0.51, 0.88)
		Lyophilisate vs. capsule	0.72 (0.54, 0.95)
		Tablet vs. capsule	0.47 (0.35, 0.62)
	AUC_{0-72} (ng·h/mL)	Granule vs. capsule	0.95 (0.80, 1.12)
		Lyophilisate vs. capsule	0.94 (0.79, 1.11)
		Tablet vs. capsule	0.65 (0.54, 0.77)
	AUC_{0-168} (ng·h/mL)	Granule vs. capsule	0.92 (0.79, 1.07)
		Lyophilisate vs. capsule	0.94 (0.81, 1.08)
		Tablet vs. capsule	0.70 (0.60, 0.81)

B.14.2.1.4 Inclusion Criteria

Healthy men aged 18 55 years.

B.14.2.1.5 Treatments

Treatment Group I

Treatment A: 600 mg HMR456 (one film coated tablet containing 200 mg + one film coated tablet containing 400 mg given together) as IR formulation under non fasting (NF) conditions (reference).

Treatment B: 600 mg HMR456 in MR formulation (matrix tablet 1) under fasting (F) and NFconditions.

Treatment C: 600 mg HMR456 in MR formulation (bilayer tablet 1) under F and NF conditions.

Treatment Group II

Treatment A: 600 mg HMR456 (one film coated tablet containing 200 mg + one film coated tablet containing 400 mg given together) as IR formulation under non fasting (NF) conditions (reference).

Treatment D: 600 mg HMR456 in MR formulation (matrix tablet 2) under F and NF conditions.

Treatment E: 600 mg HMR456 in MR formulation (bilayer tablet 2) under F and NF conditions.

MR tablet formulation 1 contains hydroxypropyl methyl cellulose, MR tablet formulation 2 contains carrageenan.

Table B.14-3

Relative bioavailability estimates and 90% confidence intervals between food conditions

Parameter	Food comparison	Ratio estimate and 90% CI
C_{max} (ng/mL)	Fed vs. fasted for capsule	2.03 (1.39, 2.96)
	Fed vs. fasted for granule	4.35 (2.99, 6.32)
	Fed vs. fasted for lyophilisate	5.04 (3.45, 7.35)
	Fed vs. fasted for tablet	5.77 (3.94, 8.47)
AUC_{0-72} (ng·h/mL)	Fed vs. fasted for capsule	2.00 (1.44, 2.76)
	Fed vs. fasted for granule	3.93 (2.85, 5.43)
	Fed vs. fasted for lyophilisate	4.31 (3.11, 5.96)
	Fed vs. fasted for tablet	4.59 (3.31, 6.36)
AUC_{0-168} (ng·h/mL)	Fed vs. fasted for capsule	2.07 (1.54, 2.77)
	Fed vs. fasted for granule	3.85 (2.84, 5.22)
	Fed vs. fasted for lyophilisate	3.69 (2.72, 4.99)
	Fed vs. fasted for tablet	3.86 (2.81, 5.31)

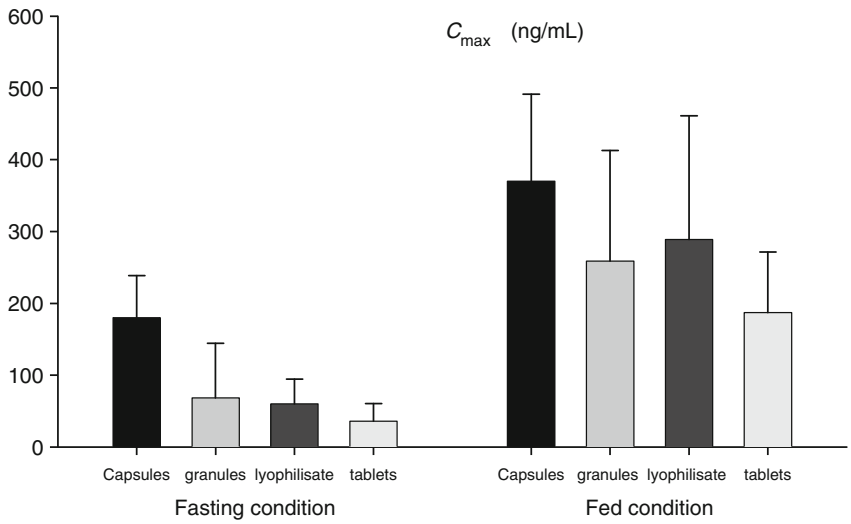


Figure B.14-1

Mean (SD) SAR001 C_{max} obtained after single oral administration to healthy young male subjects using four different formulations, in fasting or fed condition

B.14.2.1.6 Pharmacokinetic Data

Concentration of HMR123 in plasma before and at predefined times after dosing.

EVALUATION

Bioanalytical data: Individual plasma concentrations of HMR123 were tabulated together with standard descrip tive statistics for each treatment. Individual and median profiles were presented graphically.

In vivo *dissolution data:* The individual hypothetical in vivo dissolutions for the four MR formulations adminis tered under fasting and non fasting conditions were esti mated by numerical deconvolution using the individual response to the IR 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 (h) of HMR123 (time above 200, 500, 800, and 1,000 ng/mL) were calculated in

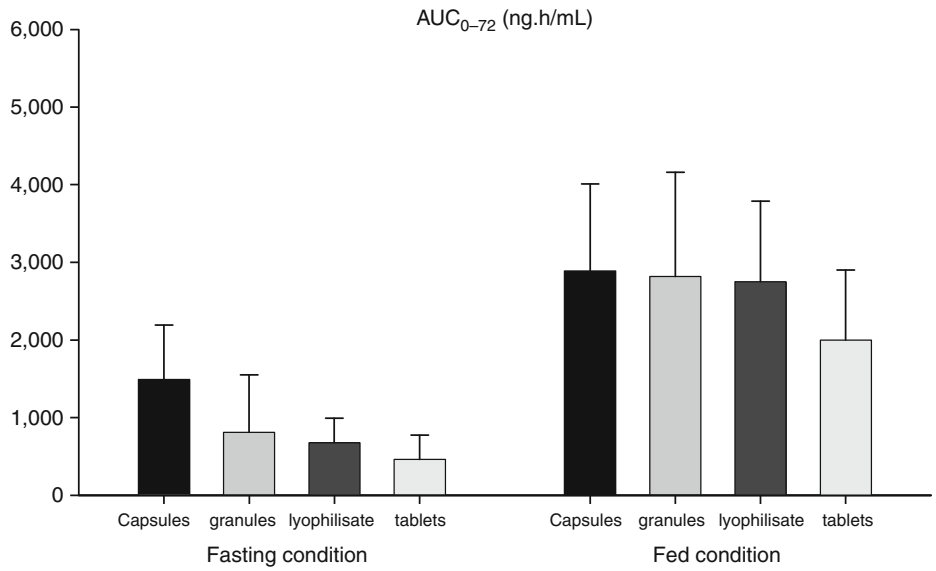


Figure B.14-2

Mean (SD) SAR001 AUC_{0-72} obtained after single oral administration to healthy young male subjects using four different formulations, in fasting or fed condition

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 are presented elsewhere.

CRITICAL ASSESSMENT OF THE METHOD

The study described here has a very complex design for its exploratory approach. It combines four different MR for mulations, each tested under fasting and non fasting con ditions, and compares the results to the IR drug product as the reference formulation in two separate study groups. The bilayer tablets combine an IR component and an MR component in one vehicle. In this project, a close cooper ation between the galenics department, analytical science department, and the clinical pharmacokinetic function (including study management, bioanalysis and PK evalu ation) was mandatory. The in vitro/in vivo correlation was done by means of the deconvolution which is an appro priate surrogate to describe the in vivo dissolution.

The mismatch of surpassing 100% absorption of the active metabolite, that we observed in our study, 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, for example, an oral solution (oral bolus input); the immediate release formulation only provides an approximation to the required properties.

MODIFICATIONS OF THE METHOD

The application of in vitro/in vivo correlation (IVIVC) and the tools for obtaining IVIVC including deconvolution are reviewed in FDA Guidance for Industry (1997).

REFERENCES AND FURTHER READING

FDA Guidance for Industry: Extended release oral dosage forms: development, evaluation, and application of in vitro/in vivo correlations. September 1997.

B.14.2.1.7 Example

To illustrate the amount of data that can be obtained using the deconvolution tool obtained from the study described above under "PROCEDURE" is presented below.

B.14.2.1.8 Results – Hypothetical In Vivo Dissolution

Deconvolution is used to evaluate in vivo drug release and drug absorption from orally administered drug formula tions (i.e., extended release) when data from a known drug

Table B.14-4

Hypothetical dissolution data for HMR123 obtained by deconvolution using Treatment A(NF) as impulse function. Median, range

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
	239.61 727.06	173.93 484.88	287.67 994.55	300.14 1,817.29	465.29 1,076.11	339.60 795.42	430.72 5,674.72	452.19 3,183.05
Maximum amount absorbed[a] (% of dose)	63.22	51.16	85.58	98.88	102.59	81.03	110.82	114.99
	42.20 128.04	30.63 85.39	50.66 175.15	52.86 320.04	81.94 189.51	59.81 140.08	75.85 999.35	79.63 560.55
Time to reach maximum amount (h)	15.00	24.00	15.00	15.00	9.00	15.00	2.50	15.00
	4.00 24.00	15.00 24.00	6.00 15.03	0.50 36.00	2.00 24.00	10.00 24.00	0.50 24.00	0.50 15.00
Time to reach 20% of maximum amount (h)	1.81	0.55	0.57	0.27	0.76	0.34	0.51	0.26
	0.43 2.64	0.33 1.58	0.26 1.25	0.05 1.64	0.28 1.92	0.16 0.81	0.10 1.54	0.12 0.31
Time to reach 40% of maximum amount (h)	2.57	2.14	0.83	0.35	0.91	0.62	0.69	0.33
	0.97 3.38	0.54 5.47	0.33 1.73	0.11 1.97	0.36 2.62	0.31 1.16	0.20 1.83	0.24 0.81
Time to reach 50% of maximum amount (h)	2.80	3.34	0.91	0.40	1.04	0.69	0.81	0.37
	1.35 4.10	0.58 7.45	0.36 2.21	0.14 2.07	0.39 2.68	0.34 1.59	0.26 1.98	0.29 1.02
Time to reach 60% of maximum amount (h)	3.57	4.54	1.06	0.44	1.54	0.94	1.04	0.41
	1.51 6.00	0.62 9.33	0.40 3.19	0.16 2.25	0.43 2.75	0.37 2.22	0.30 2.13	0.33 1.22
Time to reach 80% of maximum amount (h)	5.27	6.95	2.95	1.64	1.79	2.54	1.44	0.48
	1.80 8.61	0.71 15.28	0.46 6.17	0.22 6.18	0.53 2.87	0.44 7.96	0.40 2.64	0.42 4.49

[a]Dose calculated for 567.84 mg HMR123 (corresponding to 600 mg HMR456)

B(F/NF): 600 mg HMR456 in ER formulation (HPMC matrix tablet) under fasting (F) and non fasting (NF) conditions, respectively

C(F/NF): 600 mg HMR456 in ER formulation (HPMC, bilayer tablet) under fasting (F) and non fasting (NF) conditions, respectively

D(F/NF): 600 mg HMR456 in ER formulation (carrageenan matrix tablet) under fasting (F) and non fasting (NF) conditions, respectively

E(F/NF): 600 mg HMR456 in ER formulation (carrageenan, bilayer tablet) under fasting (F) and non fasting (NF) conditions, respectively

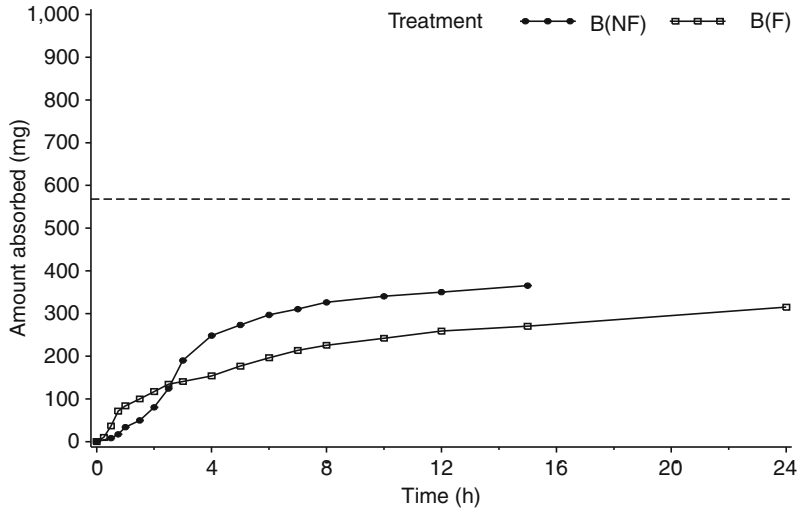


Figure B.14-3

Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments B(F) and B(NF) (mg)

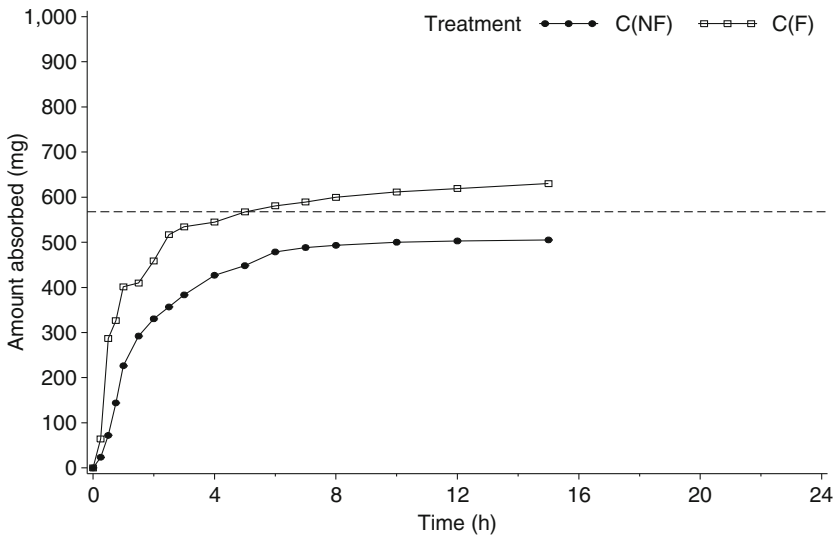


Figure B.14-4

Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments C(F) and C(NF) (mg)

input are available. The applied deconvolution method requires data from a formulation with zero order absorption as known input, for example, 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 active metabolite HMR123 obtained by deconvolution are listed in the following *Table B.14 4*.

The following *Figs. B.14 3 B.14 10* show the hypothetical geometric mean in vivo dissolution profiles for the metabolite HMR123 (absolute amount absorbed vs. time

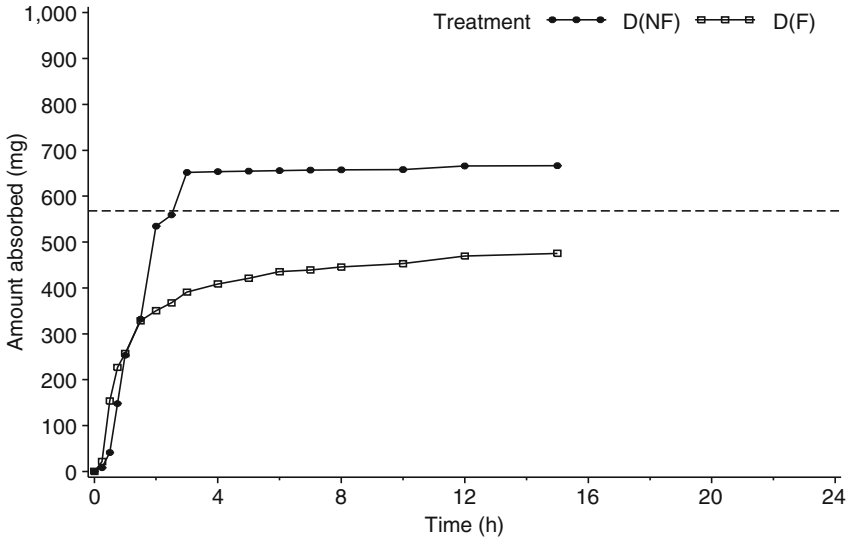


Figure B.14-5
Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments D(F) and D(NF) (mg)

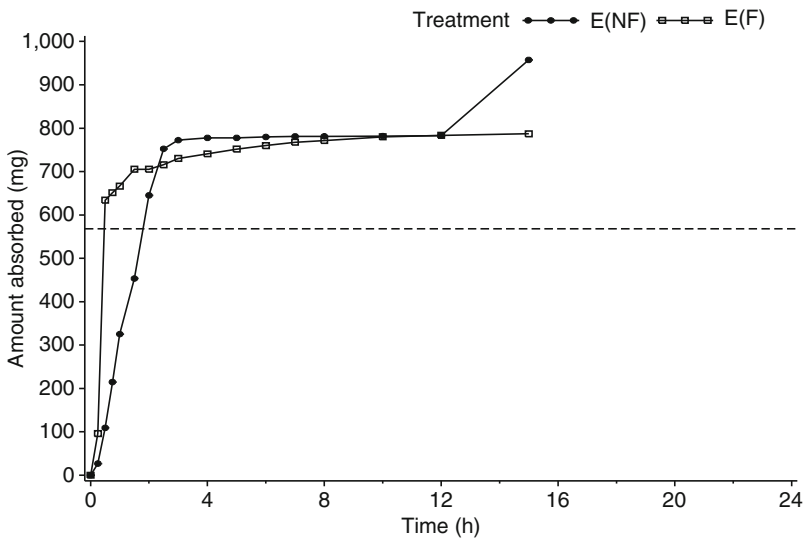


Figure B.14-6
Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments E(F) and E(NF) (mg)

as well as percentage of theoretical dose of the metabolite vs. time).

As can be seen in the above figures, as well as in *Table B.14 4*, 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). For example, the time for 50% of the maximal absorption looks much shorter (especially

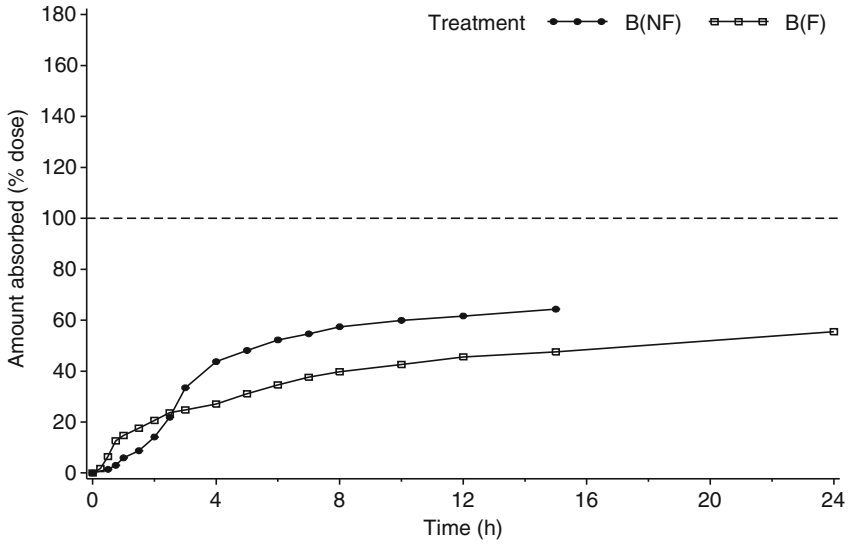


Figure B.14-7

Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments B(F) and B(NF) (%dose)

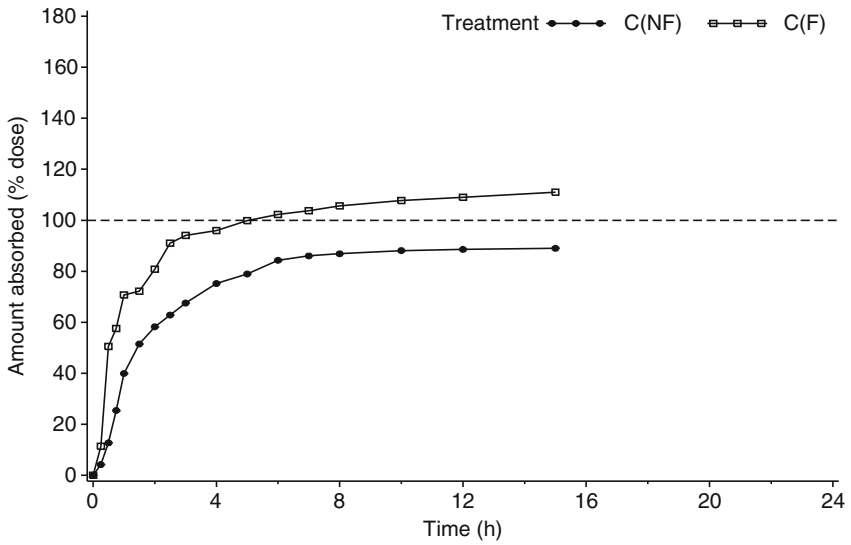


Figure B.14-8

Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments C(F) and C(NF) (%dose)

when Treatment C is compared to B). This effect was more pronounced under fasting conditions. Only with Treatment E (carrageenan bilayer tablets), the hypothetical in vivo dissolution profiles surpassed 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 nonfasting conditions.

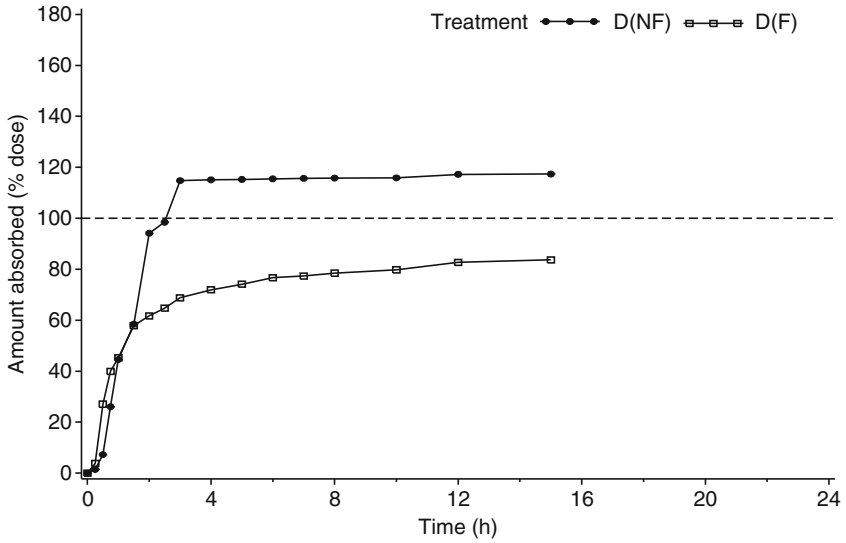


Figure B.14-9

Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments D(F) and D(NF) (%dose)

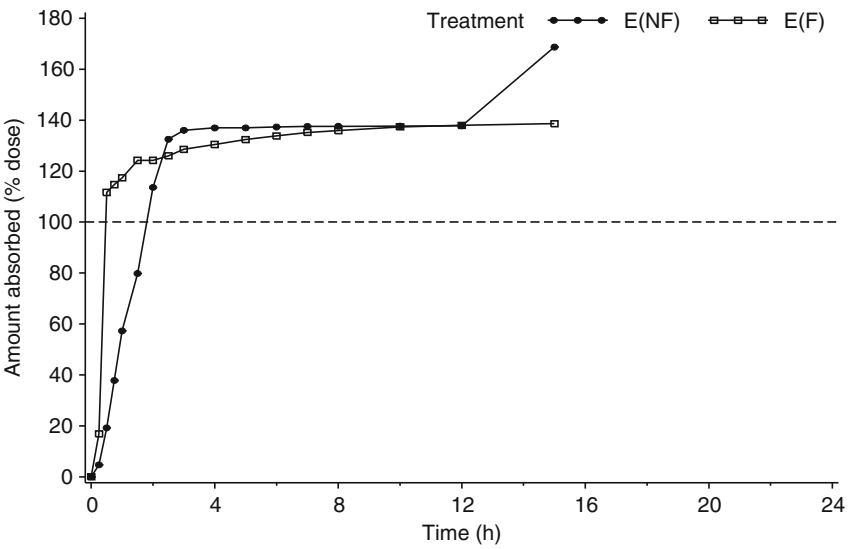


Figure B.14-10

Geometric means of the hypothetical in vivo dissolution profiles of HMR123. Treatments E(F) and E(NF) (%dose)

REFERENCES AND FURTHER READING

CPMP/EWP/560/95 Note for Guidance on the Investigation of Drug Interactions (CPMP, June 1998)

CPMP/EWP/QWP/1401/98 Note for Guidance on the Investigation of Bioavailability and Bioequivalence (CPMP, January 2002)

Guidance for Industry Scale-up and post-approval changes (SUPAC-IR): Chemistry, manufacturing, and control; in vitro dissolution testing and in vivo bioequivalence documentation. U.S. Department of Health and Human Services, Food and Drug Administration; Center for Drug Evaluation and Research (CDER). September 1995

Guidance for Industry Scale-up and post-approval changes (SUPAC-MR): Chemistry, manufacturing, and control; in vitro dissolution testing and in vivo bioequivalence documentation. U.S. Department of Health and Human Services, Food and Drug Administration; Center for Drug Evaluation and Research (CDER). September 1997

Guidance for Industry Statistical Approaches to Establishing Bioequivalence. U.S. Department of Health and Human Services, Food and Drug Administration; Center for Drug Evaluation and Research (CDER). January 2001

Guidance for Industry Waiver of in vivo bioavailability and bioequivalence studies for immediate release solid oral dosage forms containing certain active moieties/active ingredients based on the biopharmaceutics classification system (BCS). U.S. Department of Health and Human Services, Food and Drug Administration; Center for Drug Evaluation and Research (CDER). January 1999

Steinijans V-W, Hauschke D (1997) Individual bioequivalence. A European perspective. J Biopharm Stat 7(1):31–34

B.15 Absolute and Relative Bioavailability

Roland Wesch

PURPOSE AND RATIONALE

The assessment of a drug's bioavailability (BA) is the most important information on its pharmacokinetics. Consequently, numerous guidelines primarily focus on this issue as from the exposure efficacy as well as safety for the patient (Study Design et al. 2003; ICH E4: Dose Response Information to Support Drug Registration March 1994; EU CPMP: Note for Guidance on Modified Release Oral and Transdermal Dosage Forms: Section II (Pharmacokinetic and Clinical Evaluation) July 1999; EU CPMP: Note for Guidance on the Investigation of Bioavailability and Bioequivalence July 2001; US FDA Guidance for Industry: Food Effect Bioavailability and Fed Bioequivalence Studies December 2002; US FDA Guidance for Industry: Bioavailability and Bioequivalence Studies for Orally Administered Drug Products General Considerations March 2003; US FDA Guidance for Industry: Bioavailability and Bioequivalence Studies for Nasal Aerosols and Nasal Sprays for Local Action April 2003; EU CPMP: Points to Consider on the Clinical Requirements of Modified Release Products to be Submitted as a Line Extension of an Existing Marketing Authorization June 2003).

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. Absolute bioavailability is deduced from the comparison of an extravascular and an intravascular administration, i.e., AUC_{PO}/AUC_{IV}. Relative bioavailability compares the exposure following two different extravascular application forms, i.e., AUC_{IM}/AUC_{SC} or $AUC_{POtest}/AUC_{POreference}$. Extravascular routes of administration that require documentation of bioavailability and/or bioequivalence include the oral (PO), intramuscular (IM), or subcutaneous (SC) routes, and in most instances vaginal, dermal, ocular, topic, rectal, nasal, or pulmonary administration.

For absolute BA studies, the AUC after intravascular (IV or intra arterial) administration is the reference and is set to 100% availability. Factors that reduce the availability of a drug prior to entering the systemic circulation may include poor absorption from the gastrointestinal tract (Zhou 2003), an (entero)hepatic recirculation (Ezzet et al. 2001; Bergman et al. 2006), or a fast degradation prior to reaching the central compartment, the first pass effect or first pass metabolization (Kharasch et al. 2005; Zahng and Benet 2001; Chan et al. 2004; Thummel and Wilkinson 1998).

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, for example, immediate release vs. modified release solid formulation or capsule vs. tablet or tablet A vs. tablet B, etc. or they compare the disposition of different routes of administration, for example, PO vs. SC or PO vs. IV. 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. If the parent drug is pharmacodynamically inactive, details on relevant active metabolite(s) should be given.

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 the rate (C_{max}/t_{max}) and the extent (area under the plasma concentration time curve AUC).

Details on the design of and the interpretation of data from bioavailability studies are given in guidelines and guidances from ICH, FDA, or CPMP.

PROCEDURE

The design for an absolute bioavailability study is presented in Example 1. The drug in question undergoes intensive Phase II metabolism, leading to numerous conjugates, the cysteine conjugate being predominant. For the

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.15,

purposes of simplicity, the description is limited to the collection, handling, and interpretation of pharmacokinetic data although safety parameters were also in the focus.

The design for a relative bioavailability study is presented in Example 2. The drug in question had exhibited a marked positive food effect when being administered as film tablet. The purpose of this study was to compare a newly developed capsule to a pilot capsule formulation and to include an oral solution as the reference.

B.15.1 Example 1

B.15.1.1 Protocol Outline

A phase I, open label, randomized, crossover study to investigate the bioavailability, safety, tolerability, and pharmacodynamics following single oral administration of 25 mg XYZ1234 as capsule and single intravenous administration of 10 mg XYZ1234 in healthy men.

B.15.1.1.1 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 the reference formulation.

B.15.1.1.2 Study Design

This was an open label, single dose, randomized, two period crossover 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.

B.15.1.1.3 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.

B.15.1.1.4 Treatments

Regimen A (Reference Treatment):

Intravenous (IV) administration of XYZ1234 (10 mg, administered over 30 min)

Regimen B (Test Treatment):

Oral (PO) administration of XYZ1234 (25 mg, as a capsule formulation)

B.15.1.1.5 Pharmacokinetic Data

Concentrations of unconjugated XYZ1234 and Cystein (CYS) conjugated XYZ1234 in plasma were measured pre dose and at predetermined times up to 48 h 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 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 of 25 mg was selected according to the experience from the first in man study, where this dose was safe and well tolerated and was at the higher end of 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 or, in a stricter sense, bioequivalence studies are usually conducted in healthy subjects. Although the inclusion of women is now being encouraged, we enrolled only men. This study was the second clinical trial in the 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. It must be ensured that inter occasion

variability is limited to the formulations used. Typical enrolment criteria are:

- Non smoking subjects between 18 and 45 years
- Normal for weight and BMI
- (Clinically) healthy
- Not using any medication
- Massive dietary and general restrictions ("life style")
- No hypersensitivities
- No recent history or presence of any condition that might interfere with the absorption, distribution, metabolism, or elimination of the drug under investigation

In the context with the last criterion, it is important to document any adverse event during the study, especially close to the PK profiling day(s) that might affect the disposition of an orally administered drug under investi gation, for example, nausea (delaying the gastric emptying time, increasing the intestinal residence times), vomiting (erasing drug still being in the stomach, reducing the absorption from the intestine), or diarrhea (decreasing absorption from the intestine).

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 (see Example 2). Depending on the primary purpose of these investiga tions, the reference formulation can be a marketed (solid) drug product, an early clinical phase "pilot" drug product, or an oral solution/suspension.

If the drug under investigation has a toxic potential (e.g., drugs directed against cancer), BA studies have to be conducted in the patient setting the drug is intended for use.

Deviations from the high level of standardization might become necessary depending on the properties of the compound.

Crossover study designs allowing for intra individual comparisons are preferred for the purpose of bioavailability testing. Exceptions, i.e., parallel group design, might become necessary, for example, if the terminal half life of the drug exceeds 7 days. Such a long half life would translate into a washout period of five times the elimination half life, or more than 35 days, in order to avoid trough concentrations for the second trial period of more than 5% the individual maximum concentration (C_{max}) in this trial period.

Almost all clinical study types described in the PK section of this book deal in any way with bioavailability and/or bioequivalence questions. Specifics if there are any are mentioned there.

B.15.1.2 Example 1

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the pharmacoki netic 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 min was chosen.

B.15.1.2.1 Results – Pharmacokinetics

The calculated bioavailability on the 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 the basis of the AUC_{last} or $AUC_{0–inf}$ of unconjugated XYZ1234 yielded a slightly higher bioavailability of 45 47%.

A summary of the pharmacokinetic parameters in plasma is presented in *Table B.15 1*.

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 elim ination phase was observed, with a terminal elimination half life of 3.5 4 days (unconjugated XYZ1234). This long half life in combination of a washout period of a minimum of 7 days resulted in a small carry over effect in Period 2 for unconjugated XYZ1234. Correction for pre dose concen trations was not needed, as none of the individual values exceeded a 5% threshold of C_{max}. Due to a relatively high lower limit of quantitation of 10 ng/mL of the assay for conjugated XYZ1234, the terminal elimination phase for this analyte could only be reliably determined for one subject. The concentrations of conjugated XYZ1234 in plasma were five to tenfold higher than of unconjugated XYZ1234. The AUC_{last} (both analytes) and $AUC_{0–inf}$ (unconjugated XYZ1234 only) were similar after treat ment with XYZ1234 25 mg PO and XYZ1234 10 mg IV.

Results of bioavailability analysis			
Analyte	Parameter	Treatment ratio (PO/IV)	90% CI
XYZ1234 (conjugated)	AUC_{last}	0.38	0.33 0.43
XYZ1234 (unconjugated)	$AUC_{0\ inf}$	0.47	0.38 0.59
XYZ1234 (unconjugated)	AUC_{last}	0.45	0.43 0.47

Note: Data were dose corrected

Table B.15-1

Summary of the pharmacokinetic parameters in plasma

Treatment	C_{max} (ng/mL)	T_{max} [a] (h)	AUC_{last} (ng.h/mL)	AUC_{0-inf} (ng.h/mL)	$t_{½}$ (h)
Unconjugated XYZ1234					
10 mg IV	49.5 (30.4 96.7)	0.50[b] (0.50 0.58)	81.8 (42.7 149.2)	207.4 (85.1 602.4)	85 (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 (127.8 337.8)	94 (61 192)
Conjugated XYZ1234					
10 mg IV	280.8 (198.0 420.6)	0.50[b] (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]The time point 0.50 h is identical with the end of infusion of 30 min/0.5 h duration

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%.

B.15.2 Example 2

B.15.2.1 Protocol Outline

An open label, crossover study to compare bioavailability, pharmacokinetics, safety, and tolerability of three different oral formulations of 50 mg HMR123 in healthy men.

B.15.2.1.1 Primary Objective

To assess the relative bioavailability and pharmacokinetics of three oral formulations (one liquid and two capsule formulations) containing 50 mg HMR123.

B.15.2.1.2 Study Design

This was an open label, single dose, randomized, three way, three sequences (ABC, BCA, CAB), three treatments, three periods crossover study with a minimum washout period between treatments of 7 days. Each subject received a single dose of each of the three oral formulations, each of which containing 50 mg HMR123 under fasting conditions. The sequence of administration (treatments A, B, and C) was determined according to a randomization schedule.

B.15.2.1.3 Inclusion Criteria

Healthy Caucasian males, aged 18 55 years (inclusive), with a Body Mass Index between 18 and 28 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.

B.15.2.1.4 Treatments

Treatment A (reference):

Single oral dose of 50 mg solution PEG400/Water/HMR123

Treatment B (test 1):

Single oral dose of two 25 mg capsules (filled with granules) HMR123

Treatment C (test 2):

Single oral dose of two 25 mg capsules (liquid filled) HMR123

B.15.2.1.5 Pharmacokinetic Data

Concentrations of HMR123 in plasma were measured pre dose and at predetermined times up to 96 h post dose.

The primary analysis examined pharmacokinetic parameters calculated from plasma concentrations of HMR123 using non compartmental techniques.

EVALUATION

The primary analyses consisted of characterizing the bioavailability of three different oral formulations of HMR123. Determination of bioavailability was based on

the plasma concentrations. Descriptive statistics and for mal statistical analysis were used to summarize and ana lyze the pharmacokinetic parameters of HMR123.

Analysis of variance (ANOVA) was performed on the log transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and AUC_{0-inf}, with sequence, subject nested within sequence (subject (sequence), period and treatment effects as main 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. The mean square error was used to construct 90% confi dence intervals for treatment ratios. The point estimates were calculated as ratio of the antilogs of the least square means and were expressed as percentages.

In order to compare the relative bioavailability of the three oral formulations, the following ratios (point esti mates and corresponding 90% confidence intervals) were calculated using adequate contrasts: A:B, A:C, and B:C for both AUC and C_{max}.

Bioequivalence was concluded if the 90% confidence interval for the treatment ratios were fully contained within the (80 125%) acceptance range.

The secondary analyses consisted of assessing the safety and tolerability of single oral 50 mg doses of HMR123.

CRITICAL ASSESSMENT OF THE METHOD

An oral solution consisting of water, PEG400, and HMR123 was selected as the reference formulation. Oral solutions are widely accepted as the "gold standard," because they are devoid of any limitations concerning dissolution. In order to maintain a high level of standard ization single units of solid formulations are preferred, for example, one tablet or one capsule. We used two units instead, as at the time of implementation of this study, the maximal dose strength was 25 mg, and a minimum effective dose of 50 mg was anticipated. In preceding studies, single doses of up to 100 mg had proven to be safe and well tolerated by healthy male subjects.

If a subject prematurely terminates the study this was the case in our study the replacer subject has to be administered the same treatment sequence as the subject he or she replaces.

This study was the third clinical trial in the project. In the First in Man study, a film coated tablet has been used, which was dropped due to high interindividual variability in PK parameters. The second one used the test formula tion 1, compared to an oral solution. Test formulation 1 was to be dropped also due to a marked negative food effect. The test formulation 2 was the result of optimization efforts from the Galenics department. In this context please refer also to Chap. B.14, Specific Studies for Formulation Development.

B.15.2.2 Example 2

To illustrate the type of data that can be obtained using the discussed study, a high level summary of the pharmacoki netic results obtained from the study described above under "PROCEDURE" as Example 2 is presented below.

B.15.2.2.1 Results – Study Accounting

In total, 13 male subjects were enrolled in the study and received the investigational product according to the ran domization schedule. Twelve subjects completed the trial. One subject (subject no. 1xxx) was withdrawn due to adverse events on day 5 of trial period I (treatment A). He was replaced by subject no. 6xxx with the same treat ment sequence, i.e., ABC.

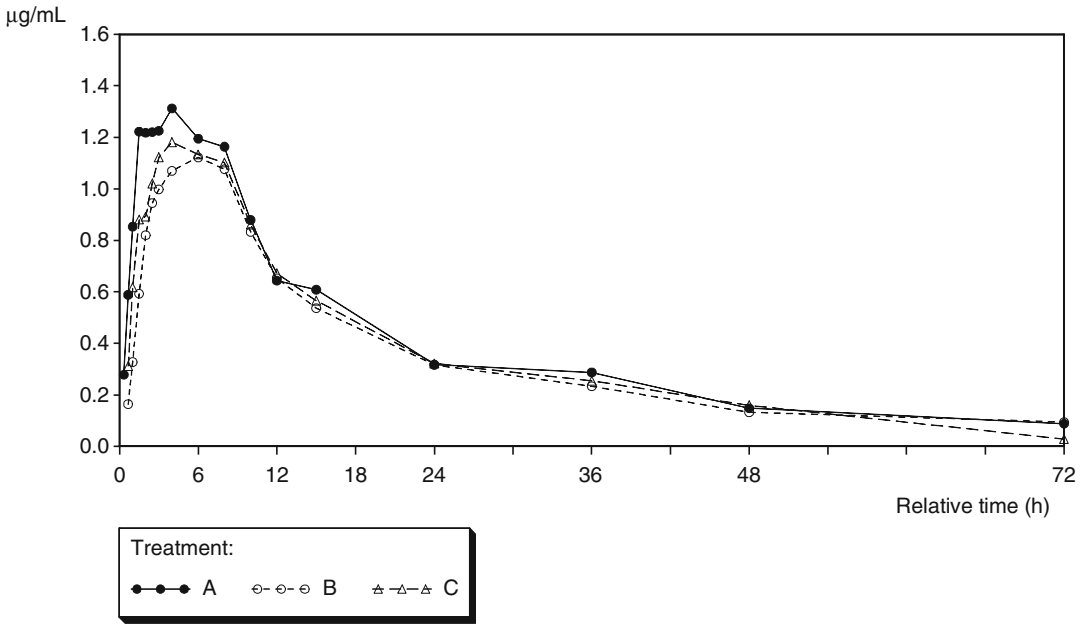
B.15.2.2.2 Results – Pharmacokinetics

All subjects who completed the study and for whom the concentrations of HMR123 were considered sufficient and interpretable by the sponsor were included in the phar macokinetic analyses. The 12 subjects who met these criteria were included in the PK analysis according to the analysis procedures described in the study specific Statistical Analysis Plan.

In *Figs. B.15 1* and *B.15 2*, the plasma concentra tion versus time profiles are given in linear and log linear presentation. For all subjects who were included in the PK analysis, the individually latest time point of quantifiable concentration T_{last} was 72 h post dose.

C_{max} and T_{max} were obtained from the highest con centration of the measured data. The apparent terminal elimination rate constants (λ_z) were determined using nonlinear regression analysis on those concentration time pairs visually assessed to be in the terminal phase. The terminal phase half life ($t_{1/2,z}$) was calculated as the ratio of ln 2 to λ_z. Areas under the curve were determined using the log/linear trapezoidal rule (linear up to the maximum concentration and log thereafter). The area up to infinity (AUC_{0-inf}) was determined by extrapolation from the last observed data point: the extrapolated area was calculated as C_{last}/λ_z.

Arithmetic and geometric means for the pharmacoki netics parameters are displayed in the table below.



Treatment A (reference): oral solution
Treatment B (test 1): capsule filled with granules
Treatment C (test 2): capsule (liquid-filled)

Figure B.15-1
HMR123 plasma concentration, linear scale

Following treatment with the solution formulation, no blood samples were available for subject 1zzz for the 15, 24, and 48 h sampling times. Consequently, a number of pharmacokinetic parameters could not be determined and this subject had to be excluded from the population used for the sensitivity analysis.

Descriptive statistics for pharmacokinetic parameters			
	Arithmetic mean (geometric mean)		
PK Parameter	Treatment A	Treatment B	Treatment C
C_{max} (µg/mL)	1.37 (1.35)	1.21 (1.15)	1.25 (1.17)
AUC_{0-t} (µg*h/mL)	26.53 (25.65)	23.97 (22.67)	24.80 (23.04)
AUC_{0-inf} (µg*h/mL)	30.06 (29.24)	26.28 (25.01)	27.35 (25.87)
$t_{1/2,z}$ (h)	21.39 (20.90)	20.61 (19.99)	20.03 (19.33)
Rate constant (1/h)	0.034 (0.033)	0.036 (0.035)	0.037 (0.036)
V_z (L)	52.16 (51.57)	60.09 (57.75)	59.33 (53.82)

Frequency tables were prepared for the blood sampling times of maximum observed plasma concentration (T_{max}) and for the last quantified plasma concentration (T_{last}).

T_{max} was observed most frequently at 4 h after treatment A (4 out of 12 subjects), at 6 h after treatment B (4 out of 12 subjects) and at 8 h after treatment C (5 out of 12 subjects). T_{last} was most frequently observed at 48 h after dosing for all three treatments (for 5 of 12 subjects each).

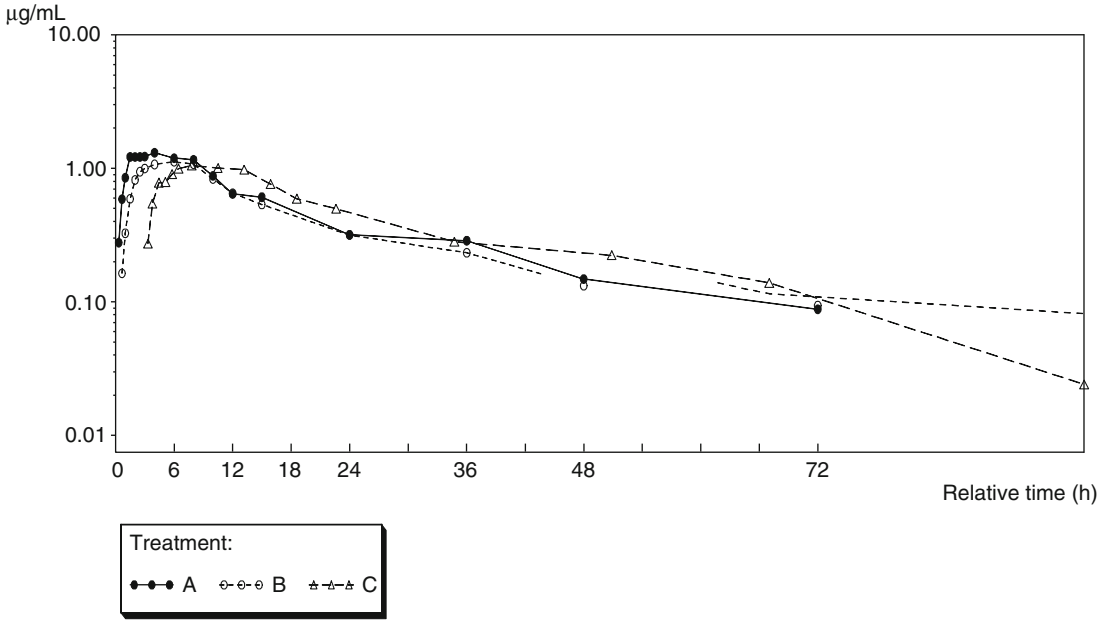
B.15.2.3 Comparison of Treatments

B.15.2.3.1 Treatment A vs. Treatment B

The 90% confidence intervals are not completely within the 0.8 1.25 range of bioequivalence and therefore no equivalence could be concluded.

B.15.2.3.2 Treatment A vs. Treatment C

The 90% confidence intervals are not completely within the 0.8 1.25 range of bioequivalence and therefore no equivalence could be concluded.



Treatment A (reference): oral solution
Treatment B (test 1): capsule 1 (filled with granules)
Treatment C (test 2): capsule 2 (liquid-filled)

Figure B.15-2
HMR123 plasma concentration, log-linear scale

B.15.2.3.3 Treatment B vs. Treatment C

The 90% confidence intervals are completely within the 0.8 1.25 range of bioequivalence and therefore equiva lence could be concluded.

only be concluded for the comparison of treatment B vs. treatment C, i.e., for both capsule formulations. The lack of equivalence between the capsule formulations and the reference oral solution probably results from an increased ratio of the treatments and not from increased variability.

						90% CI for treatment effect	
Parameter	Treatment	LS mean	Reference	LS mean	Ratio	Lower	Upper
C_{max} (µg/mL)	A	1.347	B	1.154	1.168	0.988	1.380
	A	1.347	C	1.167	1.154	0.977	1.364
	B	1.154	C	1.167	0.988	0.837	1.168
$AUC_{0\ t}$ (µg*h/mL)	A	25.649	B	22.674	1.131	0.978	1.309
	A	25.649	C	23.042	1.113	0.962	1.288
	B	22.674	C	23.042	0.984	0.851	1.138
$AUC_{0\ inf}$ (µg*h/mL)	A	29.967	B	25.948	1.155	1.003	1.330
	A	29.967	C	27.048	1.108	0.962	1.276
	B	25.948	C	27.048	0.959	0.833	1.105

The results of the sensitivity analysis for all treatment comparisons are presented below. Bioequivalence could

Generally, slightly lower C_{max} and later t_{max} values were observed for HMR123 following administration of

the capsule formulations in comparison to the solution formulation. This suggests a slightly lower rate of bioavail ability for the capsules, which would be anticipated owing to the time required for breakdown of the capsules to occur. The extent of absorption was also a little higher for the solution formulation, as evidenced by the AUC_{0-t} and AUC_{0-inf} data. After 8 h post dose, however, the median plasma concentration versus time curves were almost identical for all the three formulations investigated in this study. Statistical comparison of C_{max}, AUC_{0-t} and AUC_{0-inf} between the capsule formulations and the oral solution indicated that the confidence intervals for the parameter ratios were not fully contained within the 0.80 1.25 range: a slightly higher bioavailability was thus confirmed for the solution formulation. A statistical com parison of the two capsule administrations on the other hand showed the corresponding confidence intervals to be fully within the 0.80 1.25 range; i.e., the criteria for bio equivalence were met for these two capsule formulations.

Coefficients of variation on the primary pharmacoki netic parameters (reflecting inter subject variability) were generally about 30% for the two capsule formulations and about 25% for the oral solution. Slightly lower values were seen for the capsule filled with wet granules in comparison to the liquid filled capsules.

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B.16 Bioequivalence

Graham Lockwood · Robert Kringle

B.16.1 Introduction

Bioequivalence (BE) is a term used in pharmacokinetics to describe the similarity, or absence of any important difference, in the bioavailabilities (rate and extent of absorption) of the active ingredient/moiety between two pharmaceutical products.

Formal demonstration of BE between two drug products requires an appropriately designed study, followed by prescribed statistical data analysis procedures to quantify product comparability, along with prespecified acceptance bounds for these criteria.

Most commonly, BE is assessed in terms of comparability of the averages of pharmacokinetic parameters of the two products/formulations. Other BE approaches that additionally consider comparability of between subject variability or within subject variability of the two products have been developed (i.e., population BE and individual BE [US FDA 2001]), but are not covered in this chapter.

Extensive regulatory guidances exist regarding when and how to conduct BE studies from the (Food and Drug Administration 2000; Food and Drug Administration 2001; The European Medicines Agency 2008; Health Canada 1992, 1996; and the (Japanese Division of Drugs 1997). Although these guidelines are mostly consistent, some important differences do exist, so it is necessary to consult the relevant guidelines for the planned submission.

B.16.2 Study Designs

B.16.2.1 Standard 2×2 Crossover Design

The most common study design for a BE study is a two sequence two period two treatment crossover design (T = test formulation, R = reference formulation).

One important feature of the 2×2 crossover design is the duration of the washout between Periods 1 and 2. The purpose of the washout is to eliminate the potential influence of any residual drug concentration from Period 1 on the measured concentrations and calculated PK parameters in Period 2. Different residual effects from formulations T and R are known as "unequal carryover" and are problematic in the statistical data analysis procedures and conclusions. The best approach is to eliminate the possibility of any carryover by judicious study design (i.e., sufficient washout). A typical recommendation is that the washout should be at least five half lives of the compound.

Most regulatory guidances recommend to check for sufficiency of the washout by comparing the Period 2 predose concentration to the corresponding subject's Period 2 C_{max} value. If the Period 2 predose concentration is >5% of the Period 2 C_{max} value, then such a subject should be deleted from the data analysis.

B.16.2.2 Other Crossover Designs

When there are three formulations/treatments to be compared (e.g., two test formulations and one reference formulation), a crossover design with three periods is appropriate, using either a three sequence Latin square design, or a Latin square with all six possible sequences. The main advantage for the six sequence design is when the statistical analysis model includes an adjustment for unequal carryover. However, regulatory agencies prefer handling of carryover by proper design (see washout discussion above) rather than through statistical models that allow for unequal carryover. Therefore, a three sequence Latin square is usually preferable.

When there are four formulations/treatments to be compared, the optimal design depends on the number and type of comparisons of interest. If there are only two comparisons of interest (T1 versus R1, and T2 versus R2), then two separate 2×2 crossover designs are just as efficient (and simpler to administer in the clinic) as a single four period crossover. However, if additional comparisons are of primary interest (e.g., T1 versus T2), then a four period crossover design is more efficient than the two separate 2×2 crossover designs.

Another type of crossover design, called a replicate crossover, can be used to reduce the total number of

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required subjects. For example, instead of the 2×2 cross over design with N total subjects shown in Table B.16 1, an equally efficient design is a four period replicate design with M total subjects, where M is only half of N for the 2×2 design. One such design is shown in Table B.16 2. However, the total number of drug administrations (or clinic visits) is the same for the two designs (i.e., $2N=4M$).

Finally, for a drug that is highly variable (e.g., within subject CV > 30%), a partially replicated crossover design can be used, along with a special statistical analysis, called scaled average BE (Haidar et al. 2008). This will be discussed in more detail later in this chapter.

B.16.2.3 Parallel Designs

When the compound has a long half life, the length of each period and/or the washout may be so long that a parallel design is advantageous, in order to reduce potential dropouts and the entire study duration. However, a parallel design will require more total subjects than a crossover design.

B.16.3 Statistical Data Analysis and Interpretation

B.16.3.1 Statistical Analysis for the 2×2 Crossover

For the statistical analysis of the pharmacokinetic parameters (usually C_{max} and AUC), the conventional practice in the pharmaceutical industry is to analyze the natural log transformed values of the parameters. Pharmacokinetic rationales for the log transformation are provided in the FDA Guideline on Statistical Approaches to Bioequivalence, appendix D (US FDA 2001). The statistical analysis provides an estimate of the difference between arithmetic means ($\mu_T - \mu_R$) on the log scale, which after anti log retransformation provides an estimate of the ratio of geometric means on the original scale.

Table B.16-1
Standard 2×2 crossover design

Sequence	Number of subjects	Period 1	Washout	Period 2
T,R	N/2	T		R
R,T	N/2	R		T

Table B.16-2
A replicate crossover design

Sequence	Number of subjects	Period 1	Period 2	Period 3	Period 4
1	M/2	T	R	R	T
2	M/2	R	T	T	R

The basis for the decision regarding BE is a pair of statistical hypothesis tests, commonly known as the "two one sided testing (TOST) procedure," according to Schuirmann (1981):

$$H_{01} : \mu_T - \mu_R \leq \Delta_L \text{vs} H_{A1} : \mu_T - \mu_R > \Delta_L$$

$$H_{02} : \mu_T - \mu_R \geq \Delta_H \text{vs} H_{A2} : \mu_T - \mu_R < \Delta_H$$

where μ_T and μ_R are the unknown population means of the test and reference formulations, for the natural log transformed values, Δ_L is the lower acceptance boundary, with common default=log(0.80)= 0.223, and Δ_U is the upper acceptance boundary, with common default=log(1.25)=0.223.

The TOST procedure can be accomplished with two different, but equivalent, statistical methods. Both are based on fitting a statistical linear mixed model to the natural log transformed PK parameter values, Y_{ijk}:

$$Y_{ijk} = \mu_{(i,j)} + \gamma_i + \pi_j + \xi_{k(i)} + \varepsilon_{ijk}$$

where $\mu_{(i,j)} = \mu_R$ or μ_T, according to the formulation in the jth period (j=1 or 2) of the ith sequence (i=1 or 2), π_j is the fixed effect of the jth period, γ_i is the fixed effect of the ith sequence, $\xi_{k(i)}$ is the random between subject effect of the kth subject in the ith sequence$\sim N(0,\sigma_B^2)$, for k=1 to n_1 for sequence 1, and $k=1$ to n_2 for sequence 2, and ε_{ijk} is the random within subject effect in the jth period for the kth subject in the ith sequence$\sim N(0,\sigma_W^2)$.

To perform the TOST, one statistical method is to conduct two one sided t tests, for H_{01} and H_{02}, in the framework of the statistical model. If both null hypotheses are rejected at type 1 error rate of 0.05, then BE can be concluded. However, a more common statistical method to perform the TOST is to compute a 90% confidence interval (CI) for the difference between formulation means, in the framework of the statistical model:

$$\hat{\mu}_T - \hat{\mu}_R \pm t_{0.95,N\ 2}\hat{\sigma}_W\sqrt{2/N}$$

where $t_{0.95,\ N-2}$ is the 95th percentile of the t distribution with $N-2^{\circ}$ of freedom ($N=n_1+n_2$). The point estimate and the 90% confidence limits for the difference between formulation means are typically converted to the ratio scale by the anti log transformation. If this 90%

confidence interval on the ratio scale is entirely contained within 0.80 1.25, then BE can be concluded.

There are many commercial software packages that can be used to conduct this linear mixed model analysis to obtain the 90% confidence interval (SAS, Pharsight. WinNonlin). A software package that only allows a linear *fixed* effects model, instead of a linear *mixed* effects model, will be satisfactory (i.e., will provide results iden tical to the mixed model) as long as there are no subjects with a missing value. When one or more subjects have valid data in at least one period, but missing data in one or more periods, the linear fixed effects model will delete such subjects, whereas the linear mixed model will opti mally utilize all the available data.

EXAMPLE

The data for this example are C_{max} values from study Gen25a in the FDA database of bioequivalance studies, available on the FDA website (US FDA 2005). The study design was a 2×2 crossover of two formulations, A and B, of an antiarrhythmic compound. The number of subjects was N=24. The dataset is shown in Appendix 13.1.

The statistical analysis of $\log(C_{max})$ was conducted with SAS Proc Mixed (SAS 2008). Formulation A was considered as the reference, R. Results needed to compute the 90% confidence interval included:

$$\hat{\mu}_T - \hat{\mu}_R = -0.0127$$
$$t_{0.95,22} = 1.717$$
$$\hat{\sigma}_W = 0.109$$

Thus, the 90% CI for $\hat{\mu}_T - \hat{\mu}_R$ is

$$-0.0127 \pm 1.717 * 0.109 * \sqrt{2/24},$$

$$\text{or } -0.0666 \text{ to } 0.0412.$$

Applying the anti log transformation, the point esti mate and 90% CI for the ratio $\theta = \mu_T/\mu_R$ are:

$$pointestimate = 0.987, with\ 90\%CI = 0.936\ to\ 1.042.$$

Because this 90% CI is entirely within the conventional acceptance boundaries 0.80 1.25, BE is successfully demonstrated.

B.16.4 Sample Size Determination

An important consideration in the design of a BE study is the determination of the number of subjects, N. The choice of N is up to the sponsor. It allows the sponsor to control/minimize the probability of a type II error (prob ability that the study fails to show BE, when in fact the two formulations are truly bioequivalent). So the sponsor wants an N that provides a sufficiently high probability (power) that the study will yield a correct conclusion (demonstration of BE) when the formulations are indeed similar (i.e., the unknown true ratio of formulation means is equal to some value θ that is "close" to 1):

$$\begin{aligned}\text{Power} &= \text{Prob}\{90\%\text{CI for } \mu_T/\mu_R \text{ is wholly within} \\ &\quad 0.80 \text{ to } 1.25 | \mu_T/\mu_R = \theta\} \\ &= \text{Prob}\{(\hat{\mu}_T - \hat{\mu}_R \pm t_{0.95,N\ \ 2}\hat{\sigma}_W\sqrt{2/N}) \\ &\quad \subset (-0.223, 0.223) | \mu_T/\mu_R = \theta\}.\end{aligned}$$

Common values for the true ratio θ are 0.90, 0.95, 1.05, and 1.10 (i.e., allowing for a 5 10% true difference between formulation means). Common values for ade quate power are 80% and 90%.

One question that must be addressed is: What true ratio θ should be used to select N? Sometimes a previous/ pilot study comparing the two formulations is available, but, typically, the uncertainty associated with the point estimate is so large (i.e., the confidence interval around the point estimate is ±15% or more) that this point estimate is not very useful. Another common choice is to assume a small true difference, $\theta = 0.95$ or 1.05. However, this may often be too optimistic. Consider that, if the same formu lation was compared against itself (e.g., two different batches), there could be a true difference of several percent simply due to batch to batch manufacturing variation. So a more conservative choice would be to allow for a true difference of say 10%. The tradeoff is often between doing a larger study with more assurance of passing the BE criteria, versus doing a smaller study and running the risk that the study might need to be repeated and/or that the submission might be delayed.

Power also depends on the within subject standard deviation of the log(parameter), σ_W. Although the true value of σ_W is unknown, it can usually be estimated from previous crossover studies with the compound, as the square root of the residual variance from the ANOVA or mixed model analysis of log(parameter).

Sample size determination can be accomplished by using published tables (Hauschke et al. 1992) or commercial software (nQuery, SAS). Note that some software packages require as input the within subject coefficient of variation (CV_W) on the raw scale, rather than the within subject SD, σ_W, on the log scale. This can be calculated as

$$CV_W = \sqrt{\exp(\sigma_W^2) - 1}.$$

EXAMPLE

Assume that the within subject standard deviations of log transformed parameters from previous studies were:

$$\text{Log(AUC)}: \hat{\sigma}_W = 0.19, \text{with } 95\backslash\% \text{ CI } (0.15, 0.27)$$
$$\text{Log}(C_{max}): \hat{\sigma}_W = 0.23, \text{with} 95\backslash\% \text{CI} (0.18, 0.33).$$

The total number of subjects required for a 2×2 cross over BE study is shown in Table B.16 3, for various values of the true ratio $\theta = \mu_T/\mu_R$, various values of the true within subject standard deviation σ_W, and power of 80% and 90%.

Assuming a true σ_W of 0.25, to achieve 80% power for true ratio 0.90, 58 subjects are required; to achieve 90% power for true ratio 0.95, 38 subjects are required.

B.16.5 Onset of Action

When onset of action is a primary objective, one possibil ity for a primary endpoint is the partial AUC from zero to the population t_{max}. The planning (power and sample size) and data analysis procedures would be the same as described in Sect. B.16.3 for AUC.

Another option is to consider t_{max} as a primary end point. In general, t_{max} values (or log transformed t_{max} values) cannot be assumed to be normally distributed. Thus, instead of using the parametric statistical methods discussed so far in this chapter, it is necessary to use non parametric statistical methods. An appropriate non parametric approach is to log transform the t_{max} values, and then apply the Hodges Lehmann non parametric procedure to obtain a 90% confidence interval for the difference between formulation medians. Finally, the anti log transformation is applied to convert these confi dence limits to the ratio scale.

EXAMPLE

The data for this example are T_{max} values from Drug3 Dataset3 in the FDA database of bioequivalance studies, available on the FDA website (US FDA 2005). The complete study design was a four sequence four period crossover with two formulations, A and B, and N=36 subjects. However, to illustrate the Hodges Lehmann pro cedure for a standard 2×2 crossover, only Periods 1 and 2 from sequences ABBA or BAAB (that naturally formed a 2×2 crossover in Periods 1 and 2) alone were used for this example. This produced a 2×2 crossover design with N=18 subjects. The dataset is shown in Appendix 13.2.

To apply the Hodges Lehmann method for a 2×2 crossover, the following steps are followed:

1. Compute log(T_{max}).
2. Using the log(T_{max}) values, compute the period half differences for each subject = (Period 2 Period 1)/2.
3. Use a commercial software package for the non parametric Hodges Lehmann procedure, for parallel groups (Sequences 1 and 2 are the parallel groups), to obtain the 90% confidence for the difference between the parallel group medians (it can be shown that this is the same as the difference between the T and R medians in the crossover).

This analysis was performed with SAS Proc NPAR1WAY (SAS v9.2, 2008). Formulation B was considered as the reference, R. Results from SAS Proc NPAR1WAY were:

Point estimate of T R medians = −0.1116.

90% confidence interval (exact method): −0.3466 to 0.0589.

4. Finally, apply the anti log transformation to the above point estimate and 90% CI, to obtain the point estimate and confidence for the ratio of T_{max} medians (T/R). For this example:

$$point estimate = 0.894, with \quad 90\%\text{CI} = 0.707 to 1.061.$$

Because this 90% CI is not entirely within the conven tional acceptance boundaries 0.80 1.25, BE was not suc cessfully demonstrated.

Table B.16-3

Sample size required for 2×2 crossover BE study. Type 1 error=5%

	Power=80%			Power=90%		
$\theta = \mu_T/\mu_R$	σ_W=0.20	σ_W=0.25	σ_W=0.30	σ_W=0.20	σ_W=0.25	σ_W=0.30
0.90	38	58	82	52	80	114
0.95	20	28	40	26	38	54
1.00	16	24	34	20	30	42
1.05	18	28	40	26	38	54
1.10	32	50	70	44	68	96

B.16.6 Highly Variable Drugs

B.16.6.1 The Problem with Highly Variable Drugs

Highly variable drugs/products (HVD) have a within subject CV greater than 30%. A problem with such drugs is that the number of subjects required in a conventional 2×2 crossover can be extremely large, as shown in Table B.16 4.

One solution to reduce the number of subjects is to use a four period replicate crossover design, as described in Table B.16 2. The four period replicate crossover design requires only half as many subjects as the 2×2 crossover, but the total number of drug administrations/clinic visits will be the same as for the 2×2 crossover.

B.16.6.2 Two-Stage (Two-Group) Sequential Design

A potentially more efficient approach for HVDs is to use a two stage (two group) sequential design. This approach consists of conducting a 2×2 crossover in two sequential groups of $N1$ and $N2$ subjects, separated by an interim statistical analysis after the first group of $N1$ subjects. Based on the results of this interim analysis:

1. If BE has been demonstrated, the study is stopped after the first group.
2. If BE has not been demonstrated, then either
   (a) If BE appears to be highly unlikely (e.g., the point estimate of the ratio is outside 0.80 1.25), then the study is stopped.
   (b) Otherwise, a second group of $N2$ subjects is enrolled, and the final statistical analysis is conducted on the combined $N1+N2$ subjects.

There are several statistical issues, depending on the relevant regulatory guideline(s), that must be considered when using the two group sequential design approach. In the most rigorous application (required by the FDA guide line and the draft EMEA guideline), the two group sequential approach must be prospectively planned. The main impact is that, an adjustment must be made to the type 1 error rate at each of the two analyses (interim and final), in order to prevent inflation of the overall type 1 rate due to two "looks" at the data. One simple and conservative way to accomplish this is to construct a 95% confidence interval (instead of a 90% confidence interval) for each analysis (interim and final).

To illustrate the advantage of two group sequential approach, suppose the true within subject standard is assumed to be 0.40. According to Table B.16 4, the num ber of subjects required to have 90% power when the true ratio is a conservative 0.90 is $N=200$ subjects, for the stan dard 0.05 type 1 error rate. With a two group sequential design, sample size must be computed with a 0.025 type 1 error rate, for $N1$ of the first group, and then for $N=N1+N2$. The first group might be powered assuming a less con servative true ratio of 0.95, which would require only $N1=88$ (power=80%) or 116 (power=90%). To plan for the required $N2$, in case BE is not demonstrated after the first group of $N1$ subjects, allowing now for the more conservative true ratio 0.90, the required total sample size is $N=246$.

Some regulatory agencies do not require prospective planning, or adjustment of the type 1 rate, to use what is called an "add on" group/study of subjects when BE was not demonstrated after an initial 2×2 crossover design. To use the combined dataset (original group plus add on group), the Canadian guideline (1992a, Part A) requires two tests for consistency:

1. *Equality of residual mean squares (MSE)*: If $F=MSE_{larger}/MSE_{smaller}$ is greater than the upper 5% value of the F distribution, then the two studies cannot be combined.
2. *Formulation by study interaction*: A mixed model is fit with terms for study, subject within study, period within study, formulation, and formulation by study interaction. If the p value for the formulation by study interaction is less than 0.05, then the two studies cannot be combined.

The Japanese guideline (Japan 1997) has no require ments to enroll and combine an add on group, other than

Table B.16-4

Sample size required for 2×2 crossover BE study for HVDs. Power=90%, Type 1 error=5%

$\theta = \mu_T/\mu_R$	σ_W=0.30	σ_W=0.40	σ_W=0.50	σ_W=0.60	σ_W=0.70
0.90	114	200	312	446	608
0.95	54	96	148	212	288
1.00	42	72	112	158	216

Table B.16-5

Comparison of sample size required for BE and SABE for HVDsPower=90% for true ratio=0.90

Design	Method	σ_{WR} 0.30	0.35	0.40	0.45	0.50	0.60	0.70
Two-period	BE	114	154	200	252	312	446	608
Three-period	BE	87	117	150	192	234	336	456
	SABE	87	60	51	51	51	72	90

that the add on group of $N2$ subjects must be at least 50% of the original $N1$ subjects.

B.16.6.3 Scaled Average BE

A third and more efficient approach for HVDs is scaled average bioequivalence (SABE) (Tothfalusi et al. 2001; Haidar et al. 2008). The SABE approach has been recommended by the FDA, with rationale, study design and data analysis requirements provided in Haidar et al. (2008).

> . . . applying the conventional BE criteria to highly variable drugs/products may unnecessarily expose a large number of healthy subjects to a drug when this large number is not needed for assurance of BE.
>
> For these reasons, scientists and statisticians at the US FDA investigated various approaches available for determining BE that would reduce the sample size required for a BE study, without allowing for therapeutically inequivalent products to reach the market.

Additionally, FDA draft guidances for two drug products (Lansoprazole 2008, and Lovastatin/niacin 2008) specifically include the recommendation/option for using SABE.

Conceptually, SABE consists of expanding the default 0.80 1.25 acceptance boundaries, by a function of the within subject SD, σ_W:

Default 0.80 1.25 bounds=exp(± 0.223)
Expanded bounds=exp(± 0.223 (σ_W/σ_o)),

where σ_o is a regulatory specified constant=0.25 (Haidar et al. 2008).

The statistical analysis/testing for SABE is actually more complicated than simply computing/estimating the expanded acceptance bounds, because the uncertainty in estimating σ_W must be accounted for (Tothfalusi et al. 2001;Haidar et al. 2008). An example of the statistical analysis is included in Appendix 13.3.

The FDA recommendations for the SABE study design and data analysis are as follows (Haidar et al. 2008).

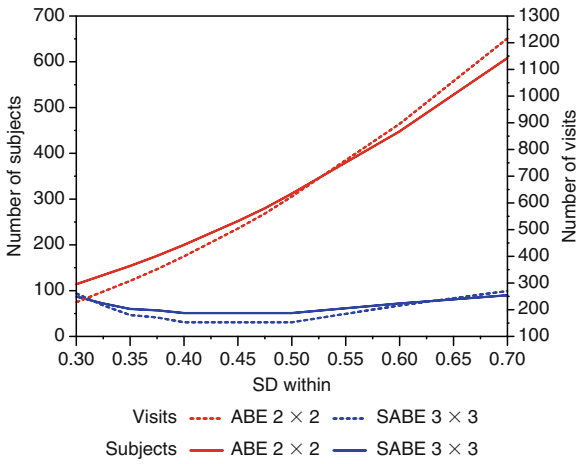

Figure B.16-1

Comparison of average BE (ABE) and SABE number of subjects and number of clinic visits Power=90% for true ratio=0.90

Study design

Three period partial replicate with three sequences RRT/RTR/TRR.

$N \geq 24$ subjects.

Data analysis

$\sigma_o=0.25$.

σ_W is estimated from the reference replicates in the mixed model = $\hat{\sigma}_{WR}$.

If $\hat{\sigma}_{WR} > 0.30$, SABE analysis can be used (otherwise, conventional BE analysis must be used).

An additional constraint is that the point estimate of $\theta = \mu_T/\mu_R$ must be within 0.80 1.25.

The benefit of SABE over conventional BE, using either a two or three period design (TRR/RTR/RRT) for conventional BE, is clearly evident in the much smaller number of required subjects, as shown in *Table B.16 5*. Sample size for SABE was determined by simulation.

For SABE, for true $\sigma_{WR}<0.40$, N increases as σ_{WR} decreases toward 0.30. This is because there is some non negligible probability that the observed $\hat{\sigma}_{WR}$ will be

$\leq$0.30, in which case the standard BE analysis with 0.80 1.25 acceptance bounds must be used instead of the SABE analysis.

For SABE with true $\sigma_{WR} > 0.50$, N increases because of the constraint on the point estimate to be within 0.80 1.25.

Fig. B.16 1 shows the sample sizes from *Table B.16 5*, along with the corresponding number of drug administrations/clinic visits. Although the SABE approach requires three periods, as opposed to only two periods with standard BE, the total number of drug administrations is still much smaller with the three period SABE approach.

Appendix B.16.1. C_{max} values from FDA database: study Gen25a

		Formulation A		Formulation B	
Subject	Sequence	Period	C_{max}	Period	C_{max}
1	2	2	4.03	1	4.00
2	1	1	2.45	2	1.96
3	2	2	1.44	1	1.21
4	1	1	1.99	2	1.41
5	2	2	2.05	1	1.87
6	1	1	3.76	2	4.58
7	2	2	2.84	1	2.37
8	1	1	3.12	2	3.04
9	2	2	1.78	1	1.70
10	2	2	2.39	1	2.58
11	1	1	2.48	2	2.22
12	1	1	2.05	2	1.87
13	2	2	1.79	1	2.47
14	2	2	1.87	1	2.06
15	1	1	2.18	2	2.26
16	1	1	1.92	2	1.99
17	1	1	1.66	2	1.93
18	2	2	2.54	1	2.13
19	2	2	1.90	1	1.98
20	1	1	1.52	2	1.81
21	1	1	1.65	2	1.58
22	1	1	1.71	2	1.60
23	2	2	1.47	1	1.62
24	2	2	1.60	1	1.68

Appendix B.16.2. T_{max} values from FDA database: Drug3 Dataset3

		Period	
Sequence	Subject	1	2
AB	1	6	4.5
AB	5	6	7.5
AB	10	3	7.5
AB	13	6	6
AB	17	4.5	4.5
AB	23	12	6
AB	25	4.5	7.5
AB	30	4.5	10.5
AB	33	9	6
BA	4	6	4.5
BA	7	4.5	4.5
BA	9	6	6
BA	16	7.5	7.5
BA	20	4.5	6
BA	21	4.5	4.5
BA	28	7.5	4.5
BA	31	6	6
BA	34	9	4.5

Appendix B.16.3. Example of SABE Analysis

The data for this example are C_{max} values from Drug7 in the FDA database of bioequivalance studies, available on the FDA website (US FDA 2005). The study design was a four sequence four period crossover with two formulations (Test and Reference), and N=22 subjects.

For the standard BE analysis, the point estimate was 1.12 with 90% CI of (0.96, 1.30), so BE was not demonstrated by the standard BE approach.

The estimated within subject SD for the reference formulation was $\hat{\sigma}_{WR} = 0.48$, so clearly this drug was highly variable. For illustration of the SABE statistical analysis/testing procedure, we re analyzed this dataset using the FDA recommended procedures (Haidar et al. 2008). Note that although this study design (four period design) is different from the FDA recommended three period design, the SABE statistical analysis and testing procedures are identical for these two designs.

The statistical test for SABE is to compute a one sided upper confidence limit for

$$(\mu_T - \mu_R)^2 - (log(1.25)/\sigma_o)^2 * \sigma^2{}_{WR},$$

on the log scale. This upper confidence limit must be less than zero to conclude BE. This upper confidence limit is calculated as (Tothfalusi et al. 2001):

$$\mathrm{UCL} = \mathrm{Em} + \mathrm{Ew} + (\mathrm{Lm} + \mathrm{Lw})^{1/2}$$

where

$$\mathrm{Em} = (\hat{\mu}_T - \hat{\mu}_R)^2$$

$$\mathrm{Ew} = -(\log(1.25)/\sigma_o)^2 \hat{\sigma}^2_{\mathrm{WR}}$$

$$\mathrm{Cm} = [\mathrm{Abs}(\hat{\mu}_{\mathrm{T}} - \hat{\mu}_{\mathrm{R}}) + t_{1\ \alpha,df1} * \mathrm{SE}]^2$$

$$\mathrm{Cw} = -(\log(1.25)/\sigma_o)^2 * df2 * \hat{\sigma}^2_{\mathrm{WR}}/\chi^2_{1\ \alpha,df2}$$

$$\mathrm{Lm} = (\mathrm{Cm} = \mathrm{Em})^2$$

$$\mathrm{Lw} = (\mathrm{Cw} = \mathrm{Ew})^2$$

All the quantities needed for calculating UCL can be obtained from a mixed effects model analysis.

Proc Mixed code in SAS®

Proc mixed;Class subj seq per trt;Model logC_{max}= seq per trt/ddfm=kr;Random trt/subject=subj (seq) type = csh;Repeated/group=trt subject=subj(seq); Estimate "diff" trt −1 1/cl alpha=0.10;

Proc Mixed results

Em=0.1095^2 Ew = $-(0.223/0.25)^{2}$*0.2277 Cm= 0.2649^2 Cw = $-(0.223/0.25)^2$ *(22−4)*0.2277/28.9

The upper 95% confidence bound UCL = −0.08, which is less than zero, so BE by the SABE approach has been demonstrated.

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B.17 Population Pharmacokinetics in Clinical Pharmacology

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B.17.1 Part 1: Role of Population Pharmacokinetics in Clinical Pharmacology

B.17.1.1 Optimizing the Dose Regimen

Clinical pharmacology is the science of drugs and their clinical use. One of the main goals of clinical pharmacology is to improve the safety and efficacy of any drug therapy. To optimize the dose regimen, the relationship between dose, concentrations, and response is investigated in more detail. One of the early results of these investigations was that most drugs obtain an optimal therapeutic response within a limited range of concentrations. To achieve the target response in each patient, his/her dose regimen should be adjusted appropriately to achieve this therapeutic target concentration range. To confirm that the target concentrations had been achieved, a few concentrations were measured per patient at predefined time points. And indeed, when clinical pharmacologists introduced such therapeutic drug monitoring (TDM) as therapy control, safety and efficacy of the drug therapy were improved.

The data obtained in TDM for a single patient, however, was too sparse for calculating his/her individual pharmacokinetic (PK) parameters. The individual clearance (CL) estimate allows the calculation of the dose rate ($\dot{D}$) required to achieve a predefined target concentration during steady state. The challenge was how to calculate the individual PK parameters with only a few observations at hand? To answer this question, a new approach had to be introduced.

The original idea of population pharmacokinetics (PopPK) was to utilize the sparse individual PK data obtained routinely in many patients to describe directly the patient population as primary unit of analysis. Each individual patient contributed only sparse and unbalanced PK data. To calculate the typical PK of the patient population, the new method had to evaluate the TDM data using a hierarchical model consisting of a subject specific level and a population or between subject level. To deal with the random effects of both, within subject variability (WSV) and between subject variability (BSV), a more complex nonlinear mixed effects modeling (NONMEM) method was required. The result of a NONMEM analysis of TDM data describing the typical PK and its variability within the target population of patients was first published by Sheiner et al. (1977).

With the PopPK results available as prior information, the limited observations per subject obtained during the TDM are evaluated using the Bayesian approach. The Bayesian approach estimates the most probable individual parameters of the patient. The obtained CL and volume of distribution (V) parameter estimates were used to calculate the maintenance dose rate, the dose interval, and the loading dose, which will achieve the optimal individual target exposure of the drug.

B.17.1.1.1 Sources of Pharmacokinetic Variability

The PopPK reasonably assumes that the PK is similar between the subjects of the target population. A common PK model would describe the subject specific PK with individual parameters changing with variable physiological conditions of the patients. On the population level, the NONMEM approach estimates the variability of the PK parameter vector between the subjects. The PK parameter vector is estimated only for the typical patient. Avoiding estimating PK parameter vectors for all individual patients dramatically reduces the total number of parameter estimates. Less parameter estimates result in more degrees of freedom in the regression and, in turn, lead to more precise parameter estimates. The individual PK estimates are modeled as a random variable with a multivariate probability density function (PDF), most often a logarithmic normal distribution function. The deviation $\eta_i = P_i - \bar{P}$ between the individual PK parameter vector P_i and the PK parameter vector $\bar{P}$ of a typical subject is obtained from a random distribution with a zero mean and a multivariate covariance matrix Ω as the random effect.

On the subject level, a single draw of a vector P_i from the parameter distribution is used to calculate the subject

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specific model prediction $C_{\text{pred}}(t_j, P_i)$ at time t_j. The deviation $\varepsilon_{i,j} = C_{i,\text{obs}}(t_j) C_{i,\text{pred}}(t_j, P_i)$ between the individual observations and their model predictions is obtained from a random individual error distribution. Most often, a normal distribution with a mean of zero and a covariance matrix of σ is used for the individual ε error. Compartment models are most often used to describe the PK on the subject level. All deviations η_i and $\varepsilon_{i,j}$ are independent, identically distributed random variables. When fixed effect parameters $\bar{P}$ and random effect parameters Ω and σ are used in a model, the model is called a mixed effect model. Because the subject specific PK is nonlinear with time, the following nonlinear mixed effect model (NONMEM) is used:

$$\begin{aligned} \text{Subject} &: C_{i,\text{obs}}(t_j) = C_{\text{pred}}(t_j, P_i) + \varepsilon_{i,j} \\ & \varepsilon_{i,j} \sim N(0, \sigma) \\ \text{Population} &: P_i = \bar{P} e^{\eta_i} \\ & \eta_i \sim N(0, \Omega) \end{aligned} \qquad (B.17.1)$$

Generally, we have to differentiate between the real human physiological system and the virtual PopPK model. The observed system response to a dose will be compared with the predicted model response to the same dose. From a variety of models, the best model is chosen, when the model predictions describe the observed data as best as possible using a minimum number of parameters (Boeckmann et al. 1994).

B.17.1.1.2 The Population Approach

The situation in large clinical studies is similar as in TDM. We investigate the drug effects, i.e., the efficacy and adverse events (AE), in a large number of patients. These patients are sampled from the total population of patients with the target disease. The typical values and the variability of the drug properties of the sample are assumed to be representative for the whole patient population.

The population approach uses a statistical model to describe the typical behavior of the patient population. The mean and variability of the model parameter of a population can be estimated in a single evaluation step. The parameter error decreases with increasing number of patients. Increasing the number of blood samples taken per patient is less important, because an individual parameter does not need to be estimated.

To optimize the drug treatment, we need to know the relationship between the exposure and the drug effects to select the right dose. Again, we need to know the individual PK in a large number of patients and we should apply a TDM like approach.

However, in phase I of the drug development (DD), the first studies are most often performed in healthy volunteers instead of patients in the target population. At least in the first in man (FIM) study, there is no prior information on the PK in man. To estimate the individual PK parameter in this situation, a dense sample regimen is required. The burden of a dense sample regimen may only be justified in healthy volunteers.

In contrast to TDM and to the large clinical trials, we obtain many PK observations per subject in the few subjects enrolled in a phase I study. Classically, the PK data of each subject is separately evaluated in the first step, and the group mean parameter and its variance is calculated in the second step. At the beginning of phase I, the use of low starting doses leads frequently to a variable number of concentrations below the limit of quantification (LOQ). At the same sampling time, some subjects may have concentration values below LOQ, while others have a concentration which can be precisely measured. As a result, the amount of PK data per subject differs between subjects. Analyzing such unbalanced data leads to a variable precision of the individual parameter estimates. Therefore, the second step requires calculating a weighted mean for the typical PK parameter estimate.

In instances when phase I data are strongly unbalanced, an additional late terminal PK phase may be observed in only a fraction of the study population. In the other subjects, the concentrations of the late terminal PK phase cannot be observed, because they are below LOQ. Only the use of the NONMEM approach allows the appropriate summary of such data using a common PK compartment model for all the subjects (Schoemaker and Cohen 1996).

B.17.1.1.3 Prior Knowledge for Individual PK Estimates

With the availability of a PopPK model as prior knowledge for the Bayesian approach, even sparse individual data are sufficient to calculate the most probable estimate of the individual PK parameters. The Bayesian approach can be applied even in subjects with fewer observations than PK parameters in the model, i.e., when the classical two step approach would fail (Sheiner and Beal 1982).

Without any observations, the most probable estimate of the individual PK parameter in the ith subject is given as the fixed effect parameter vector $P_i = \bar{P}$ of the

typical patient. With a single observation $C_{obs}(t_{i,1})$, the fixed effect parameter vector may result in a model prediction $C(t_{i,j}, P_i)$ far away from the observed concentration $C_{obs}(t_{i,1})$. Allowing some deviation $\eta_i = P_i - \bar{P}$ for the individual parameter P_i will probably reduce the deviation between the observation and its prediction $\varepsilon_i = C_{obs}(t_{i,j}) - C(t_{i,j}, P_i)$. In the case of several observations obtained in the ith subject, we are looking for a compromise between two squared sums, i.e., the sum of the weighted deviation $z_{\eta i} = \eta_i/\omega$ for each PK parameter and the sum of the weighted deviation $z_{\varepsilon i} = \varepsilon_i/\sigma$ for each observation.

We can iteratively estimate the individual PK parameter vector P_i by maximizing the joint probability for the η vector and ϵ vector, which is given as:

$$-\log(\text{Likelihood}) \sim \sum_i \left(\frac{lnP_i - ln\bar{P}}{\omega}\right)^2 + \sum_{i,j} \left(\frac{C_{obs}(t_{i,j}) - C(t_{i,j}, P_i)}{\sigma}\right)^2 \quad (B.17.2)$$

Both summands consist of dimensionless, z transformations as typical variables of standardized probability distributions (see Eq. B.17.13). The sum of squared z variables is χ^2 distributed:

$$\chi^2 = \sum_i z_{\eta_i}^2 + \sum_{i,j} z_{\varepsilon_{ij}}^2 \quad (B.17.3)$$

The best Bayesian estimator of the individual PK parameter vector is defined by the minimal χ^2 value and can be estimated using maximum likelihood estimation, e.g., the post hoc estimate in the NONMEM program.

B.17.1.2 Relationship Between Dose and Clinical Success

B.17.1.2.1 Confirming Efficacy of a Drug

When two groups of patients were randomly selected from the same target population and treated with a dose of either a drug or a placebo, we may observe the target drug effect in both groups. The efficacy of the drug is confirmed only if the efficacy was significantly higher in the drug group than in the placebo group.

If several doses of the drug are tested, the classical statistic approach will sequentially test each drug dose group against the single placebo group. The NULL hypothesis H0 assumes no drug effect at all. This approach requires no modeling of a possible drug effect. The size of any drug exposure plays no role using these tests.

B.17.1.2.2 Relationship Between Concentration and Clinical Success

In contrast to the classical statistical approach, a dose dependent drug effect is assumed in DD. Using PopPK and the post hoc Bayesian approach, as a first step we can calculate the relationship between the dose regimen and the concentrations. In the second step, we build a relationship between the concentrations and the drug effects.

In the case of a long term multiple dose treatment, the drug exposure is approximated by the concentration at steady state (C_{ss}). The relationship between the dose rate $\dot{D}$ and C_{ss} is a simple algebraic proportionality with the CL/F random variable as the proportionality constant:

$$\dot{D} = C_{ss,i} CL/F \quad (B.17.4)$$

During steady state, the elimination rate must be equal to the drug input rate. Equation B.17.4 is the basis of the TDM and is generally used to calculate the maintenance dose leading to the target concentration C_{ss}.

After treating with a fixed dose rate, let n_1 patients out of a sample of N patients reach the success criteria. Then, the observed success rate of n_1/N is the expectation value E of the true success probability P_{succ} in the sample of patients, conditional for the given $\dot{D}$, the CL/F distribution, and the attendant circumstances (... of the drug investigation.

$$E(P_{succ}(\dot{D}, CL/F, ...)) = \frac{n_1}{N} \quad (B.17.5)$$

The success rate $P_{obs} = \frac{n_1}{N}$ observed in the treatment group is the expectation value of the true success probability P_{succ} in the target population. P_{succ} is caused by the sum of two effects, i.e., the placebo effect P_0 and the drug effect P_D. The drug effect P_D cannot be observed alone, but an expectation value of the placebo effect P_0 is observed directly in the placebo control group.

Even the highest possible dose rate may still fail to achieve the desired clinical effect in all patients. Such patients, summarized as non responders, limit the response rate in the study population to a maximum response rate P_{max}.

The relationship between drug exposure and the true success rate P_{succ} must describe the sigmoid transition

between both boundary limits, i.e., between the minimum success rates P_0 and the maximum response rate P_{max} (Collett 2003).

$$P_{succ} = P_0 + (P_{max} - P_0)P_D(x)$$
$$x = \left\{ C_{ss} = \frac{\dot{D}}{CL/F} \right\} \qquad (B.17.6)$$

$P_D(x)$ is the probability distribution describing the frac tion of patients responding to a specific C_{ss} level of the drug. The following sigmoid Hill function is frequently used for describing the relationship between the probabil ity $P_D(x)$ of a response to the drug level:

$$P_D(x) = \frac{x^S}{x^S + x_{50}^S} \qquad (B.17.7)$$

x_{50} is the predictor variable at half maximum drug response and S is the slope of the transition function. $P_D(x)$ describes the transition from zero to unity, i.e., from no drug response when the exposure is small $x \to -\infty$ to full drug response when the exposure is high $x \to +\infty$. When no information on CL/F is avail able, the Hill equation is often used with the dose rate alone as the predictor variable. The PopPK model enables us to obtain a more subject specific exposure estimate. During steady state after multiple dose therapy, C_{ss} as the ratio between the dose rate and the individual CL/F value is the better predictor variable.

As will be shown below, the Hill equation is closely related to the logistic distribution. The odds ratio, i.e., the ratio between success rate p and failure rate $1-p$ is defined as:

$$\text{odds} = \frac{p}{1-p} \qquad (B.17.8)$$

Inserting the Hill equation 14.7 for $p = P_D(x)$ in the odds ratio and doing some algebraic manipulations, we achieve an S power function of the normalized predictor x/x_{50} given in the following equation:

$$\text{odds} = \left(\frac{x}{x_{50}}\right)^S \qquad (B.17.9)$$

The logarithm of the odds ratio is a random variable, which follows a logistic distribution.

$$\text{logit}(p) = \log(\text{odds}) \qquad (B.17.10)$$

The logit(P_D)for the drug effect is a linear logarithmic function with the individual $x = C_{ss,i}$ as predictor. The logistic distribution uses $x_{50} = EC_{50}$ as location and the inverse slope as scale parameter ω_S.

$$\text{logit}(P_D) = \frac{\log(C_{ss,i}) - \log(EC_{50})}{\omega_S} \text{ with } \omega_S = \frac{1}{S} \qquad (B.17.11)$$

The link function between the linear predictor function logit(P_D)and the observed success rate P_{succ} is given as:

$$P_{succ}(x) = P_0 + \frac{P_{max} - P_0}{1 + e - \text{logit}(P_D(x))} \qquad (B.17.12)$$

The parameters P_0, P_{max}, EC_{50}, and ω_S can be estimated simultaneously using software like NONMEM (Boeckmann et al. 1994), allowing maximum likelihood estimation for a binomial likelihood function B(N, P_{succ}).

B.17.1.2.3 Success Rate Depending on *CL/F* and Dose Rate

Dimensionless, standardized Z random variables are use ful for random distributions such as the normal distribu tion and the logistic distribution.

$$z = \frac{x - \mu}{\sigma} \qquad (B.17.13)$$

where z is an element of a random variable Z, which has a zero mean and a unit variance, and is the inverse func tion of a standard probability distribution. The standard distribution may be the normal or logistic distribution and z is called the probit and logit, respectively.

$$\text{Prob}(Z \le z) = p \qquad (B.17.14)$$

The probabilities p of the standardized random distribu tions of the z values are tabulated in the statistical litera ture. Using the standard random distribution of z, we can easily obtain the random distribution of the x random variable:

$$x = \mu + z\sigma \qquad (B.17.15)$$

A logarithmic z transformation is apparent already in Eq. B.17.11 for the logit of the drug effect P_D. A loga rithmic z transformation is also apparent in Eq. B.17.2 for the logarithmic standard normal distribution for the PK parameters (left summand). For the CL/F parameter, the logarithmic z transformation is given as:

$$\text{probit}(CL/F) = \frac{\log(CL/F) - \log(\overline{CL/F})}{\omega_{CL}} \qquad (B.17.16)$$

An analytical expression for the inverse function of a standard normal distribution, i.e., the probit, is still missing in statistical literature. Only statistical tables are available for probits.

The therapeutic goal consists of achieving a predefined fraction of the maximum possible drug effect:

$$\text{Prob}(\text{logit}(P_D) \leq 1.39) = 80\% \qquad (B.17.17)$$

in a predefined fraction of the patient population:

$$\text{Prob}\big(\text{probit}(P_{CL/F}) \leq 0.84\big) = 80\% \qquad (B.17.18)$$

To select an appropriate maintenance dose rate for the target population in order to obtain this therapeutic goal, the following two random distributions are involved:

- The logistic distribution of the fraction of the maximum drug response rate P_D with EC_{50} as location, ω_S as scale parameter and with the individual C_{ss} levels as predictor and
- The logarithmic normal distribution of the frequency of the individual CL/F with $\overline{CL/F}$ as location and ω_{CL} as scale parameter

The logarithmic transformation of the dosing rule for the maintenance dose given in Eq. B.17.4 results in a sum of the logarithm of the two random variables $C_{ss,i}$ and $(CL/F)_i$ as follows:

$$\log(\dot{D})_i = \log(C_{ss})_i + \log(CL/F)_i \qquad (B.17.19)$$

Using Eq. B.17.19, both random variables can be expressed as a function of their logit/probit, their mean, and their variance.

$$\log(\dot{D}) = \log(EC_{50}) + \text{logit}(P_D)\omega_S + \log(\overline{CL/F}) + \text{probit}(P_{CL/F})\omega_{CL/F} \qquad (B.17.20)$$

The inverse functions, i.e., the $\text{logit}(P_D)$ and the $\text{probit}(P_{(CL/F)})$ are given in Eqs. B.17.11 and B.17.16, respectively.

Rearranging Eq. B.17.20, a linear relationship between the $\text{logit}(P_D)$ and $\text{probit}(P_{CL/F})$ with a negative slope is obtained for any fixed dose rate $\dot{D}$.

$$\text{logit}(P_D) = \frac{1}{\omega_S}\log\left(\frac{\dot{D}}{EC_{50}\,\overline{CL/F}}\right) - \frac{\omega_{CL/F}}{\omega_S}\text{probit}(P_{CL/F}) \qquad (B.17.21)$$

The location $\overline{CL/F}$ and the scale $\omega_{CL/F}$ of the CL/F distribution is one of the most important results of a PopPK analysis. Furthermore, the PopPK model is the prerequisite for applying the Bayesian (post hoc) approach, which allows the calculation of the individual steady state concentration C_{ss}. The individual C_{ss} is required to estimate the relationship between C_{ss} and the responder rate, leading to the important estimates of the drug potency EC_{50}, the maximum drug effect P_{max}, and the scale ω_S of the responder rate.

B.17.1.3 Conclusion

The PopPK is always the method of choice, when we need to know the PK in many subjects. The PopPK approach avoids the need to repeat an extensive PK sampling in many subjects. This has several advantages:

1. Drawing numerous blood samples is an unnecessary burden for the patient.
2. The extensive investigation of each subject within a large study population is an unnecessary waste of resources in terms of cost and time for the investigator.

At the end of a successful DD, the pooled PopPK analysis of all the data obtained during DD will enable us to find:

1. The target concentration range associated with the target drug effect and
2. The optimal dose regimen to achieve this target concentration range

Such optimal dose selection should improve the probability of a successful outcome of a confirmatory phase III study and improve the clinical use of new drugs.

B.17.2 Part 2: Clinical Use of Leflunomide

B.17.2.1 Introduction

Leflunomide has been used for almost 12 years in the treatment of patients with rheumatoid arthritis (RA). During the DD, the efficacy of doses between 5 and 25 mg daily (QD) was tested. In a dose finding Phase IIb study, three QD doses of 5, 10, and 25 mg were tested against placebo. Each dose group enrolled approximately 100 patients. The study duration was 6 months. The clinical success rates in the 25 and 10 mg dose groups were significantly higher than the success rate in the placebo group. The success rate in the 5 mg group was not different from the placebo group. Because the frequency of increased liver enzymes was slightly increased in the 25 mg group, a slightly reduced dose of 20 mg QD was tested in Phase III (Weber and Harnisch 1997; Rozman 2002).

The study design in phase III allowed the physicians to reduce the dose from 20 to 10 mg daily. However, at the end of the study only a small subgroup of patients were treated with 10 mg. To clarify the potential role of a 10 mg dose, a postmarketed study tested efficacy and safety of 10 mg against 20 mg in two groups of 200 patients. The study

duration was 12 months. This randomized, controlled trial showed numerically (not significantly) better efficacy and fewer dropouts due to lack of efficacy or adverse events in patients receiving 20 mg QD compared with 10 mg QD. The outcome of the postmarketing study confirmed the recommendation that the maintenance dose is 10 20 mg QD (Horn and Oed 2003; Poor and Strand 2004).

The PopPK model obtained during the DD was reconfirmed using the new PK data of the postmarketed study (Weber and Rueppel 2004). The relationship between C_{ss} and clinical outcome has been reevaluated using the pooled Phase II and Phase III clinical data (Weber and Harnisch 2002). In the following section, the fixed effect and random effect models for the apparent clearance *CL/F* was used to investigate the predicted prob ability of success for 10 and 20 mg daily doses in the target population of patients with RA.

We used the PopPK approach and the extended clin ical data to investigate the relationship between the dose rate and the binary clinical outcome.

The following steps led to a scientifically justified dose recommendation:

1. The first step was to build a refined PopPK model quantifying the fixed and random effects on the parameter model.
2. The full power of the approach was obtained, when the results of the PopPK analysis were further used as prior knowledge for the Bayesian approach to estimate the individual PK in all patients of the clinical study.
3. The individual PK estimates allowed us to investigate the relationship between the probit of the *CL/F* distri bution and the logit of the probability of success con ditional on the dose rate.
   - Based on the relationship between the probit of the *CL/F* distribution and the logit of the probability of success conditional on the dose rate given in Eq. B.17.21, a dose recommendation was calcu lated to obtain 80% of the maximum drug effect in 80% of the patients (see Eqs. B.17.17 and B.17.18).

B.17.2.2 Population Pharmacokinetics of Leflunomide

B.17.2.2.1 Subject Level of the Pharmacokinetic Model

The PK response to a single extra vasal drug input (po) is often described by a simple Bateman function:

$$C(t) = \frac{\text{Dose} \times F}{CL} \frac{\left(e^{-t/\tau_c} - e^{-t/\tau_a}\right)}{\tau_c - \tau_a} + \varepsilon$$
$$\tau_c = \frac{V/F}{CL/F} \qquad (B.17.22)$$

The residual error ε contained an additive $\sigma_{\text{mg/L}}$ and a relative error term $\sigma_{\%}$.

The PK parameter vector consists of an absorption part defined by the bioavailability F and the absorption time constant τ_a and an elimination part defined by CL and V. However, when data are not obtained from inves tigating the absorption or the elimination separately, we cannot differentiate between the time constants τ_a and τ_e. However, two time constants τ_1 and τ_2 can be estimated. The terminal half life is given as:

$$t_{1/2,z} = \max(\tau_1, \tau_2)\log(2) \qquad (B.17.23)$$

For leflunomide, the terminal half life is approximately 2 weeks. The initial half life is approximately 1 h. Because the intestinal passage after oral intake is no longer than 2 or 3 days, an absorption time constant of 2 weeks is impossible, i.e., the 1 h half life must be the absorption half life. As a consequence, the PK of leflunomide must be elimination rate limited, i.e., the terminal half life reflects the elimination time constant τ_e.

B.17.2.2.2 Population Level of the Pharmacokinetic Model

The absorption rate constant was estimated as $\tau_a \approx 1$ h using Phase I data and was fixed in the following analysis. A bivariate log normal distribution was used for describ ing the distribution of the two system parameters, i.e., *CL/F* and *V/F* (apparent volume of distribution), in the study population.

$$(CL/F)_i = \overline{CL/F}\left(\frac{\text{LBM}}{50\,\text{kg}}\right)^{3/4}(1 + \theta_{\text{SMOK}}) \times e^{\eta_{CLi}}$$
$$(V/F)_i = \overline{V/F}\frac{\text{LBM}}{50\,\text{kg}} \times e^{\eta_{Vi}}$$
$$\vec{\eta}_i \in N(0, \Omega) \qquad (B.17.24)$$

The η elements are independent, identically distributed ran dom variables of mean 0 and variance Ω. Covariates like body size, given as lean body mass (LBM), or smoking status θ_{SMOK} may displace the location of the median of the multivariate probability density function of the PK param eter. The NONMEM approach (Boeckmann et al. 1994)

and a one compartment model with first order input function was used to estimate the PopPK parameter (Fig. B.17 1).

B.17.2.2.3 PopPK Model Development

Three PopPK models shown in Table B.17 1 were fitted to the data using the PK parameter model given in Eq. B.17.24.

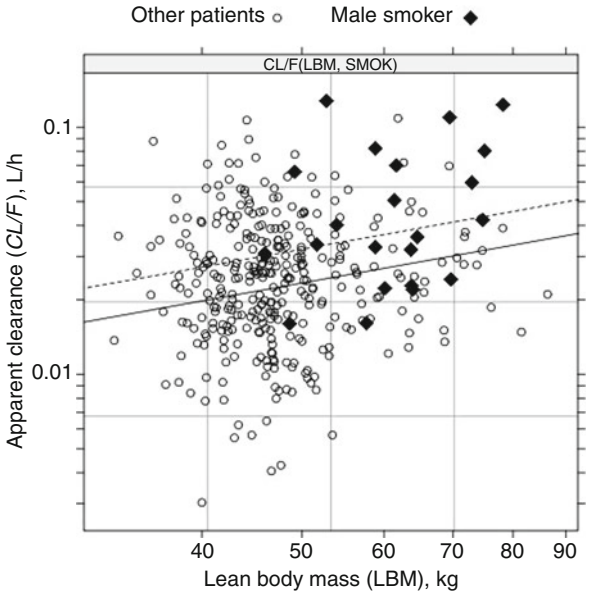

Figure B.17-1

Fixed effects of LBM and SMOKing status on *CL/F*. Only a marginal fraction of the *CL* variability was explained by LBM and SMOKing status. The *CL/F* dependency on LBM is given as a *solid* and *broken* lines for the nonsmokers and smokers, respectively. LBM was higher in male patients than in female patients. The combined fixed effect of LBM and SMOKing status on *CL/F* result in higher *CL/F* values in male smokers than in all other patients: other patients *circle* and male smoker *filled rhombus*

Table B.17-1

Covariate models tested

Model	Covariate	Added fixed effect
1	Base	No covariate
2	+LBM	LBM on *CL/F* and V/F
3	+SMOK	SMOK on *CL/F*

Table B.17 2 shows the individual NONMEM results of the parameter estimates.

The $\overline{CL/F}$ estimate for the +SMOK model in Table B.17 2 is provided for nonsmokers (see Eq. B.17.24). The $\overline{CL/F}$ estimate for smokers was increased by $\theta_{\mathrm{SMOK}} = 36.9\%$.

Only a negligible variability part of the random effect ω_{CL} could be explained by the LBM and SMOK covariates. However, LBM alone explained a moderate part of the variability of the random effect ω_V on *V/F*. The ω_V was reduced from 28.7% in the base model to 21.4% in the +LBM model.

B.17.2.2.4 Subpopulations

LBM included in the fixed effects on *CL/F* and *V/F* was the most important covariate improving the NONMEM fit. Extending the fixed effects on *CL/F* by smoking status further improved the fit slightly. The linear relationship between the logarithm of *CL/F* and LBM is highlighted in Fig. B.17 1. The line for smokers was shifted by a factor of 1.37 to higher values compared to nonsmokers.

LBM is calculated using the sex, height, and weight of a patient (Rowland and Tozer 1989). LBM is distinctly higher in male than in female subjects (see Table B.17 3), resulting in a 28.8% higher $\overline{CL/F}$ estimate for male patients. The SMOK effect on top of the LBM effect in male smokers increased $\overline{CL/F}$ by 69.9% compared to female nonsmokers. The $\overline{CL/F}$ estimate for male smokers was distinctly higher than for all other subgroups. However, only 6% of the study population were male smokers, while the majority, i.e., approximately two thirds of the total RA study population, were female nonsmokers. The clinical relevance of the increase in the *CL/F* depends on the given dose rate. If the dose rate generated C_{ss} values in the majority of patients that were linked closely to the maximum efficacy, almost all patients will benefit from the full drug effect.

B.17.2.3 Comparing Clinical Outcome Between 10 and 20 mg

B.17.2.3.1 Relationship Between C_{ss} and Clinical Outcome

The individual C_{ss} was calculated using the sparse individual PK data, the individual dose history, and the model as prior knowledge for the post hoc estimation. Clinical

Table B.17-2

PopPK parameters used in Eq. B.17.24 for the Bateman-function (see Eq. B.17.22) with τ_a = 1 h fixed)

	ΔOF	$\overline{CL/F}$ L/h	$\overline{V/F}$ L	$\omega_{CL\%}$	$\omega_{V\%}$	$\omega_{CL,V}$	$\sigma_{\%}$	$\sigma_{mg/L}$
Base	0.0	0.0245	12.1	61.4	28.7	0.204	13.5	5.5
+LBM	87.1	0.0252	12.5	61.0	21.4	0.139	13.4	6.3
+SMOK	16.5	0.0234	12.5	59.5	21.4	0.151	13.4	6.3

Table B.17-3

Fixed effects by SEX and SMOKing status

	Subgroup	$\overline{LBM}$ kg	$\overline{CL/F}$ L/h	$Fcl_{LBM,SMOK}$	$N_{sub\text{-}group}$	Fraction
1	Female nonsmoker	45.8	0.0224	0.936	233	0.654
2	Female smoker	45.0	0.0286	1.265	60	0.169
3	Male nonsmoker	64.2	0.0273	1.206	41	0.115
4	Male smoker	61.5	0.0381	1.600	22	0.062

outcome defined as the success rate was evaluated using a binary yes or no ACR20 variable (Arnett et al. 1988) at the final visits after 1 year of drug treatment. The relation ship between concentration C_{ss} and clinical outcome (see Eqs. B.17.6, B.17.7, B.17.11, and B.17.12) was reported previously (Weber and Harnisch 2002). The parameters for the concentration effect relationship are shown in *Table B.17 4*.

Table B.17-4

Parameter (median and 95% confidence interval [CI]) of the expected success rate P_{succ} (ACR20), (see Eq. B.17.12)

	Median	CI_{95}	CI_{95+}
EC_{50}	11.0	9.4	12.9
ω_S	0.394	0.324	0.479
P_{max}	0.622	0.536	0.708
$P_{0,\ placebo}$	0.265	0.219	0.317

B.17.2.3.2 Dose Recommendation

The logit(P_D) of the responder rate given in Eq. B.17.21 is a linear function on the probit($P_{CL/F}$) values of the *CL/F* frequency with a negative slope. The ratio between the dose rate and the mean *CL/F* of the treated patient popu lation determines the intercept of the lines in the quantile QQ plot (see *Fig. B.17 2*). Changing the dose rate leads to a parallel shift of the lines with unchanged slope. A horizontal dotted line marks the logit(P_D) for 80% of the maximum drug effect:

$$\text{Prob}(\text{logit}(P_D) \leq 1.39) = 80\% \qquad (B.17.25)$$

The intersection between the logit(P_D = 80%) dotted line and the probit($P_{CL/F}$) linear functions for 10 and 20 mg doses are indicated by a dotted line and a solid vertical line, respectively. Depending on the given dose, i.e., 10 or 20 mg QD, 80% of the maximum drug effect is achieved in the percentage of patients shown below:

$$\begin{aligned}\text{Prob}\big(\text{probit}(P_{CL/F}) \leq -0.185 | \dot{D} = 10\,\text{mg/day}\big) &= 42.6\% \\ \text{Prob}\big(\text{probit}(P_{CL/F}) \leq +0.943 | \dot{D} = 20\,\text{mg/day}\big) &= 82.7\%\end{aligned} \qquad (B.17.26)$$

The therapeutic target was to achieve 80% of the maximum drug effect in at least 80% of the patient pop ulation. Our model showed that using 20 mg QD will achieve a probability of a positive ACR20 which is equal or higher than 80% of the maximum drug effect in 82.7% of the patient population. In contrast, 10 mg QD failed to achieve the therapeutic target, because only 42.6% of the patients will achieve the target of 80% of the maximum drug efficacy (*Fig. B.17 2*).

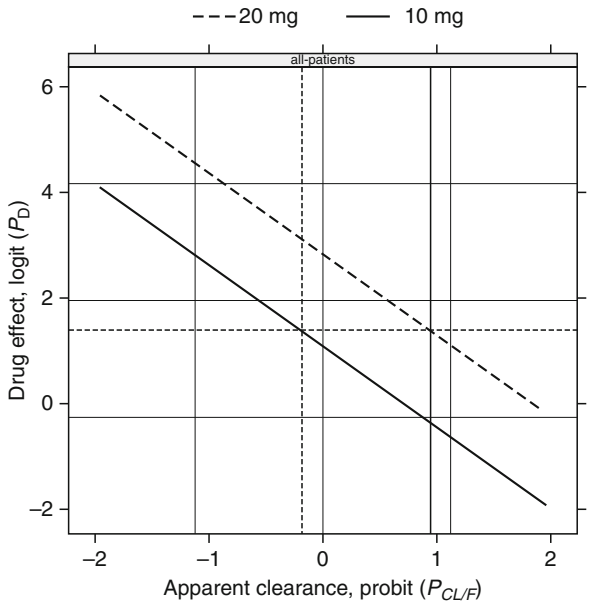

Figure B.17-2

Logit of the probability of obtaining a drug effect P_D dependent on the probit ($P_{CL/F}$) of the distribution conditional on the given dose rate. The *horizontal broken line* marks 80% of maximum drug effect. The two vertical lines are the probits($P_{CL/F}$) for 42.6% and 82.7% for the 10-mg and the 20-mg QD dose, respectively, with at least 80% of maximum drug effect

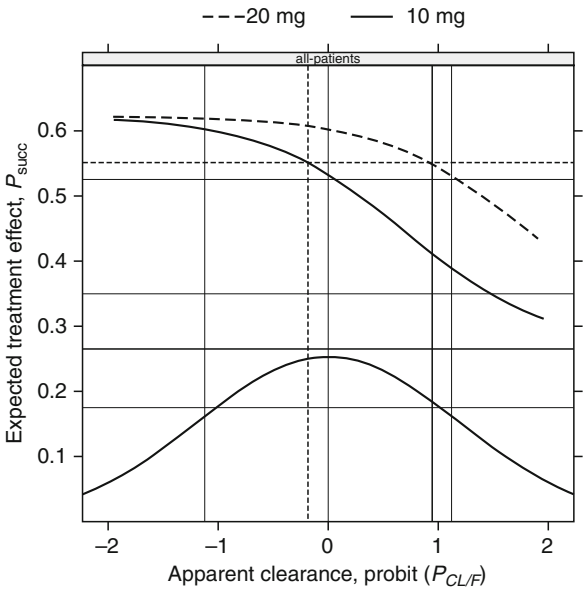

Figure B.17-3

Expected probability of success P_{succ} dependent and on the probit($P_{CL/F}$) of the distribution, conditional on the given dose rate. The *horizontal broken line* marks the target effect of $P_{succ} \geq 55.1\%$ corresponding to 80% of the maximum drug effect. The *horizontal solid line* marks the placebo effect. The two vertical lines are the probits($P_{CL/F}$) for 42.6% and 82.7% for the 10-mg and the 20-mg QD dose, respectively, with at least 80% of maximum drug effect. The *bell-shaped curve* at the bottom is the standard normal distribution of the total sample of patients with RA

Lack of efficacy led to a decreased compliance of the patients and was more frequent in the 10 mg QD than in the 20 mg QD group. Therefore, 20 mg QD is the preferred dose regimen leading to a higher fraction of patients obtaining the full drug effect than those using 10 mg QD.

B.17.2.3.3 Expected Success Rate

The relationship between the expected probability of suc cess and the probit of the *CL/F* distribution based on the given dose is shown in *Fig. B.17 3*. The dotted and solid vertical lines again indicate the probits of the *CL/F* prob ability distribution for achieving the therapeutic success target as seen in *Fig. B.17 2*.

The maximum drug effect is given as the difference between the maximum success rate in the drug and pla cebo treatment groups. According to Eq. B.17.6, 80% of the maximum drug effect $P_{max} - P_0$ translates to an expectation of P_{succ} of 55.06% for the observed success probability.

B.17.2.3.4 Relevance of Covariates?

Although the NONMEM fit improved significantly when LBM and SMOK were introduced as fixed effects on the *CL/F* model, the variability in *CL/F* was only negligibly reduced. The logit(P_D) (see Eq. B.17.21) is inversely related to the mean $\overline{CL/F}$. A shift of the $\overline{CL/F}$ location to larger values in smokers results in lower C_{ss} concentra tions and in turn to lower success rates for a given dose rate. The therapeutic 80% target is therefore achieved in a smaller fraction of the patient population of smokers:

$$\begin{aligned}\text{Prob}\big(\text{probit}(P_{CL/F}) \leq -0.70 | \dot{D} = 10\,\text{mg/day}\big) &= 24.7\% \\ \text{Prob}\big(\text{probit}(P_{CL/F}) \leq +0.43 | \dot{D} = 20\,\text{mg/day}\big) &= 66.7\%\end{aligned} \quad (B.17.27)$$

The extent of lowering the probability of success P_D in smokers depends on the chosen dose rate. Using a dose of

20 mg QD or more, the majority (66.7%) of the subpop ulation of smokers remains in the range of at least 80% probability of success.

A dose rate of 20 mg QD reaches the C_{ss} range, where saturation of the relationship between C_{ss} and probability of success is obtained. In this saturation range, the clinical efficacy after a moderately reduced exposure will remain unchanged and close to P_{max}.

The combined covariate effect LBM and SMOK on *CL/F* is especially pronounced in male smokers. The LBM of male patients (≈63 kg) was higher than female patients (≈45 kg, see *Table B.17 3*). The *CL/F* is increased by a higher LBM and the effect of the smoking status.

The relationship between the expected probability of success and the probit of the *CL/F* distribution conditional on the given dose is shown for male smokers in (*Fig. B.17 4*). The dotted and solid vertical lines again indicate the probits for achieving the therapeutic target as seen in Eq. B.17.17 and Eq. B.17.18. The male smoker patient subgroup had the smallest fraction of patients achieving the therapeutic target:

$$\begin{aligned}\text{Prob}\big(\text{probit}(P_{CL/F}) \le -0.950 | \dot{D} = 10\,\text{mg/day}\big) &= 17.1\% \\ \text{Prob}\big(\text{probit}(P_{CL/F}) \le +0.178 | \dot{D} = 20\,\text{mg/day}\big) &= 54.4\%\end{aligned} \qquad (B.17.28)$$

The largest shift of the location by factor 1.6 to higher $\overline{CL/F}$ values was observed for in a small subgroup of 22 (6% of 356 patients) male smokers. The large shift in $\overline{CL/F}$ in the subgroup of male smokers translates to a distinctly smaller fraction of patients achieving the ther apeutic target effect. Using 10 and 20 mg QD in male smokers only 17.1% and 54.4%, respectively, will achieve at least a probability of success of 80%.

A dose rate of 10 mg QD or even lower is no longer suitable in male smokers. In contrast, 20 mg QD is still effective in 54.4% of male smokers. In the event of a lack of efficacy, this small subgroup of male smokers may benefit from even higher doses than 20 mg QD.

B.17.3 Conclusion

A high LBM and smoking status shift the *CL/F* distribu tion to higher values and reduce the responsiveness to a given leflunomide dose. Based on the relationship between the probit of the *CL/F* distribution and the logit of the probability of success, which is conditional on the dose rate, a scientifically justified dose recommenda tion was obtained. A recommended dose should achieve the therapeutic target to obtain at least 80% of the max imum drug effect in at least 80% of target patient population.

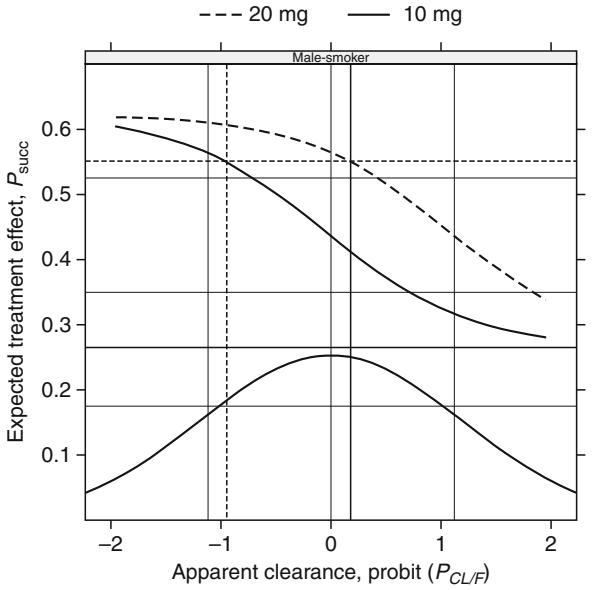

Figure B.17-4

Expected probability of success P_{succ} dependent and on the probit($P_{CL/F}$) of the distribution, conditional on the given dose rate in male smokers. The *horizontal broken line* marks the target effect of $P_{succ} \ge 55.1\%$ corresponding to 80% of the maximum drug effect. The two vertical lines are at the probits($P_{CL/F}$) for 17.1% and 54.4% for the 10-mg and the 20-mg QD dose, respectively, with at least 80% of maximum drug effect. The *bell-shaped curve* at the bottom is the standard normal distribution of the subgroup of male smokers. The fraction of male smokers was only 6.2% of the total sample of patients with RA

Our dose recommendation for leflunomide is as follows:

- 20 mg daily as maintenance dose achieved the thera peutic target in the overall target patient population.
- 10 mg daily achieved the therapeutic target effect only in 42.6% of the overall target patient population. In case of any serious AE, the dose can be reduced to 10 mg daily to exploit the remaining drug efficacy.
- The largest *CL/F* values were found in the subgroup of male smokers, 10 mg daily failed to achieve the

therapeutic target; at least 20 mg daily is recommended.
- When male smokers fail to show efficacy, an increase of the dose to at least 20 mg daily or even more is highly recommended.

B.17.4 Part 3: Population Pharmacokinetics of Dronedarone

B.17.4.1 Introduction and Objectives

During the DD, Phase I studies allow the achievement of a complete (PK) profile in a few dozen healthy volunteers by means of rich sampling schemes (up to 20 samples over 24 h). In the later stage of DD (Phase III studies), the drug is administered for several months (depending on its indication) to hundreds (or even thousands) of patients in a realistic clinical situation, i.e., to patients with different characteristics and/or pathologies. In the latter case, samples are collected very sparsely due to obvious ethical and practical reasons.

By means of modeling techniques such as the (PopPK) approach, one can retrieve the same PK information from Phase III studies as that obtained during Phase I studies. PopPK also provides an explanation for the potential sources of variability between patients, hence increasing the knowledge of the new compound.

Dronedarone (Multaq®) is a new drug which has been developed for the treatment of atrial fibrillation. During its development, several Phase III studies were performed to evaluate the efficacy and safety of dronedarone. More than 800 patients in three of these studies (Singh et al. 2007; Køber et al. 2008) were included in a PopPK analysis.

The objectives of this analysis were to evaluate the PK properties of dronedarone in patients and to provide an assessment of the dronedarone PK variability based on the investigation of the influence of several potential covariates. The PopPK model was also used to provide individual estimates of plasma clearance and exposure, as measured by the area under the plasma concentration time curve (AUC_{0-12}), and the maximum (C_{max}) and minimum (C_{trough}) concentrations in the dosing interval at steady state.

Based on the results of the PopPK analysis, a simulation was performed to assess whether the PK properties of dronedarone remained comparable in a specific subpopulation of elderly female patients with low body weight.

B.17.4.2 Step 1: Population Pharmacokinetic Analysis

B.17.4.2.1 Materials and Methods

Data from a total of 849 patients who had PK information during 12 months of dronedarone 400 mg twice daily dosing were included in the analysis. For two of the three Phase III studies, five trough samples per patient were collected on Day 7 ± 2, Day 21 ± 3, Month 4 ± 5 days, Month 9 ± 5 days, and Month 12 ± 5 days. For the last study, dronedarone plasma levels were collected as close as possible to the assessment of the 12 lead ECG at Months 1 and 6 in about 50% of patients. In addition, several blood samples (pre dose, 2, 4, 6, 8, 10, and 12 h post dose) were to be taken in a subgroup of about 30 patients at Month 1.

The NONMEM software (version V level 1.1) was used to model the plasma concentration data. All runs were performed using the first order conditional estimation (with interaction) method.

Once outliers were detected (and possibly excluded from the database) from a preliminary model, the total dataset was split into both a model building (70%) and a validation (30%) dataset by means of a random procedure.

Initially, the population parameters (fixed and random effects) together with the individual estimates were computed assuming no dependency between pharmacokinetic parameters and covariates. Different structural PK models (one and two compartment models, with and without lag time) and different residual error models (additive, proportional, power, and combined) were evaluated.

The relationship between the individual estimates and the covariates was investigated. Based on the knowledge of the compound, the following variables were tested as potential model covariates:

- Demographic characteristics, such as body weight, age, height, sex, and race (coded as a binary variable 0 for Caucasians and 1 for non Caucasians).
- Renal function was initially coded as a binary variable as a function of creatinine clearance CL_{CR}, which was classified as normal ($CL_{CR} \geq 80$ mL/min) or impaired ($CL_{CR} < 80$ mL/min) renal function; finally, CL_{CR} was tested as a continuous variable.

- CHF degree as described by the New York Heart Association (NYHA) score, coded as a binary variable (0 for the NYHA score 0 and 1 for the NYHA scores I to IV).
- Co administration of CYP3A4 inhibitors, coded as a binary variable (1 if either a moderate and/or a strong inhibitor was co administered during at least 80% of the dronedarone treatment duration, and 0 otherwise).

The selected covariates were added individually to the model and tested for statistical significance. Only the covariates providing a significant change ($p < 0.001$) in the objective function (OFV) when introduced in the model were retained in the analysis. The population parameters were re estimated considering the relationship with the covariates. Before validation, verification of the model was performed by examination of the good ness of fit plots and by estimation of several quality criteria.

The validation of the PopPK model predictive ability was performed in the validation dataset using different approaches. The robustness of the final model and the accuracy of parameter estimates (computation of standard error of estimates) were assessed using a boot strap method performed with Wings for NONMEM.

Finally, exposure variables in the dosing interval, such as AUC_{0-12}, C_{max} and C_{trough} were calculated from indi vidual PK parameters obtained using the final model for the total dataset.

B.17.4.2.2 Results

Prior to model selection, detection of outliers led to the deletion of 126 samples and consequent exclusion of 10 patients. The total dataset was then composed of 839 patients (2,786 samples). This was divided into a validation dataset of 250 patients and a model building dataset of 589 patients.

Data were best fitted by a two compartment model without lag time, with an additive residual error model. The interindividual variability of the parameters (absorption constant k_a, central and peripheral volumes *V2/F* and *V3/F*, and elimination and intercompartmental clearances *CL/F* and *Q/F*) was modeled with an exponential error model.

Inter patient variability in dronedarone clearance, central volume, and peripheral volume were about 30%, 110% and 70%, respectively. The residual (intraindividual) variability was about 14 ng/mL.

The significant covariates explaining dronedarone pharmacokinetic variability in patients were sex, weight, and age. The impact of each covariate, considered without the influence of the other covariates, is given below. Median values for body weight and age in the total dataset were 83 kg and 65 years, respectively.

- For *CL/F*, the direct relationship with WGT and the relationship with age could be expressed as:

$$\mathrm{CL}/F(\mathrm{L}) = [290 \times (\mathrm{WGT(kg)}/83)^{**}0.528]* (\mathrm{AGE(years)})/(\mathrm{AGE} - 12.9)$$

- The difference for *CL/F* between male and female patients is quantified as:

$$CL/F(\mathrm{L/h})\text{for female patients} = 0.843*CL/F(\mathrm{L/h})\text{of male patients}$$

The impact of covariates on PK parameters is presented below:

- *CL/F* was 16% lower in female (244 L/h) compared to male (290 L/h) patients.
- *CL/F* was lower for a lower weight; thus, for a 65 year old (median age) patient:
  - For a 59 kg patient (5th percentile), *CL/F* = 302 L/h (17% compared to median)
  - For an 83 kg patient (median value), *CL/F* = 362 L/h
  - For a 113 kg patient (95th percentile), *CL/F* = 426 L/h (+18% compared to median)
- *CL/F* was lower for older patients; thus, for an 83 kg (~median weight) patient:
  - For a 47 year old patient (5th percentile), *CL/F* = 400 L/h (+10% compared to median)
  - For a 65 year old patient (median value), *CL/F* = 362 L/h
  - For an 82 year old patient (95th percentile), *CL/F* = 344 L/h (5% compared to median)

CL_{CR} (tested as a binary variable i.e., classified as normal or impaired renal function, or as a continuous covariate), degree of congestive heart failure (as measured by NYHA score), race (classified as Caucasian or non Caucasian), concomitant intake of CYP3A4 inhibitors, and height did not influence dronedarone PK in this patient population.

No important systematic deviations or major bias in any of the goodness of fit plots were observed.

Final population PK parameters and their relationship to covariates are presented in *Table B.17 5*.

Individual exposure variables (AUC_{0-12}, C_{max} and C_{trough}) were then calculated for all patients. Mean (SD) values together with ratios are presented in *Table B.17 6*.

Table B.17-5

Dronedarone population PK model

Parameter	Estimate	%RSE	95%CI
$\theta_{(1)}$ (intercept) in [$CL/F = \theta_{(1)}$ * WGT/83], L/h	290	3.93%	[268 312]
$\theta_{(6)}$ in [$CL/F = \theta_{(1)}$ * (WGT/83) ** $\theta_{(6)}$]	0.528	14.0%	[0.383 0.673]
$\theta_{(7)}$ Gender effect on *CL/F* for female	0.843	3.32%	[0.788 0.898]
$\theta_{(8)}$ in [*CL/F* = TVCL * AGE/($\theta_{(8)}$ + AGE)]	12.9	12.9%	[16.2 to 9.65]
Central volume *V2/F* [$\theta_{(2)}$], L	3,140	6.43%	[2,740 3,540]
Inter-compartmental clearance Q/F [$\theta_{(3)}$], L/h	316	4.75%	[287 345]
Peripheral volume *V3/F* [$\theta_{(4)}$], L	6,340	7.82%	[5,370 7,310]
Absorption rate constant k_a [$\theta_{(5)}$], h^{-1}	0.291 FIXED	Not applicable	
Interindividual variability (CV%)			
CL/F	30.2%	7.15%	[28.0 32.2%]
V2/F	110%	11.8%	[96.1 122%]
V3/F	70.4%	11.2%	[62.1 77.7%]
Residual variability $Y = C_{Ipred} + \varepsilon$			
σ (SE[ε] in ng/mL)	$2.01.10^{-4}$ (14.2)	2.00%	[$1.93.10^{-4}$ $2.09.10^{-4}$]

F, bioavailability. %RSE, Percentage of relative standard error (100% × SE/estimate). θ and ω are the PopPK parameters (θ) and the variance of their associated interindividual variability (ω). ε and σ are the residual (intraindividual) error variable (ε) and its associated variance (σ), respectively.

Table B.17-6

Dronedarone steady-state exposure parameters

Parameter	All patients	Males (M)	Females (F)	Age (years)		Body weight (kg)		
	n = 839	*n* = 604	*n* = 235	<65 (Y) *n* = 390	≥65 (E) *n* = 449	<50 (A) *n* = 6	50 100 (B) *n* = 700	≥100 © *n* = 133
C_{max} (ng/mL)	121 (39.4)	112 (32.1)	144 (46.6)	109 (29.1)	131 (44.0)	159 (31.6)	125 (40.5)	99.9 (23.0)
		F/M = 1.29		E/Y = 1.20		A/B = 1.27		C/B = 0.799
C_{trough} (ng/mL)	77.0 (32.0)	69.7 (26.8)	95.8 (36.6)	67.1 (24.5)	85.7 (35.2)	122 (27.0)	80.1 (32.4)	58.8 (21.5)
		F/M = 1.37		E/Y = 1.28		A/B = 1.52		C/B = 0.734
$AUC_{0\ 12}$ (ng.h/mL)	1,230 (412)	1,131 (335)	1,483 (480)	1,096 (302)	1,345 (458)	1,749 (352)	1,271 (420)	988 (247)
		F/M = 1.31		E/Y = 1.23		A/B = 1.38		C/B = 0.777

As shown in *Table B.17 6* and *Fig. B.17 5*, a trend to higher Dronedarone $AUC_{0–12}$ values was observed in females compared to males. Similarly, elderly (>65 years) or low body weight (<50 kg) patients had higher exposures than younger patients or patients with higher body weight. The same trends were observed for C_{max} and C_{trough}.

The question was then raised to evaluate the impact of the combination of these three factors on the PK behavior of dronedarone. Even if more than 830 patients were included in the PopPK analysis, only 6 patients were elderly females (>65 years) with low body weight (<50 kg); since no clear decision could be made on such a small number of patients, simulation was the best method to evaluate the real impact of these three factors on the PK of dronedarone.

B.17.4.3 Step 2: Simulation of the Pharmacokinetic Properties of Dronedarone in a Specific Subpopulation

B.17.4.3.1 Materials and Methods

The objective of the current simulation was to estimate the exposure of dronedarone (as measured by steady state $AUC_{0–12}$, C_{max} and C_{trough} values) in a virtual population

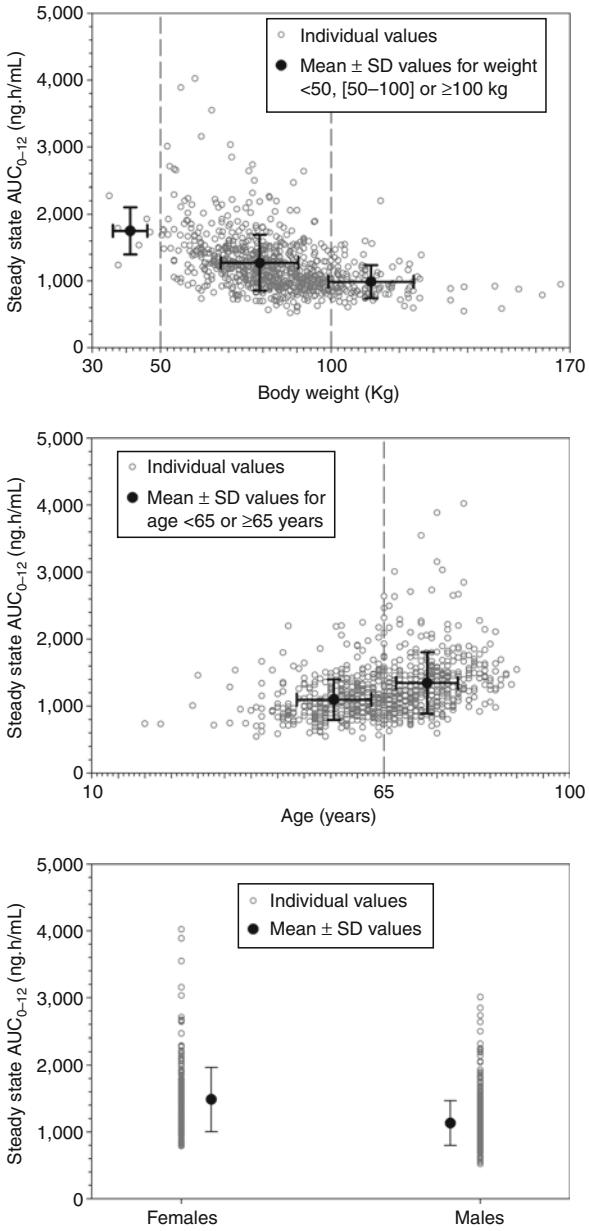

Figure B.17-5

Individual AUC_{0-12} values versus covariates included in the PopPK analysis

of patients treated with dronedarone, especially in elderly female patients with low body weight (<50 kg). To maximize the results, the minimal age of this subpopulation was set at 75 years.

To estimate exposure parameters, a complete PK profile was simulated in virtual patients at steady state using TS2 software (Pharsight Corporation). Patients were assumed to be similar to those included in the Phase III studies in terms of covariates.

The simulation process consisted of a drug model, namely the one obtained in the PopPK analysis, a covariate model, and a simulation design.

Covariate Model

The three covariates included in the PopPK model (body weight, age, and sex) were included in the covariate model. Standard inputs of TS2 software, i.e., mean, SD, minimum, maximum values, were computed on continuous (weight and age) for males and females separately. The correlation coefficient of weight versus age was also computed by sex.

Simulation Design

About 2,000 elderly females with low body weight were simulated to better explore the PK properties of dronedarone in this subpopulation. For comparison, a virtual set of 5,000 patients with similar demographic characteristics as those included in the PopPK analysis was simulated. In both cases, only one replicate was simulated.

Steady state was assumed to be reached for all patients including extreme ones at Day 50. Patients then received a daily 400 mg BID dronedarone dose for 50 days, and a rich sampling was performed at Day 50: samples were collected before, 0.25 and 0.5 h after drug intake, then each hour up to 12 h. These 14 samples per patient allowed an accurate estimation of steady state exposure variables.

The following steady state exposure variables were simulated:

- Area under the plasma concentration versus time curve in the dosing interval, AUC_{0-12}
- Maximum plasma concentration in the dosing interval, C_{max}
- Minimum plasma concentration in the dosing interval, C_{trough}

For validation purposes, simulated concentration versus time curves were compared graphically with the typical patient's curve obtained in the PopPK analysis. Basic descriptive statistics (mean, SD, minimum, and maximum values) of the simulated exposure variables were compared with those estimated in the PopPK analysis.

B.17.4.3.2 Results

A first set of 5,000 patients with covariates distribution identical to the one of the PopPK analysis was

simulated and their exposure variables were estimated. From simulated concentrations versus time curves, 5th, 50th, and 95th percentiles of concentration values were calculated for males and females separately. The typical male or female patient's concentration versus time curve from the PopPK analysis was then plotted for comparison (see *Fig. B.17 6*). Very close concentration time curves were obtained, indicating a correct transcription of the PopPK model into the simulation model.

A second subset of patients, composed only of females with body weight $\leq$50 kg and age $\geq$75 years, was also simulated with the same dosing regimen (400 mg BID for 50 days). Due to the structure of the correlation matrix of

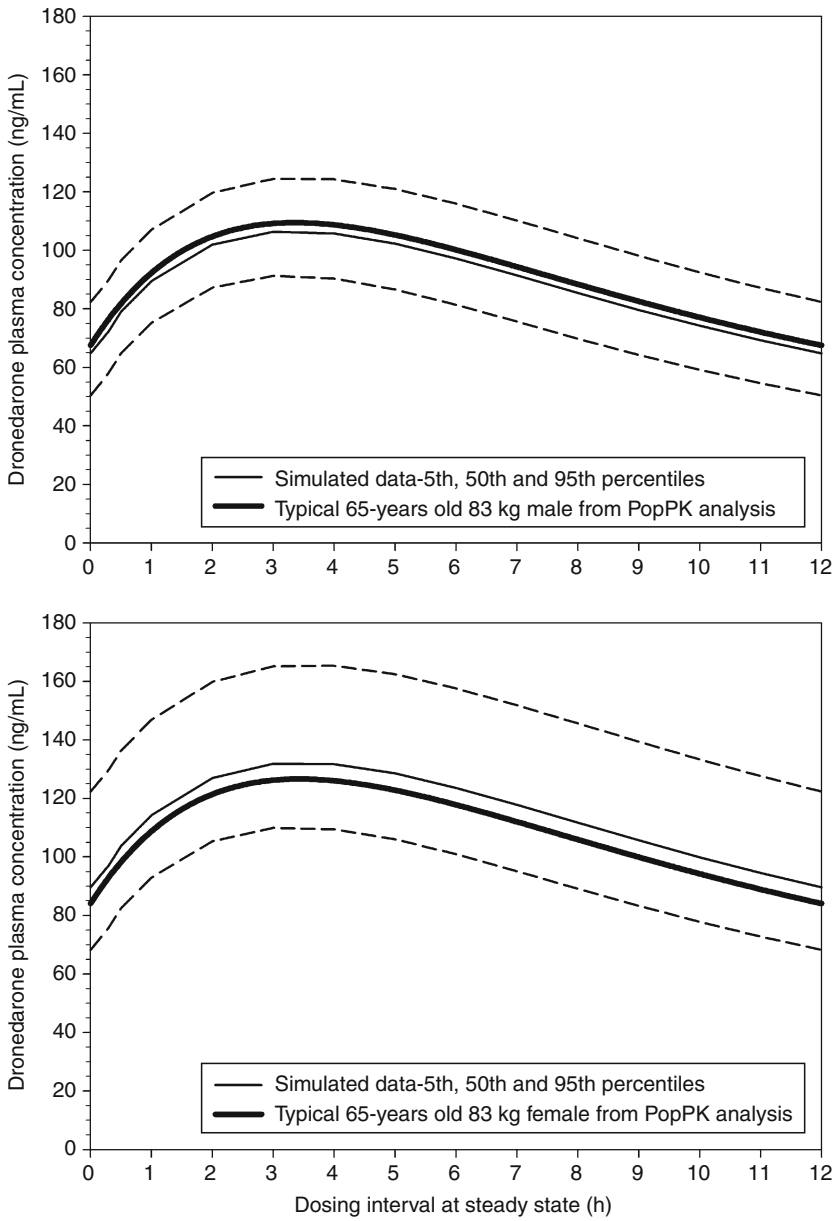

Figure B.17-6

Validation of the PopPK model transcription Males (*top*) and females (*bottom*)

covariates, it was not possible to obtain 2,000 virtual patients; a total of 1,954 patients were available. Fifth, 50th, and 95th percentiles of concentration versus time curves obtained in simulated female patients with weight ≤50 kg and age ≥75 years are presented in *Fig. B.17 7*. For comparison, 5th, 50th, and 95th percentiles of concentration versus time curves obtained in patients whose age and weight ranged as in the PopPK analysis (i.e., 20 ≤ age ≤ 90 years and 35 ≤ body weight ≤ 167 kg) are also represented.

Exposure variables computed from the simulated concentrations versus time curves (see *Fig. B.17 7*) are presented in *Table B.17 7* and represented in *Fig. B.17 8*.

B.17.4.4 Conclusions

This example illustrates the benefits of the modeling and simulation techniques in the late DD phases.

The population approach first allows estimating individual PK parameters in a large number of patients with sparse data (a median of 5 samples collected over one treatment year in more than 800 patients). As compared to classical PK analyses carried out in the early DD stages on a small number of healthy volunteers (standardized in terms of demographic characteristics), population PK allows taking the inter patient differences into account by the exploration of the relationships between covariates and PK parameters.

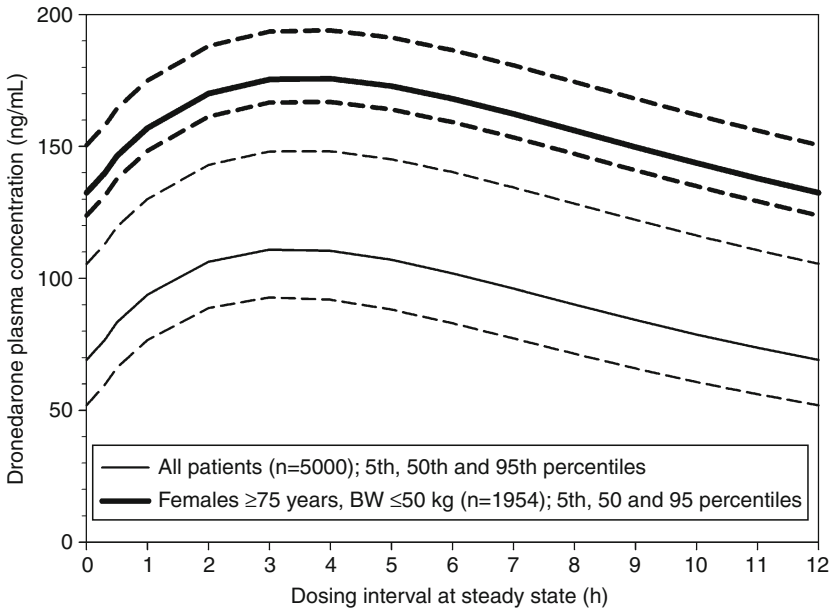

Figure B.17-7

Simulated concentration versus time curves in the dosing interval

Table B.17-7

Mean (CV%) steady-state dronedarone exposures in either simulated patients with the same covariates range than in the PopPK analysis, or in simulated old (>75 years) female patients with low body weight (<50 kg)

Type of patients	*n*	C_{max} (ng/mL) Mean (CV%)	AUC_{0-12} (ng.h/mL) Mean (CV%)	C_{trough} (ng/mL) Mean (CV%)
All patients	5,000	114 (15.1%)	1,165 (17.8%)	72.5 (23.0%)
Females ≥ 75 years and BW ≤ 50 kg	1,954	178 (4.77%)	1,926 (5.28%)	134 (6.21%)
Females ≥ 75 years BW ≤ 50 kg versus all patients		1.56	1.65	1.86

N/A, not applicable.

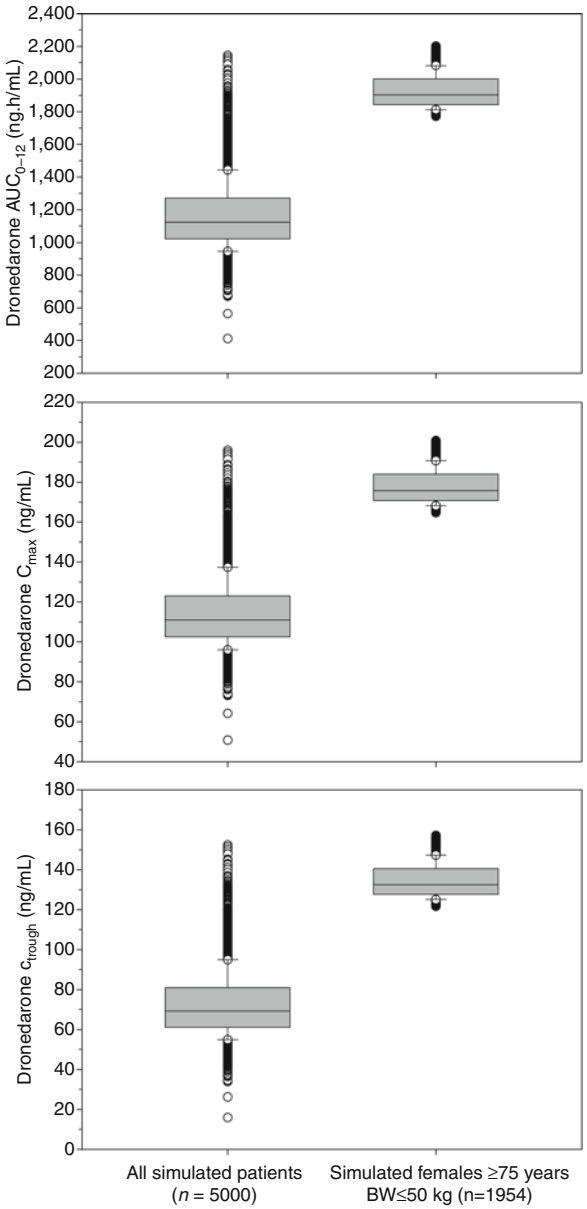

Figure B.17-8

Exposure variables obtained in all patients or in a subset of females with weight <50 kg and age >75 years

Utilizing the PopPK model, by means of simulation techniques, then allows the exploration of PK properties of a drug in special subpopulations, such as elderly females with low body weight in this analysis.

From PK parameters, individual exposure variables can be calculated and then used to study the potential relationship with some safety or efficacy endpoints. This particular subgroup of elderly female patients with relatively low body weight did not present with any excess incidence of key safety parameters, including cardiovas cular death and death from any causes, compared to the overall population during clinical development of dronedarone.

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B.18 Bioanalysis of Clinical Studies

Joern Krause · Ronald Schmidt

PURPOSE AND RATIONALE

Powerful analytical techniques are one key requirement for the successful drug research and drug development. The concentration of drugs, prodrugs, and metabolites has to be determined in very diverse matrices such as plasma (blood), urine, feces, and also in different organ tissues (depending on the nature of drug and on the targeted organs).

A wide set of analytical tools has been used for this purpose. Gas chromatography (GC) in combination with different detection techniques such as mass spectrometry, flame ionization detector (FID), (Jennings 1987) as well as liquid chromatography (LC) in combination with UV or fluorescence detection and others have been successfully used for this purpose(Dorschel et al. 1989; Chu et al. 1999; Jin et al. 2004; Abu Qare and Abou Donia 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 for example, 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.

The technique, which is currently used predominantly, is liquid chromatography coupled with mass spectrometry (LC MS or LC MS/MS). Consequently, this chapter will describe the use of LC MS in detail. However it should be mentioned here, that other very powerful techniques such as immunoassays, ELISAs, and chromogenic enzyme assays are used as well, and these types of bioassays are coming more and more into focus since biomolecules are being developed in a rapidly increasing number, which fuels the need for assays that specifically allow the sensitive analysis of biomolecules. The increasing number of biomolecules such as proteins in drug development also brought the possibility of analyzing lyze these compounds by LC MS/MS into focus. The possibilities and limitations of LC MS/MS in this application are also discussed in this chapter.

PROCEDURE

B.18.1 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; Robb et al. 2000; Raffaelli and Saba 2003) as a detection system for liquid chromatography. These techniques, established in the late 1980s (Lim and Lord 2002; Dole et al. 1968; Whitehouse et al. 1985; Cole 1997; Gaskell 1997; Robb et al. 2000) have very rapidly become the method of choice for drug quantification throughout the drug research community and in the pharmaceutical industry (Tiller et al. 2003; Hopfgartner and Bourgogne 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 $[M+Na]^+$, $[M+NH_4]^+$, and other adducts. In addition to that and depending on the size of the molecules, multiple charging might also occur (Lim and Lord 2002; Cole 1997; Gaskell 1997).

B.18.2 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 quadrupole mass spectrometer. This type of mass analyzer has several key advantages for the analysis of biological

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samples in combination with liquid chromatography. Triple quadrupole mass analyzers allow for the selection of one or several analytes, which can be filtered in the first quadrupole, allowing only ions of a certain mass to charge ratio to pass this first quadrupole (first mass filter). In the 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 the 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 every analyte, which can be integrated and processed (Venn 2000; Willoughby et al. 1998).

Besides the triple quadrupole 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 quadrupole 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.

A new technique introduced recently is the "FAIMS" (High **F**ield **A**symmetric waveform **I**on **M**obility **S**pectrometer) technology (Guevremont 2004). The FAIMS interface works in combination with ESI and APCI ion probes in order to increase selectivity in challenging assays in combination with LC MS/MS. FAIMS is an atmospheric pressure ion separation technology. In FAIMS, the ions are separated according to their properties while drifting in very high electric fields. Simply stated, each type of ion has an ion mobility, which is a constant in low electric fields. At high fields the mobility of each ion deviates from its low field value. The extent of that deviation is the key to ion separation in FAIMS. Using FAIMS can help to overcome selectivity issues that are not solvable by LC MS/MS alone. Although signal intensities with FAIMS are typically lower, the elimination/reduction of chemical noise might still provide a better sensitivity through an improved signal to noise ratio.

B.18.3 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 and compensate for matrix effects and recovery variations.

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 separate the isotope distribution of the internal standard from the natural isotope distribution of the analyte. The replacement of six ^{12}C or six ^{1}H atoms by ^{13}C or ^{2}H is usually a good choice for most pharmaceutical analytes ($m/z < 500$). Such isotopes are deemed ideal as internal standards since they will have the best probability for similar 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 chromatography to the best possible extent (Venn 2000). Caution should be applied when dealing with deuterated (^{2}H) standards. In some cases, a small shift in retention time is observed, which can limit the effect of such a standard. Unfortunately, the synthesis of labeled 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 fluctuations in the 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.

The use of an analogue compound as internal standard will be the method of choice when it comes to the analysis of biomolecules such as proteins by LC MS/MS, since a stable isotope labeled standard is typically not available due to the fact that a large number of for example, hydrogen atoms would need to be replaced by deuterium in order to achieve a mass difference that is large enough in

order to prevent an overlay of the natural isotopic distri bution with the stable isotope pattern. An iodine labeled compound would be an option to overcome this hurdle. Otherwise, an analogue standard will be the choice, which for example, has a difference in one amino acid.

B.18.4 LC Conditions

In drug analysis, LC MS usually means reversed phase liquid chromatography (RPLC) coupled to mass spectrom etry. Although normal phase LC can be used as well (espe cially in combination with atmospheric pressure chemical ionization APCI), predominantly RPLC 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 ammo nium 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 phase LC columns). Other vol atile buffers can be used as well. Nonvolatile buffers (e.g., 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 triethylamine 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 min) in order to realize a short analysis time and high sample throughput. A chromatographic sep aration 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 5,000 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 is in use. However, C_{18} reversed phase columns are probably the basic standard columns, which are in use (Venn 2000; Willoughby et al. 1998).

The newest developments in LC techniques have significantly shortened analysis time without compromis ing chromatographic resolution or sensitivity. For that purpose, short LC columns with small particles (e.g., C_{18} columns with 50 mm length, a particle size of 1.8 µm, and an inner diameter of about 3 mm) are used. These kind of columns are used at higher temperatures of about 60°C in order to reduce back pressure. Using these techniques, analysis times per sample can be reduced to less then 2 min in many cases, allowing for high sample throughput. This type of LC, often referred to as "UPLC" or "Rapid Resolution HPLC" (Hüsgen 2006; Swartz 2005) needs special LC equipment in order to keep up with the higher pressure (>400 bar) that is caused by the higher flow rates.

B.18.5 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; Chambers et al. 2007).

(a) Protein precipitation/dilution (PP)
(b) Solid phase extraction (SPE)
(c) Liquid liquid extraction (LLE)

B.18.6 Protein Precipitation

Protein precipitation is a very simple method of sample preparation. The sample (typically a plasma or urine sam ple) is spiked with internal standard solution and analyte solution in case of the calibration standard or quality con trol (QC) . 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 acetoni trile or methanol), which leads to protein precipitation. Samples are typically centrifuged after this step and the resulting supernatant is either analyzed directly by LC MS/MS, or a dilution step is implemented prior to sample analysis (Beck et al. 2004; de Jonge et al. 2004; Viberg et al. 2004; Crommentuin et al. 2004; Stovkis et al. 2004a; Hou et al. 2004). In many cases, this kind of sample preparation proved to be sufficient. However, more advanced sample clean up might be necessary, depending on the matrix and analytes that need to be handled.

B.18.7 Solid-Phase Extraction (SPE)/Mixed Mode SPE

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 et al. 2004; Pichini et al. 2004; Ding and Neue 1999). However, using SPE might not always be sufficient in order to remove endogenous matrix constituents (such as proteins and phospholipids) or exogenous compounds (e.g., drug dosing vehicles such as PEG 400 or Tween 80), which can lead to ion suppression or ion enhancement effects during LC MS/MS analysis. Mixed mode SPE uses a dual retention mechanism to extract ionizable drugs from biological fluids. This approach allows a rigorous interference elution procedure to be used, selectively removing interfering compounds from the SPE column, prior to elution of drugs of interest. Mixed mode SPE can significantly improve sample clean up compared to SPE based on a single retention mechanism (Chambers et al. 2007). However, the more complicated sample work up for mixed mode SPE is not in general justified. It should be considered on an assay by assay basis.

B.18.8 Liquid–Liquid Extraction (LLE)

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., pK_a; 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 the 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, for example, 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 et al. 2004b; Bonato et al. 2003; Baker et al. 2004; Xia et al. 1999; Laurito et al. 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 and Neubert 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 and Neubert 2004; Boner et al. 2003; Barratè et al. 2004; Bogialli et al. 2003; Hows et al. 2004; Ito et al. 2004). Recovery considerations are of special importance in these cases in order to get an idea on the completeness of the compound extraction procedure.

B.18.9 Eleven Steps on 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 lower limit of quantification (LLOQ) will be required for the assay and 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, APPI, and also the combination with FAIMS if available. 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, and source position) 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.

B.18.10 Sample Preparation for "Large Molecules"

Large molecules (MW > 500 Da) are becoming more and more important in research and development of drug candidates as well as biomarker candidates (van den Broek 2008; Ezan 2009). Peptides, proteins, and certain sugars are usually analyzed by immunological methods due to their high sensitivity and rapid sample throughput, but one major disadvantage is their potential of cross reactivity (e.g., metabolites, other compounds). Mass spectrometry is much more selective but cannot compete with the sensitivity of the immunological methods in general. However, mass spectrometrical methods are increasing in the literature since the sensitivity of immunoassays is not always required and new LC MS/MS instruments are performing much better (Zhang 2008). Nano LC in combination with LC MS/MS results in an increase of sensitivity, but with longer run times as compared to the usual HPLC methods.

Sample clean up of large molecules is rather challenging (Yang 2007). Proteins and peptides adsorb on various surfaces during sample extraction and inside the LC MS/MS systems. It should be tested, if dilutions of the stock solution are linear and if the analyte binds to extraction vials (usually glass or polypropylene) or pipette tips. Binding inside the LC MS/MS systems results in a severe carry over problem. The adsorption can be controlled by using other solvents, pH, surfactants, ionic strength, other material (glass < > polypropylene), or addition of serum albumin. For compounds, which bind to polypropylene tubes and glass tubes, low binding polypropylene might be an option. The lower the concentration of the protein or peptides in aqueous solutions, the higher is the adsorption rate to tube surfaces in general.

The major extraction techniques for small molecules (protein precipitation, solid phase extraction, and liquid liquid extraction) can be used for large molecules, but the use of organic solvents can result in an unintentional precipitation and loss of the analyte. In addition to the three basic extraction methods, immunoaffinity purification is frequently used to extract and clean the analyte from biological matrices (Dubois 2008; Thevis 2008; Whiteaker 2007). In many cases, an antibody that is directed against the analyte is immobilized on a surface (e.g., magnetic beads) or on a gel matrix. The biological sample will be incubated with the antibody that binds the analyte. After several washing steps at physiological pH, the analyte is eluted from the matrix with an acidic pH. The resulting eluate can be used directly for LC MS/MS analysis or will be further cleaned up by SPE, PP, LLE, TFC (turbo flow chromatography) or a 2D aproach. Since the presence of "antidrug antibodies" in the biological matrix can reduce the unbound fraction of the analyte/drug, immunoaffinity purification extracts only the non antibody bound fraction of the analyte/drug. Depending

on the selectivity of the antibody, structural analogues of the analyte, ^{127}labeled analyte or stable isotopic labeled internal standard (SILS) can be used as internal standard. A second approach is the quantification of an antibody itself where a receptor has been coupled to magnetic beads (Dubois 2008).

The most sensitive triple quadrupole mass spectrom eters are limited to 1,250 1,500 m/z, but most proteins and peptides have a molecular mass above 1,500 Da. One solution is the detection of multiply charged ions (e.g., $[M+3H]^{3+}$ to $[M+8H]^{8+}$) of the intact protein or an enzymatic digestion of the protein. The first approach may be used with smaller peptides (MW < 7,000 10,000 Da) and the second approach must be used with peptides and proteins with a MW > 10,000 Da. During the enzymatic digestion, the peptide is incubated with a proteolytic enzyme (e.g., trypsin, pepsine, Lys C, Glu C, or others) under its specific cleavage conditions and the much smaller pep tide fragment can be quantified by LC MS/MS usually with an increased sensitivity. The optimal internal stan dard is of course the stable isotopic labeled internal standard to cover the whole cleavage process, but another possibility is to add an SILS for the smaller peptide frag ment after the enzymatic digestion has been performed. In the second way, the SILS does not cover the enzymatic reaction.

B.18.11 Sample Preparation from "Dried Blood Spots"

Another method of sample preparation for LC MS/MS analysis is the so called dried blood spot (DBS) technique. In this technique, a droplet of blood is spotted onto a paper card and dried. Small pieces of paper can be cut out of these dried blood spots and the analyte might be extracted from these dried blood spots using an appropri ate solvent. This technique is currently under investigation in many pharmaceutical companies and it is already very likely that this technique will replace at least some part of the classical blood sampling as it is currently done on a routine basis. There are several potential advantages of this technique: Only a very low volume of sample is needed (only one or a few droplets of blood per sampling time compared to typically 1 or 1.5 mL of blood per time point). Another advantage might be the sample handling, since the resulting papers are a lot easier to store, handle, and ship compared with frozen blood samples. Moreover, the DBS technique might also allow the sampling of other populations such as children, where it otherwise might be difficult for ethical reasons. The number of experiments and analyses per subject might potentially also be increased due to the low volume of blood that is consumed. Prelim inary tests also indicate that samples might even be more stable in dried blood spots than in frozen plasma. However, there are also limitations: Due to the limited amount of blood per blood spot the sensitivity of a DBS assay is typically not as good as the corresponding plasma assay. In addition to that, a thorough validation needs to be done in order to cover variability induced by different types of sampling cards and a potentially uneven distribu tion of blood over the dried blood spot.

EVALUATION

B.18.12 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 consid ered to be mandatory. During the validation procedure, the assay is evaluated with respect to the overall per formance. The following tests might be considered as a standard set of tests for the validation of a bioanalytical method (Shah et al. 2000; EEC Guidance on Validation 1994, 1996; FDA 2001; Viswanathan et al. 2007; Shah 2007). However, additional tests or modifications of tests might be necessary since the regulatory requirements are not identical in all countries, although the guidances from the FDA are widely accepted:

- Determination of the regression model and weighting factor
- Assessment of assay accuracy and precision (assay variability)
- Analyte stability in plasma at 37°C over 24 h (or other appropriate temperature)
- Investigation of ionization efficiency (effect of analyte matrix on ionization)
- Efficiency of extraction procedure
- Assay specificity and selectivity
- Test of matrix variability (different lots of plasma or other analytical matrix)
- Test of assay robustness (largest batch size)
- Stability of analyte in stock solutions and working solutions
- Whole blood stability of test compound
- Vacutainer effect/container binding (e.g., investigation of potential adsorption effects)
- Stability over typically three or more freeze thaw cycles
- Show the ability to dilute samples with blank matrix in order to extend the calibration range

- Characterization of autosampler carry over
- Effect of hemolysis on analysis (in case of plasma samples)
- Multicomponent analysis test in order to show that different analytes do not influence each other
- Assess the effect of potential co medications on the analysis of the compound of interest
- Signal to noise ratio (5:1 for lowest calibration stan dard or better)
- Long term frozen stability of spiked samples
- Incurred sample reproducibility (ISR)
- Incurred sample long term frozen stability

CRITICAL ASSESSMENT OF THE METHOD

B.18.13 General Acceptance Criteria

B.18.13.1 Accuracy and Precision

Accuracy is defined as the percent difference (%D) from nominal at each concentration level, according to the following equation:

$$\%\mathrm{D} = \frac{(\text{observed conc.} - \text{expected conc.}) \times 100}{\text{expected conc.}}$$

Mean percent differences (M%D) are reported for all data sets.

Precision is defined as the percent coefficient of vari ation (%CV) for each data set and will be calculated by the following equation:

$$\%\mathrm{CV} = \frac{\text{standard deviation} \times 100}{\text{mean}}$$

%CV values will be reported for all data sets.

B.18.13.2 Assay Variability

No more than 33.3% of individual validation samples within a given concentration level may be greater than ±15.0% of nominal, except at the LLOQ, where the accep tance criterion is ±20.0% of nominal. The point estimates for accuracy (bias) and variance (precision) for each val idation level cannot be greater than ±15.0%, except at the LLOQ where the acceptance criterion is ±20.0%. Within run, between run, and total variances are estimated by equating observed and expected mean squares with a one way random effects analysis of variance (ANOVA) for each concentration separately. A statistical test (Lund) for outliers can be performed and points determined to be outliers can be removed prior to ANOVA analysis. If an outlier is observed, data should be reported with and without the outlier.

B.18.13.3 Assay Robustness

If a data point appears to be anomalous and if an n of six or greater per observation is obtained, the Lund test for outliers will be performed and points determined to be outliers will be removed prior to calculation of mean sta tistics. No more than 33.3% of individual validation sam ples within a given concentration level may be greater than ±15.0% of nominal, except at the LLOQ where the accep tance criterion is ±20.0% of nominal. The mean estimates for accuracy (bias) and variance (precision) for each vali dation level cannot be greater than ±15.0%, except at the LLOQ where the acceptance criterion is ±20.0%.

B.18.13.4 Matrix Variability

The acceptance criteria for the matrix variability test apply to each individual lot. For the individual lot, no more than 33.3% of individual samples can be greater than ±20.0%, that is, no more than two out of the six within a lot can be outside the specifications. The mean, SD, %CV, and M%D will be calculated for each matrix lot. The point estimates for accuracy (bias) and variance (precision) cannot be greater than ±20.0%. If a data point appears to be anom alous, the Lund test for outliers will be performed and points determined to be outliers will be removed prior to calculation of mean statistics.

The acceptance criteria for the analysis of the blank samples from the six individuals are based on the raw peak areas found at the retention times of the analyte and internal standard. No more than 10.0% of the blank sam ples can have raw peak areas that are greater than 20.0% of the average peak area of the analyte in the LLOQ valida tion samples.

B.18.13.5 Acceptance Criteria for Stability, Dilution, Hemolysis, and Concomitant Medications

For the tests listed below, no more than 33.3% of individ ual validation samples within a given concentration level could have been greater than ±15.0% of nominal, except at the LLOQ where the acceptance criteria is ±20.0% of

nominal. The point estimates for accuracy (bias) and variance (precision) for each validation level should not be greater than ±15.0%, except at the LLOQ where the acceptance criterion is ±20.0%.

- Stability in stock solutions
- Freeze/thaw stability
- Stability at 37°C for 24 h
- Frozen stability
- Stability in extraction buffer
- Processed sample stability
- Dilution
- Hemolysis
- Specificity against concomitant medications
- Binding
- Multicomponent analysis (if applicable)

B.18.13.6 Blood Stability

The limit of stability is estimated as the time at which the 90% two sided lower confidence bound for the estimated stability from time zero intersects the lower specification limit of 85%. If blood cell partitioning is high, an upper specification limit of 115% will be considered in the event that significant blood cell lysis occurs.

B.18.13.7 Vacutainer Effect

At each nominal concentration, the blood collection tubes will be determined to be equivalent to the polypropylene (control) tubes if the 90% confidence interval for the estimated ratio falls entirely within the 15% specification limits (0.85, 1.15).

B.18.13.8 Autosampler Carryover

No more than 33.3% (i.e., two out of six) of individual LLOQ 1 samples (injected immediately after a HIGH QC sample) should be greater than ±20.0% of the nominal. The point estimates for accuracy (bias) and precision (variance) for all LLOQ 1 samples may not be greater than ±20.0%.

B.18.13.9 Process Efficiency

There is no fixed criterion for extraction (process) efficiency as well as the matrix effect. However, both effects should be determined and they should be on a constant level over the entire concentration range. Matrix effects might be acceptable to some extent when stable isotope labeled standards are used, since this should correct for these effects.

B.18.13.10 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 if the analyte and the corresponding internal standard show different matrix effects. Such matrix effects are typically well compensated when a stable isotope internal standard is used. In case of analogue compounds, which are often used in case that no stable isotope labeled standard is available, the likelihood of an uncompensated matrix effect is a lot higher. For this reason, the usage of stable isotope labeled internal standards is becoming more and more a routine procedure.

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 analytical response for the analyte and internal standard 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 dependent. 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 et al. 2003; Pascoe et al. 2001; Annesley 2003; Hopfgartner 2003; Schuhmacher et al. 2003; King et al. 2000; Liang et al. 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.

B.18.13.11 Sample Analysis (Routine Application of the Assay)

B.18.13.11.1 General Considerations

In general, clinical studies can be divided into two groups from a bioanalytical point of view:

1. Early clinical studies (Phase I and II). In these kind of studies, samples are usually identified by subject and time.
2. Late clinical studies (Phase III), where samples are usually identified as those belonging to the bioanalytical labs by a barcode. This could be described as "nonspecific" labeling.

Both types of studies are typically double blind studies, which mean that it is not clear to the analytical lab, which study subjects got which treatment (placebo/verum). However, in early studies, the bioanalysts are often unblinded prematurely in order to allow for an efficient time saving bioanalysis. Unblinding allows the bioanalytical laboratory to analyze samples of one subject in one batch in the order in which they were obtained (PK profile). In case of these studies it is also possible to analyze placebo samples in separate analytical runs (batches) in order to minimize the risk of contamination during sample work up and analysis. In addition to that, the number of samples to be analyzed might be reduced quite a bit, since this approach allows to analyze only selected samples of placebo subjects. This can very well lower the number of samples to be analyzed by 30% in a typical Phase I study. Typically, only the pre dose sample and the sample at the expected t_{max} would be analyzed. This is especially useful and essential, when only limited time is available for analysis, for example in dose escalation studies, where bioanalytical results are mandatory in order to calculate PK parameters such as AUC, C_{max}, and elimination half life before the clinical study can proceed with the next higher dose level. It should be mentioned here, that all samples from a placebo subject should be analyzed in case that concentration level above LLOQ is observed for a placebo study specimen.

In late stage clinical studies (Phase III), samples are usually labeled in an unspecific way (barcoded) and the bioanalytical lab is not unblinded. In these cases, all samples need to be analyzed and all data will be reported. Separation of placebos and verum samples is not possible. Special attention has to be paid in these studies. A potential swapping of samples is almost impossible to identify for the bioanalytical lab. A second important issue might arise from carry over effects: When samples are analyzed in profile, there will be no huge differences in concentration from one time point to the next one (typically). In case of blind studies this might be different. It could happen that a sample with very low concentration is analyzed right after a sample with a concentration above the upper limit of quantification (ULOQ). In these cases, it might be necessary to reanalyze not only the ULOQ sample but also the one after that.

B.18.13.11.2 Setting Up Analytical Runs

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 the 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; two to three times the LLOQ, 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 $\pm 15\%$ of the nominal value. However, in typical assays, it is considered to be acceptable, if 75% of the standards are within the $\pm 15\%$ criteria. Outliers will not be used for the calculation of the calibration curve. Not all standards at one concentration should be excluded. A similar criterion is applied for the quality control samples: two out of three of the quality control samples should be within $\pm 15\%$ of their nominal value. In any case, at least 50% of the QCs at one level must be acceptable. This leads to the conclusion, that it is not advisable to use odd numbers of QC sample sets, since three QC sets will still fail the criteria in case that more than one QC at one level is out of criteria.

Special consideration should be given to the placing of quality control samples (concentration levels): Especially in cases were most of the unknown samples have concen tration levels between the same two QCs. In these cases, it can be necessary to revalidate the assay with a narrower calibration range, or additional QC levels might be intro duced in order to better characterize the calibration in the concentration range of interest (Viswanathan et al. 2007; Shah 2007).

Processing of analytical data such as chromatograms is another new and very critical quality criterion. Usually, data are processed by integration of chromatograms and subsequently by applying a standard regression and weighting to the data. For these steps, the same parame ters, , which were established during method development and method validation should be used. However, it might be necessary to change integration parameters on a run by run basis in order to achieve proper integration of all data. If this is done, it is considered to be mandatory to document the data before and after manual modification of the integration. Moreover, it is typically not accepted if more than 30% of the samples in a run are modified. In order to avoid problems in routine analysis, the quality of chromatography (e.g., peak shape) should be an impor tant point to consider during method development.

B.18.14 Bioequivalence Studies

Special consideration should be given to the conduct of clinical bioequivalence studies. Although these clinical studies are in general non GLP studies, they are conducted in a way that implies the same procedures as GLP studies. This specifically includes a quality assurance review in order to guarantee that these studies are conducted in agreement with the relevant regulations. Further special requirements are the following:

- Majority of sample concentration data must fall into the calibration range of the assay (only limited dilu tion is acceptable).
- Serial chromatograms of at least 20% of all specimens must be included in the report.
- All chromatograms of calibration samples, quality controls, and blanks need to be reported.
- Samples from one subject should be analyzed together (not be split between batches or even instruments).
- Each reanalysis needs to be adequately documented, especially any repetition for pharmacokinetic rea sons, in order to avoid the possibility of a bias intro duced by that reanalysis. This is valid also for other clinical studies.

B.18.15 Incurred Sample Reproducibility (ISR)

A rather new topic, which is now already considered to be a regulatory requirement for GLP studies as well as clinical studies is the conduct of the so called ISR Test "incurred sample reproducibility Test" and also ISA and ISS (incurred sample accuracy and incurred sample stability) (Viswanathan et al. 2007; Shah 2007). The term "Incurred samples" describes samples that are obtained after actual dosing of the compound of interest. ISR samples are natural samples that are not artificially prepared (spiked). In fact, incurred samples are nothing else but regular specimens from a GLP study or clinical study.

The samples that are used during method validation are typically spiked samples, which do not contain any metab olites of the drug administered. In addition to that, vari ability in the matrix of patients might be high, which could lead to analytical problems as well. For these reasons, it is now considered to be mandatory to perform the ISR.

Assessment of incurred specimen reproducibility (ISR) is usually performed for each analyte and matrix (plasma, serum, urine, etc.) in several studies and with the respective validated assay:

- Primary rodent toxicology study (typically rat) (Depending on the assay validation strategy, at the time of the exploratory non GLP study only an explor atory assay may be available.) with an exploratory assay
- Primary non rodent toxicology study (typically dog) with an exploratory assay
- Primary rodent GLP toxicology study (typically rat)
- Primary non rodent GLP toxicology study (typically dog)
- All first in man studies with healthy volunteers single dose
- All first in man studies with healthy volunteers repeated dose
- All studies with special patient populations, for exam ple, renally or hepatically impaired patients
- All drug drug interaction studies
- All bioequivalence studies

For ISR testing, 5% of the total numbers of study samples of each matrix are used, but no fewer than 30 samples of each matrix are taken into consideration . Based on the precision of the validated assay a statistical approach may result in a different number of study samples, which should be used for ISR testing. ISR testing is described in the study plan and reported in the bioanalytical phase report for the study.

The ISR is performed during or at the end of the study and the same validated assay (in case of primary non GLP studies this could be exploratory assays) have to be used for the selected ISR samples and the study samples. The selected samples for ISR must have an acceptable analyt ical result, which has been obtained without dilution of the respective sample and the result should be within three times the LLOQ and the ULOQ of the validated assay. A sufficient volume to perform the ISR should be left and samples must be within the documented stability period. Additionally, the selected samples should originate from different animals or subjects and should represent the pharmacokinetic profile of the respective analyte. The ISR concentrations are only used for ISR assessment and are not used for pharmacokinetic or toxicokinetic analysis.

$$\%\mathrm{D} = \frac{(\text{repeat conc.} - \text{original conc.}) \times 100}{\text{mean conc.}}$$

After acceptance of the run acceptance criteria for calibra tion and quality control samples, no more than 33.3% of the ISR samples from each study can have a concentration greater than ±20% (physicochemical) or ±30% (bioas say) of the initial concentration. Any individual ISR sam ple with a concentration outside of the acceptance limit will not be reanalyzed, provided that the total number of ISR samples failing does not exceed 33.3%. However, in those cases where more than 33.3% of ISR results exceed the acceptance limit, an investigation into the cause of ISR failure will be initiated. The impact of ISR failure on existing specimen data must be assessed and documented.

B.18.16 Incurred Sample Accuracy (ISA)

Assessment of short term incurred specimen analyte sta bility (ISA) is usually performed for each analyte and matrix (plasma, serum, urine, etc.) in several studies and with the respective validated assay:

- Primary rodent toxicology study (typically rat) (Depending on the assay validation strategy, at the time of the exploratory non GLP study only an explor atory assay may be available) with an exploratory assay
- Primary non rodent toxicology study (typically dog) with an exploratory assay
- Primary rodent GLP toxicology study (typically rat)
- Primary non rodent GLP toxicology study (typically dog)
- All first in man studies with healthy volunteers single dose
- All first in man studies with healthy volunteers repeated dose
- All studies with special patient populations, for exam ple, renally or hepatically impaired patients
- All drug drug interaction studies

Due to limited volume of study samples, the investigation of short term stability of incurred specimen stability on individual incurred samples is not feasible and therefore a pooled stability sample is used for ISA assessment instead. ISA testing is described in the stability study plan and reported in the stability report.

The ISA is performed at the end of the study, which includes acceptance of all study concentration data of the respective matrix and successful ISR testing with the same validated assay (in case of primary non GLP studies this could be exploratory assays) as was used for study samples and ISR assessment. The selected samples for ISA must have an acceptable analytical result and must be representative of the study design (multiple time points, dosing days and dose groups, multiple sub jects/animals, dosed subjects/animals only, impaired subjects only, etc.). As far as the study and ISR are accepted, the ISA samples are pooled to one bulk stability sample. The concentration of the bulk stability samples should be within the LLOQ and the ULOQ of the vali dated assay.

The ISA samples must be transferred into the same type of tubes, which are used for the long term storage of the study samples. Replicate aliquots ($n = 9$) of the freshly prepared pooled bulk stability sample should be stored frozen (t_{0h} at approximately −20°C or other validated storage temperature) and the remainder stored for 24 h at room temperature (t_{24h}). Both the t_{0h} and t_{24h} samples are analyzed within the same run to reduce inter assay variability.

After acceptance of the run acceptance criteria for calibration and quality control samples, the mean ratio of data at t_{24h} versus the data at t_{0h} and its 90% confidence interval will be calculated using Fieller's theorem for each study type. Incurred short term specimen analyte stability will be assured if the 90% confidence interval for the estimated ratio falls entirely within the 15% specification limits (85%, 115%).

The results of short term incurred specimen stability including the statistical results are documented in the stability report.

B.18.17 Incurred Sample Stability (ISS)

Assessment of long term incurred specimen analyte stability (ISS) is usually performed for each analyte and matrix (plasma, serum, urine, etc.) in all human repeated dose studies and with the respective validated assay. The ISS of incurred animal specimens is not routinely performed unless the ISA in animal species indicate analyte instability.

Due to limited volume of study samples, the investigation of long term stability of incurred specimen stability on individual incurred samples is not feasible and therefore a pooled stability sample is used for ISS assessment instead. ISS testing is described in the stability study plan and reported in the stability report.

The ISS begins at the end of the first in men study that includes acceptance of all study concentration data of the respective matrix and successful ISR testing with the same validated assay as was used for study samples and ISR assessment. The samples for ISS are usually selected from the highest dose group on the last day of dosing as these samples most likely represent steady state concentrations of analyte and metabolites.

As far as the study and the ISR are accepted, the ISS samples are pooled to one bulk stability sample. The concentration of the bulk stability samples should be within the LLOQ and the ULOQ of the validated assay. In principle, the same bulk solution for ISA testing can be used for ISS testing, as far as the necessary volume of the bulk sample can be used as well as for ISA and ISS investigation.

Replicate aliquots ($n = 6–9$) of the freshly prepared pooled bulk stability sample should be analyzed immediately (t_{0h}). The remaining ISS bulk samples must be transferred into the same type of tubes, which are used for the long term storage of the study samples and should be stored frozen at approximately −20°C or/and at other appropriate temperatures (−80°C or below −130°C). After 1, 3, 6, 12, 18, and 24 months replicate stability samples ($n = 6–9$ per time point) will be removed from frozen storage and analyzed with the same assay as for all other time points. A minimum of seven time points are required to provide sufficient statistical power to demonstrate stability. Depending on the matrix, the time points for ISS testing may vary.

After acceptance of the run acceptance criteria for calibration and quality control samples, the data should be analyzed by regression analysis. The two sided 90% confidence intervals for the mean concentrations will be calculated at each time point, within the regression framework. The limit of stability will be estimated as the last time (within the range of observed time points) at which the 90% two sided lower confidence interval for estimated stability from time t_{0h} is contained within the 15% specification limits (85%, 115%).

The results of long term incurred specimen stability and the statistical results including detailed statistical methodology are documented in the stability report.

MODIFICATIONS OF THE METHOD

The following table lists a selection of method changes that are often required during drug development. These changes usually require some degree of method revalidation. The listed activities are only a guide. In all cases, judgment of the bioanalyst will be the key factor to determine the necessary steps in order to cover the required method change.

Change to validated method	Test required
Reduce LLOQ of assay, while maintaining similar dynamic range of assay (e.g., change a 1 to 500 ng/mL assay to a 0.1 to 50 ng/mL assay)	Perform full assay validation, including calibration model, core studies, and all satellite studies. Perform and report as a new study
Increase LLOQ of assay, while maintaining similar dynamic range of assay (e.g., change a 1 to 500 ng/mL assay to a 10 to 5,000 ng/mL assay)	Perform calibration model, assay variability test, autosampler test, and dilution test
Verification of analyst competency to perform assay (multiple species of same assay are deemed equivalent)	Assay verification test, for example, run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)
Verification of instrument (same platform, e.g., API4000 to API4000) to perform assay	Assay verification test, for example, run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)
Verification of instrument (new platform, e.g., API4000 to API5000 or API5000 to Quantum Ultra)	Full assay variability with statistical analysis. Consideration being given to performing calibration model if significant differences in sensitivity observed between platforms
Change of autosampler	Perform autosampler test
Changes of assay consumable (tube type and or vendor, solvent vendor, plate vendor, etc.)	Assay verification test, for example, run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)

Additional freeze/thaw stability	Perform freeze/thaw stability test
Additional biological fluid stability	Perform additional biological fluid stability test
Additional processed stability	Perform additional processed sample stability
Additional dilution factor required	Perform dilution factor test
Additional major concomitant medication	Perform concomitant medication test
Chromatographic modification to resolve in vivo interference (gradient, flow or modification of column geometry such as length, diameter, or particle size)	Assay verification test, for example, run full calibration curve plus six replicates at all validation levels (LLOQ, LOW QC, MID QC, HIGH QC)
Change to assay specificity (e.g., chromatographic or extraction chemistry)	Full assay variability test with statistical analysis and full matrix variability test
Increase signal to noise (e.g., increase injection volume)	Assay robustness test
Decrease signal to noise (e.g., reduce injection volume)	Full assay variability with statistical analysis
Increase run size	Assay robustness test
Additional anticoagulant (same species)	Full calibration curve with three to six replicates at the LOW AND HIGH validation levels against a calibration curve prepared in the original anticoagulant
Additional nonclinical species (same matrix)	Perform partial assay validation, including core study and all appropriate satellite studies. Perform and report as a new study
Additional matrix (same species)	Perform full assay validation, including calibration model, core studies, and all satellite studies. Perform and report as a new study
Physiological proxy matrices	Full assay variability with validated matrix as calibration samples and physiological proxy matrix as validation samples. It also includes statistical analysis
Addition of metabolite	Perform full assay validation, including calibration model, core studies, and all satellite studies. Perform and report as a new study
Effect of stable-labeled analyte (e.g., ^{2}H, ^{15}N, or ^{13}C) on validated assay	Full assay variability with statistical analysis and crossover test
Effect of radio-labeled analyte (^{14}C) on validated assay	Use ^{14}C standard as test article. Alternative approaches, such as ion summing, and theoretical correction factor may be used when proven
Change SRM transition in assay (either for analyte or stable-labeled internal standard)	Full assay variability with statistical analysis. Full matrix variability
Transfer of assay from site to site	Perform full assay validation, including calibration model, core studies, and all satellite studies. Perform and report as a new study

REFERENCES AND FURTHER READING

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B.19 Biomarker Definition and Validation During Drug Development

Sibylle Hess · Marie-Laure Ozoux · Martin Gerl

B.19.1 Introduction

Clinicians have used numerous biomarkers over the past centuries to monitor disease progression, therapeutic pro gress, and efficacy of interventions. Imaging biomarkers (X ray), biophysical biomarkers (blood pressure, pulmo nary function test), and biochemical biomarkers (glucose, insulin) have been applied. The majority of biomarkers are biological molecules found in blood, urine, and other body fluids such as bile, cerebrospinal fluid, sputum, etc. or are quantified in tissues.

In pharmaceutical companies, the strategic use of bio markers in drug development was controversial for quite a while. Recently, a wide variety of biomarkers and com binations thereof have been recognized. The US Food and Drug Administration (FDA) states that "biomarkers are the future of drug development." They link the mechanis tic, reductionist science that has been carried out to the whole organism. If we are going to link basic science to human outcomes, we are going to have to use biomarkers; this includes new biomarkers and also networks and path ways. They will not necessarily be single markers, but a set, a pathway, a designation, or signature. This will be the future of medicine (Woodcock 2009). With the concept of personalized medicine and targeted therapies, biomarkers can be a valuable tool for making informed decisions throughout the whole drug development process.

B.19.2 Biomarker Definitions

The following definition of biomarkers came out of the April 1999 "Biomarkers and Surrogate Endpoints: Advancing Clinical Research and Applications" consensus conference held by the FDA, the National Institute of Health (NIH), academia, and industry (Atkinson et al. 2001; Wagner 2008).

A *biomarker* is "a characteristic that is objectively mea sured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention."

A *clinical endpoint* quantifies a characteristic related to how a patient feels, functions, or survives. Clinical end points are distinct measurements or analyses of disease characteristics observed in a study or a clinical trial that reflects the effect of a therapeutic intervention. Clinical endpoints are the most credible characteristics used in the assessment of the benefits and risks of a therapeutic inter vention in randomized clinical trials.

A *surrogate endpoint* (*SEP*) is a biomarker that is intended to substitute for a clinical endpoint and expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysio logic, or other scientific evidence.

There are relatively few biomarkers that qualify for surrogate endpoints: tumor imaging in oncology, low density lipoprotein (LDL) cholesterol in heart disease, glycosylated hemoglobin A1C (HbA1C) in diabetic con trol, blood pressure in hypertension, plasma testosterone in prostate cancer, and CD4 count and RNA viral load in HIV AIDS (Lathia et al. 2009).

In 2009, Lathia et al. conducted a simple survey based on drug information from WebMD, on the availability of medicines (www.webmd.com). They compared the num ber of available therapies for conditions with surrogate endpoints with the number of therapies available for conditions with efficient clinical endpoints, and also with those for which there are no efficient clinical end points. "Efficient" clinical endpoints were defined as those enabling proof of concept in studies of <6 weeks and pivotal trials of <6 months of dosing. The median number of products approved by the FDA per indication where clinical endpoints are efficient was 42; where sur rogates exist, the median number of products was 61; and where no surrogates exist and clinical endpoints are inefficient, the median number of products was 0. The authors concluded that the existence of either an efficient clinical endpoint or a surrogate clinical endpoint can be an enabling factor for success in the drug development process, where >80% of new drugs fail during clinical phases.

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.19,

B.19.3 Current Status and Trends in Biomarker Science

The rising costs of drug development and the challenge of facing new and re emerging diseases are putting high pressure in the selection of suitable drug candidates.

According to some estimates, it costs over 800 million dollars to develop a new drug (Tufts Cent. Study Drug Dev 2001). Over the past 20 years, the success rate of drug development has declined. There are several reasons for that, including work on less validated, novel targets with limited availability of appropriate animal (disease) models and some focus on chronic diseases which require long term treatments. Almost 50% of late phase clinical trials fail, mostly in differentiating the test drug from placebo, i.e., establishing efficacy. Thus, the main goal of bio marker driven drug development is to predict drug efficacy more quickly and to "kill early" a drug candidate, if necessary.

The failure to customize therapy and, most impor tantly, also to predict adverse drug reactions (ADRs) results in failure of most drug candidates entering Phase I clinical testing to finally reach the patient (Kola and Landis 2004). As a consequence, health authorities notice a depress ing number of drug approvals over the past decade (Owens 2007). Knowledge obtained before treatment of a drug's likely efficacy, metabolism, and toxicity could greatly reduce the costs and mortality from ADRs.

There is a wide consensus that drug development can be accelerated and late phase trial success rate can be improved by the proper selection and application of bio markers. The FDA has recognized this and states that "it is imperative that biomarker development be accelerated along with therapeutics" (J. Woodcock, Director of the Center for Drug Evaluation and Research, FDA, February 5, 2009). This is not an easy task as there are various obstacles: complicated biology, technology, and assay lim itations, lack of standards, commercialization, and ethical issues.

Biomarkers can be influential in every phase of drug development, from drug discovery and preclinical evalu ation through each phase of clinical trials and into post marketing studies (*Fig. B.19 1*).

Back to the fourteenth century or earlier, practitioners would regularly inspect the color and sediment of a patient's urine ("uroscopy") and make a diagnosis based on what they observed. Physicians were able to diagnose diabetes centuries ago by using patient charac teristics (thirsty patient who urinated a lot) and by tasting the urine of patients. If the urine was sweet, it signaled

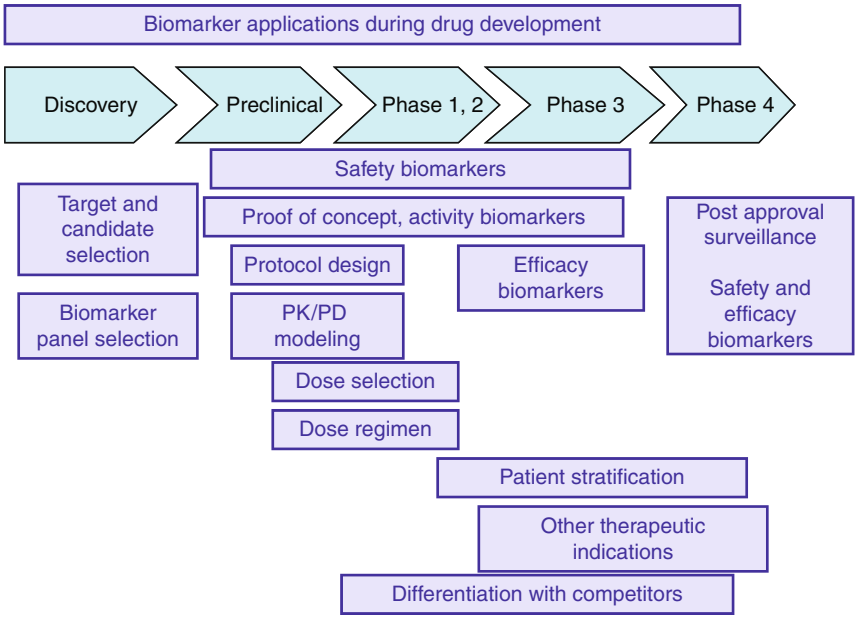

Figure B.19-1

Biomarker application during various phases in drug development. PK, pharmacokinetic (study of how the body absorbs, distributes, breaks down, and eliminates drugs). PD, pharmacodynamic (study of the biochemical and physiological effects of drugs and the mechanisms of drug action and the relationship between drug concentration and effect)

classical diabetes, which was termed diabetes mellitus (meaning "honey" in reference to its sweetness) by the English physician Thomas Willis in 1675 (Molnár 2004).

Originally, the term "biomarker" referred to such physiological indicators as body temperature, blood pres sure, or heart rate that signaled an imbalance in the body.

Today, a biomarker can be a blood , or urine based test, a test to monitor lung function, the measurement of blood pressure, a gene sequence or mutation, mRNA expression profile, the quantification of a tissue protein, circulating tumor cells, and numerous imaging methods such as computed tomography (CT) or magnetic reso nance imaging (MRI) among others.

The concept of "translational medicine" is defined by improving the "translation" of early biomedical research findings into later stages of development. Biomarkers can be essential tools because they can potentially bridge ani mal and human data, guide dose adjustments, guard safety in animal models and in early clinical development, and establish initial proof of efficacy in proof of concept stud ies. Establishing the latter is the most risk reducing step in drug development, since it provides initial indication that the concept underlying the choice of target provides ben efit to the target population. After this initial indication of efficacy (and also safety), larger and thus very costly trials are needed to confirm the results in powered, placebo controlled studies (Aronson et al. 2008; van Gool et al. 2009). The hope is to reduce attrition, particularly at the later, more costly stages of drug development. In many cases, biomarkers used in animal experiments help to predict efficacy and safety in man (Clayton et al. 2006). The assessment of these key indicators which are needed for the translational prediction is not easy, since some biomarkers do not exist in humans (e.g., certain hor mones), or cannot be measured ethically (e.g., serial brain slices).

Many pharmaceutical companies have begun to move from the study of single genes, single mRNA transcripts, single proteins and single metabolites to studies that encompass entire genomes, transcriptomes, proteomes, and metabolomes and invest in "omics" technologies: genomics, transcriptomics, proteomics, and metabolomics (Holmes et al. 2008; Lin et al. 2009; Ross 2009).

These high throughput technologies drive the science of "individualized" or "personalized" or "targeted" ther apy and tackle the approximately 20,000 30,000 genes which translate to roughly 200,000 different proteins in humans. An estimated 15 million loci are present along the genome where a single base can differ between indi viduals or between populations. More than 3 million such locations, known as single nucleotide polymorphism (SNP), have been identified. There are an estimated 100,000 splice variants of messenger RNA transcripts.

The Human Proteome Organization (HUPO, Montreal, QC, Canada) reported approximately 10,000 core proteins in human plasma; of these, only 10% can be effectively sampled with current approaches (Rifai et al. 2006). It is expected that the proteome (the whole of the expressed proteins of an individual at a given time) and also the metabolome (the collection of all metabolites of an organ ism at a given time) are unique for each and every indi vidual and have the potential to truly individualize patient care. Because the level of metabolites responds to environ mental effects (e.g., nutrition levels, stress, exercise, etc.), it can provide a sensitive measure of current physiological activity. The challenge is, by using biomarkers, to differ entiate and group patient populations, having multiple medical conditions, being treated with multiple medica tions and being influenced by a variety of diets and body mass indices among other factors.

The "omics" approach enables the detection of small changes of molecules in tissue and fluid composition by using powerful and sophisticated methodologies such as multiplex technologies with protein arrays and multiple reaction monitoring (MS), gel electrophoresis, liquid and gas chromatography, nuclear magnetic resonance (NMR) spectroscopy, and liquid chromatography with electrochem ical detection, Several assay formats are used to quantify protein biomarkers in (pre)clinical samples; enzyme linked immunosorbent assay (ELISA) based immunoassays are still the golden standard and have been the workhorse of protein measurement for more than half a century, with hundreds of assays available, predominantly on the diagnostic market. Immunoassays (planar or suspension microsphere) that pro vide multiple, parallel measurements on the same specimen (multiplex assays) have been developed during the last years, resulting in an increase of the number of biomarker measurements per sample, especially in the diagnostic and prognostic field.

B.19.3.1 Biomarkers Are Currently Used in:

B.19.3.1.1 Target Discovery

Biomarkers have been used to identify targets for therapy such as vascular endothelial growth factor (VEGF) and its receptor (VEGFR), human epidermal growth factor recep tor 2 (HER 2), the oncogene ras, etc. For example, HER2 is frequently amplified in breast cancer and is associated with poor prognosis. This correlation between biomarker and clinical outcome was the rationale for the development

of anti HER2 therapy. Sophisticated methods such as focused RNA interference (RNAi) screening, kinome wide inhibitor profiling (kinome: activity of all kinases of whole cell lysates) mass spectrometry (MS) based phosphoproteomics, and analysis of proteome microarrays are used. Some of these biomarkers may also be applicable for dose selection.

B.19.3.1.2 Diagnostic/Disease Identification

A "diagnostic" biomarker is directly linked to the etiology of the disease, e.g., elevated blood glucose concentration for the diagnosis of type 2 diabetes (T2DM); a tumor biopsy showing overexpression of a growth factor recep tor, or a tumor derived protein in the blood (e.g., carcinoembryonic antigen=CEA).

B.19.3.1.3 Patient-Selection

Depending on the purpose, biomarkers of disease severity, or pharmacogenetic/pharmacogenomic biomarkers can be used to identify patients who are more or less likely to respond (or experience adverse effects). Thus, a personal ized approach for drug delivery is facilitated. For example, for the development of an osteoarthritis (OA) drug, indi viduals with "low grade" OA versus individuals with "medium" or "high grade" OA can be selected. Populations of good versus poor responders can be identified to maxi mize a drug effect, such as individuals with variations in certain cytochrome P450 (CYP450) family of enzymes, for example CYP2C9 for warfarin or codeine treatment (Meckley et al. 2010; Sindrup and Brosen 1995).

B.19.3.1.4 Disease Severity

A disease severity biomarker classifies and evaluates the extent of the disease and gives a quantitative indication of the stage of the disease (e.g., elevated brain natriuretic peptide (BNP) in heart failure).

B.19.3.1.5 Dose-Selection

Many clinical trials fail as a result of inappropriate dosing. For most development programs, dosing decisions are guided by allometric scaling (extrapolation from effective dosages in animals to those judged equivalent in humans). Biomarkers that enable, for example, quantification of receptor occupancy by a drug during multiple time points in the dosing cycle could support right dosing. One can use such a biomarker to avoid doses above those needed to saturate a receptor, and, with a sufficiently sensitive assay, those below that required to achieve some receptor occu pancy. Very often, pharmacodynamic biomarkers are used as an indirect guide to dose selection. A dose that triggers a pharmacodynamic biomarker can be different from that required for therapeutic effect.

B.19.3.1.6 Efficacy Biomarkers/Predictive Biomarkers/Pharmacological Response

These biomarkers are used to monitor clinical responses and beneficial effects of therapeutic intervention strate gies. Unlike pharmacodynamic biomarkers, these bio markers of disease progression monitor events that lay in a series along the chain of events that link modulation of the drug target with the pathophysiological consequences (i.e., symptoms or disease progression) for which the treatment is intended. LDL is an efficacy marker for statin therapy, glucose levels for insulin therapy and plasma norepinephrine is a marker of increased or reduced sym pathetic outflow used for neurological therapies.

B.19.3.1.7 Toxicity/Safety

Toxicity and unacceptable breaches of clinical safety are the highest causes of attrition during development of novel chemical entities. Safety issues also account for more than 90% of withdrawals of marketed pharmaceuti cals, and ADRs account for more than 100,000 deaths, hospitalizations, and 100 billion dollars in healthcare costs in the USA each year (Bond et al. 2006). Therefore, there is a need for biomarkers that can identify, prior to dosing, individuals at risk for toxic side effects. Many biomarkers are already in use such as alanine aminotrans ferase (ALT) and bilirubin to assess liver injury; serum creatinine as a measure of renal function, or troponin quantification to monitor potential adverse cardiac effects or myocardial infarction.

B.19.4 Biomarker Assay Development and Validation

Assay development and validation for biomarkers tends to be more complicated than those for drug molecules

because of several factors: heterogeneity of the endogenous biomarkers (i.e., variable glycosylation, protein variant form), lack of well characterized reference substance, low in vivo concentration range (or the opposite: high in vivo concentration range, depending on the status of the disease), matrix interference variable with the disease status, instability of the biomarker, sensitivity to the process of sample collection and processing, etc. (*Table B.19 1*). The extent of a biomarker's analytical validation should be dictated by the purpose of its use (*Table B.19 2*). For instance, an assay can be used to measure the effect of a drug on a particular biomarker with adequate precision and accuracy in a proof of concept study but the same assay will be inappropriate for an early cancer screening program because of not enough sensitivity (Hodgson et al. 2009).

Once the biomarkers are selected in discovery using "omics" approaches (genomics, proteomics, and metabolomics), extensive analytical work is needed to qualify them as supporting tools for drug development and later, for diagnostic purposes.

Several authors propose strategies to tailor the analytical validation to the purpose of the biomarker (Hodgson et al. 2009; Lee et al. 2006).

In the following section, the plan to develop and validate an immunoassay for a biomarker (Hodgson et al. 2009; Lee et al. 2006, 2009) is described.

B.19.4.1 Development of Assay

The selection of reagents (capture and detection antibodies), standard reference, and/or the selection of RUO kits to quantify a biomarker is the preliminary step of each assay development. Depending on the "fit for purpose," the choice is based on the assay sensitivity, reproducibility, specificity, and recovery, in addition to the stability information.

In a preliminary step, the sensitivity of the assay is based on the dynamic range of the standards. An estimate of the reproducibility is calculated using the same standards on at least three different batches. The specificity is

Table B.19-1

Application of method validation of biomarkers from drug development versus drug analysis

	Biomarker for drug development study	Drug analysis
Intended application	Safety, activity proof of concept, efficacy, PK/PD modeling	Pharmacokinetic analysis
Method types	(1) Qualitative assays	Quantitative assays
	(2) Quasi-quantitative assays	
	(3) Relative quantitative assays	
	(4) Quantitative assays	
Reference standard	Many are not well characterized, RUO kits	Well defined, under GMP
Analytes	Endogenous biomarkers, may exist under several isoforms, active and inactive configuration	Exogenous drug, well defined
Method and reagent source	Developed in house, RUO kits	Developed internally
Assay selectivity and specificity	May be not specific, matrix effect	Specific
Calibrator matrix	Substituted matrix (buffer, depleted biological matrix, matrix from different species)	In study matrix
Validation samples (VS) Quality controls (QC)	VS and QCs made by spiking reference standard into buffer or matrix, or by pooling actual samples at low and or high level Four to six levels	Four to five VS levels made in study matrix Three QCs made in study matrix
Accuracy	Mostly relative accuracy QC in every run	Absolute accuracy QC in every run
Assay acceptance criteria	Confidence intervalle or a variant of four to six X rule for each run	Confidence intervalle or a variant of four to six X rule for each run

RUO research use only kit

Table B.19-2

Extent of validation (level of complexity: 1 3) based on fit-for-purpose approach

	(1) Exploratory evaluation	(2) Dose and dosing regimen selection/proof of concept	(3) Safety/diagnosis
Choice of matrix	Based on feasible conditions	Define the best matrix (minimum interferences and analyte stability)	(2)+as close as possible to analyte production
Sample collection and processing	Based on feasible conditions	Establish short-term, bench top, freeze/thaw stability, optimize conditions and effects on assays	(2)+Long-term stability
Documentation	No validation plan required	Validation plan required	Validation plan required
	Use the technical worksheet of RUO kit or detailed assay description for in house assay	Validation report required for RUO and in-house assays	Validation report required for RUO and in-house assays
Standard reference	Limited characterization, stability initiated	Well described, establish change control from batch to batch	(2) GMP
Calibrators	Use RUO standards, or six nonzero standards	Minimum of six nonzero standards	Minimum of 6 non zero standards
Dynamic range	Use three validation runs to define LLOQ and ULOQ based on standards	Use at least six validation runs LLOQ and ULOQ are defined on accuracy and precision of independent validation samples	(2)+Sensitivity is the critical parameter
Standard fitting	Point to point is authorized	The best fitting is defined on at least three runs	(2)
Reagents	Limited characterization, stability initiated	Well characterized	(2)+stability evaluation
Accuracy	Defined on VS or controls provided in RUO kits, on at least three independent runs	Defined on four to six VS levels, using at least six independent runs	(2)
Precision	Defined on VS or controls provided in RUO kits, on at least three independent runs	Defined on four to six VS levels, using at least six independent runs	(2)
Sensitivity	Defined on RUO kit worksheet or defined as the lowest standard for in house assay	Based on independent VS samples, assayed on at least six independent runs	Adequate sensitivity must be achieved to make a difference between normal and pathological population
Dilution effect	On VS and actual samples	(1)	(1)
Selectivity and specificity	Use RUO worksheet information	Towards other isoforms, endogenous ligands, and co-medications	Toward other isoforms, endogenous ligands, and co-medications
	For in house assays, test the selectivity and specificity toward tested drug		
Biomarker stability	One cycle of freeze/thaw Stability at room temperature at +4°C up to 4 h a least	Three cycles of freeze/thaw Stability at room temperature and at 4°C up to 24 h	(2)+2 year stability On actual samples
	Dilution effect	Stability during extraction process when needed	
	Short-term stability		
	On VS or actual samples for a period bracketing the interval between sample collection and analysis	Stability at −20°C and −80°C	
		1 year stability	
		On VS and/or actual samples	

tested by spiking buffer samples with the co administered drug and other available isoforms of the biomarker or endogenous pro biomarkers or products of degradation.

Then the recovery is tested using different sources of biomarkers, when available, samples spiked in buffer and in study matrix at low, mid, and high levels. The recovery should be the same in buffer and in study matrix.

During the assay development, it is also important to test the matrix effect. The biomarker can circulate as a free protein or can be bound to its target or other transporter proteins, for instance. In the latter case, the sample processing may affect its binding and makes it less or more accessible for capture , and detection antibodies with an impact on assay reproducibility and recovery. This matrix effect can be evaluated at early stage by diluting the same sample at different dilutions. Either the dilution has no effect on the recovery, or it can happen that the recovery increases in parallel with the dilution of the sample, demonstrating a matrix effect (e.g., insulin like growth factor1 [IGF 1] in monkey serum) (*Fig. B.19 2*). In such a case, it is recommended that the dilution at which there is no more effect on the recovery be evaluated and this dilution be used as the minimum dilution factor (MDF).

B.19.4.2 Calibration Curve

At least six nonzero standards should be prepared either in in study matrix or buffer. Anchoring points (lower than the low limit of quantification or higher than the upper limit of quantification) may be used to improve the overall curve fit. The fitting of the standard curve should be evaluated using at least three independent runs. The choice is based on the back calculated concentration of each standard.

B.19.4.3 Validation Samples and Quality Controls Samples

A minimum of four validation levels should be used. One validation level should be at the proposed LLOQ of the assay and one should be at the upper boundary ($>0.75\times$ ULOQ) of the standard curve (HIGH QC). The remaining two validation levels should be placed at the low end (LOW QC $\leq 3\times$ LLOQ) and the middle of the calibration range (MID QC). When needed, a super high validation sample should be added to mimic the expected concen tration in in study samples. This sample must be diluted before analysis.

For assay variability studies, all four validation levels (LLOQ, LOW, MID, and HIGH levels) should be used. For matrix variability and satellite studies, different validation levels will be utilized as defined below. During routine analysis, QCs used will be at the LOW, MID, and HIGH levels.

Validation samples should be prepared in free in study matrix. If no free matrix is available or cannot be prepared, it is recommended to use in study matrix spiked with biomarker or diluted with standard dilution buffer taking into account the endogenous predetermined

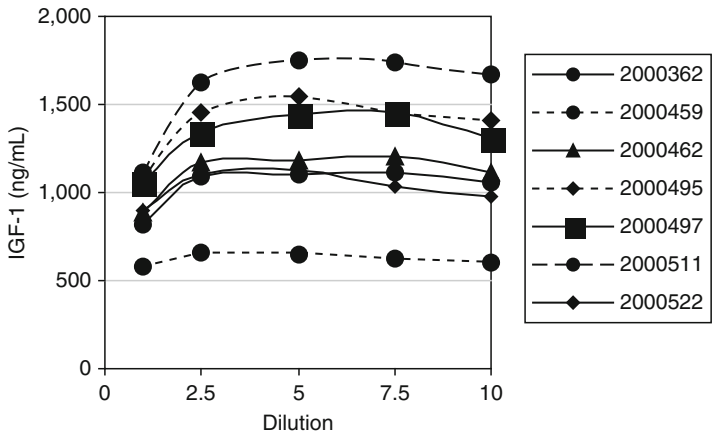

Figure B.19-2

Evaluation of matrix effect and minimum dilution factor. Seven individual monkey sera were analyzed after serial dilution in buffer. An MDF equal to 5 was used for further assay validation

biomarker level. In that case, only a relative accuracy can be determined.

The target concentrations of these validation samples/quality controls should be determined in at least two independent runs with three determinations per level in each run.

B.19.4.4 Assay Variability Study

The assay variability study should contain six different runs, each containing a full calibration curve, and triplicate validation samples at each validation level. In general, sources of variability such as instruments and analysts may be introduced when appropriate.

Large run size evaluation:

In order to estimate if a bias occurs from the beginning to the end of a run, this run should contain a full calibration curve and then several sets of validation samples at all levels.

B.19.4.5 Variability of Endogenous Levels

A minimum of ten individual lots of matrix from male and female origin should be analyzed (at least from five males and five females). When available, the matrix from target population should be evaluated.

B.19.4.6 Stability of Reference Standard and Working Solutions

Generally, the stability of reference standards is provided by the manufacturer. Working solution is prepared by diluting the reference standard.

The stability of the working solution must be evaluated : If stability information is not available, buffer samples or appropriate matrix samples will be prepared from working solutions at the LOW and HIGH QC levels and analyzed on the day of preparation ($t=0$) against a freshly prepared calibration curve (from separately prepared working solutions). The remaining stability working solutions will be stored at the temperature defined in the method until needed for analysis. After approximately 1 and 3 months storage (depending on the use), buffer samples or appropriate matrix samples ($n=3$ to 6) will be prepared from the stability working solutions and analyzed against a calibration curve freshly prepared from working solutions.

B.19.4.7 Freeze/Thaw Stability

Validation samples at the LOW and HIGH levels ($n=3$ to 6 per level) should be analyzed up to three freeze/thaw cycles against a freshly prepared calibration curve. Each freeze cycle should last at least 12 h at approximately 20°C and thawing time should be 1 h. The process for thawing samples (i.e., unassisted in the refrigerator or at room temperature, assisted in a heated water bath, etc.) must be described and followed. It is recommended that these studies be conducted in tubes that are intended for long term storage of samples.

B.19.4.8 Stability in Matrix at 37°C

Stability at 37°C will be assessed, as appropriate. The duration will be defined according to the sample process under evaluation. Samples at the LOW and HIGH levels ($n=3$ to 6 per level) should be analyzed after incubation at 37°C. It is recommended that these studies be conducted in tubes that are intended for long term storage of samples.

B.19.4.9 Frozen Stability

Samples at the LOW and HIGH QC levels ($n=3$ to 6 per level) should be analyzed after storage at −20°C and/or 80°C for 1 and 3 months against a freshly prepared calibration curve.

Additional time points and/or temperatures may be conducted if required. It is recommended that these studies be conducted in tubes that are intended for long term storage of samples.

B.19.4.10 Long-Term Frozen Stability

When appropriate, the long term frozen evaluation should be performed in quality controls for animal matrix and samples from dosed subjects should also be evaluated for human matrix. The long term stability should be assessed up to 2 years in human matrix. In animal matrix, the duration should be dependent on study design and planning.

B.19.4.11 Processed Sample Stability

When appropriate, extracted validation samples at the LOW and HIGH levels ($n=3$ to 6/level/time point) should

be analyzed after appropriate storage temperature and storage time against a calibration curve within validated stability limits.

B.19.4.12 Stability in Extraction Buffer

This experiment is appropriate and recommended when matrix pH is modified toward extreme values (<pH 3 or >pH 9) during sample processing. The stability of analyte (s) at the LOW and HIGH concentrations (n=3 to 6/level) in buffer diluted matrix should be tested after approxi mately 3 h storage at ambient temperature and analyzed against a calibration curve within validated stability limits.

B.19.4.13 Dilution

The ability to quantify biomarker concentrations above the ULOQ should be evaluated using dilution levels that are intended for routine analysis (e.g., from 1:2 to 1:500 or higher if appropriate with n=3 to 6 per level). The highest dilution should be conducted at a concentration equiva lent to the calibration range multiplied by the maximum dilution factor (referred to as the SUPER HIGH VS). If the solubility factors do not permit this evaluation, the highest possible concentration should be used. When the range of dilution is validated, a dilution factor within this range can be used in routine analysis.

B.19.4.14 Influence of Anticoagulant

Validation samples at the LOW and HIGH QC levels (n=3 to 6/level/anticoagulant) using matrix containing different anticoagulants (e.g., Heparin, citrate, etc.) should be ana lyzed against a calibration curve used during the validation.

B.19.4.15 Autosampler Carryover

Validation samples at the LLOQ (n=12) and SUPER HIGH level (SHVS) level (n=6), as defined in Sect. B.19.4.13 should be analyzed against a calibration curve. The order of sample processing in the autosampler should be as follows: Calibration curve, SHQC, LLOQ 1, LLOQ 2, SHQC, LLOQ 1, LLOQ 2, etc. This study may be performed as part of the assay validation study or in pre validation. If it is conducted in pre validation, documen tation should be included as an appendix to the validation report. Other validation sample levels between HIGH and SUPER HIGH level may be evaluated if necessary.

B.19.4.16 Multi-component Analysis

In assays that quantitate multiple biomarkers, samples (n = 3 to 6 per component) containing one biomarker at the LLOQ level and the other biomarker(s) at the HIGH level should be analyzed. A set of samples should be used for each biomarker quantitated within the assay.

B.19.4.17 Specificity Against Xenobiotics

Validation samples at the LOW and HIGH levels (n=3 to 6 per level) use matrix containing first the drug of interest and then, xenobiotics. The co administered drugs which are already defined in the clinical development plan should be analyzed and controlled for no interference.

B.19.5 Examples of Biomarkers Application During Drug Development

B.19.5.1 Characterization of Animal Models Using Clinically Validated Biomarkers

The use of validated and predictive pharmacological models is prerequisite for unequivocal characterization of exploratory drugs. Biomarkers can be used to describe disease progression in a given model and show the validity of the model, particularly when clinical evidence of the selected biomarker or a panel of biomarkers is given.

The Biomarkers can serve two purposes:

1. They describe the disease model and demonstrate the validity of the selected model for the intended therapy.
2. They indicate therapeutic efficacy of a new drug in a model which is comparable to human disease, at least in respect to the markers that have been selected to “diagnose” the disease progression.

The Zucker diabetic fatty (ZDF/Gmi fa/fa) male rat model is a rodent model for insulin dependent diabetes mellitus (IDDM), which is frequently used for evalua tion of anti diabetic and anti obesity drugs (Peterson 2001). The rats become hyperglycemic and hypertrigly ceridemic with a predictable progression from the predia betic to the diabetic state. The ZDF rat may be appropriate

for addressing the pathophysiology of the human non insulin dependent diabetes mellitus (NIDDM) due to a defect in insulin secretion (Dolan et al. 1997) and their state of hyperglycemia is greater than in other NIDDM models.

While the male homocygote ZDF (obese) rats develop NIDDM, the heterocygotic (lean) rats do not develop the diabetic state and are used as lean ("healthy") controls.

In order to monitor disease progression in this animal model, a panel of established "clinical biomarkers" and exploratory biomarkers was analyzed in an internal pilot study. The selected biomarkers comprised blood glucose, HbA1c, free fatty acids (FFA), triglycerides, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), insulin, leptin, and malone dialdehyde. Because an integrated interpretation of more individual datasets is multi dimensional and therefore complex, we applied principal component analysis (PCA) and presented the result in specific plots, where each animal occupied a specific position within a spread sheet, which was defined by the integrated result of all analyzed variables (*Fig. B.19 3*). Animals clustering at a specific position within the spread sheet can be considered identical, at least in respect to the analyzed variables and the physiological condition which is expressed by these variables. It is inter esting to see that lean and obese rats cluster in different, but rather narrow clusters even at an age of 6 weeks. At this age, there is no visible difference between the homocygotic and heterocygotic animals and disease progression in the homocygotic (obese) animals has not yet started. The selected biomarker panel therefore is able to discriminate between different physiological conditions of the obese and lean animals.

The analysis of the biomarker panel was repeated at weeks 8, 10, 13, 16, and 19. The spontaneous progression to overt diabetes at the age of 7 12 weeks was reported (s.a.). A plateau of disease progression beginning at older ages of about 18 weeks is observed when the diabetic condition itself may be associated with a catabolic lipid metabolism (Peterson et al. 1990; Dohm et al. 1993; Sundell et al. 1992; Kuhlmann et al. 2003).

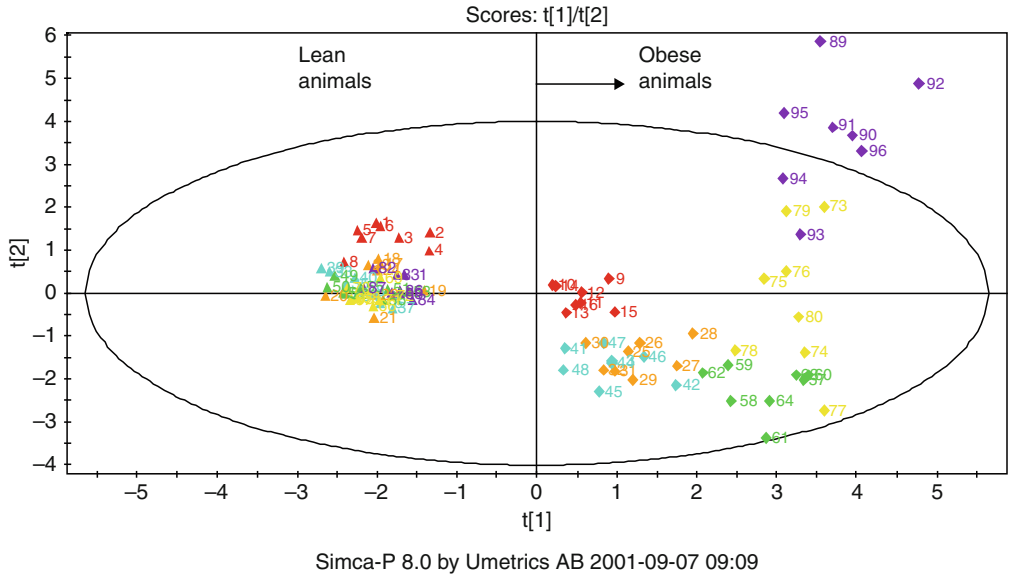

Figure B.19-3

Comparison of lean (*triangles*) and obese ZDF rats (*diamonds*) based on a principle component analysis of a biomarker panel comprised of blood glucose, HbA1c, FFA, triglycerides, cholesterol, HDL, LDL, insulin, leptin, and malone-di-aldehyde. Results of animals with different ages (eight animals per group) are plotted in different grey grading. Lean animals cluster on the left side of the spread sheet, After 8 weeks of age, there is a constant position of the results in the scatter plot irrespective of age. Animals which are 6 weeks old (animals 1 8) constitute a separated group. Obese animals cluster on the right side of the plot. Samples for analysis were taken after different ages from the animals: 6 weeks (animals 9 16), 8 weeks (numbers 23 30), 10 weeks (numbers 42 49), 13 weeks (numbers 57 64), 16 weeks (numbers 73 80), and 19 weeks (numbers 89 96, all in the right upper quarter)

In the healthy control group (lean), all animals older than 6 weeks cluster at the same position throughout the observation period of 18 weeks, indicating a stable physiological condition. The obese ZDF rats, however, take up new positions in the spread sheet, but not in a synchronized way. In *Fig. B.19 4*, only the obese animals are depicted. There seems to be a new cluster with all 8 and 10 weeks old animals, but also some older rats. This is a first demonstration that physiological chances and disease progression follows different speed in different animals. Most of the animals older than 13 weeks take positions in the spread sheet which are outside of this second cluster and a left shift is observed for most of the 18 weeks old animals. In *Fig. B.19 5*, the different speeds of disease progression are highlighted by lines, which follow the positions of three selected animals.

The demonstrated approach to characterize and validate a disease model can serve several purposes:

- It demonstrates the biological variability/inter individual variability of the model.
- It can be used to define the ideal time point for treatment.
- Knowing the variability of the model helps in data interpretation, PK/PD modeling, and estimation of group sizes.
- It can be used to identify the marker mostly impacted by disease progression (= principle component).
- It can be used to identify the marker which is mostly impacted by the drug.

Due to the fact that the selection of the applied biomarkers is driven by their clinical relevance or their involvement in the pathophysiology of the human disease, there is a rationale to speculate that disease progression in the animals as monitored by the biomarkers is highly comparable to the human situation. Of much higher importance, however, is the high probability that drugs that influence the selected biomarker panel and avoid the shift in the cluster in the spread sheet have a good chance to positively influence the human physiology and prevent disease progression. Therefore, these kinds of investigations contribute to early drug development because they provide a basis for candidate comparison and selection for further development which is based on scientific evidence.

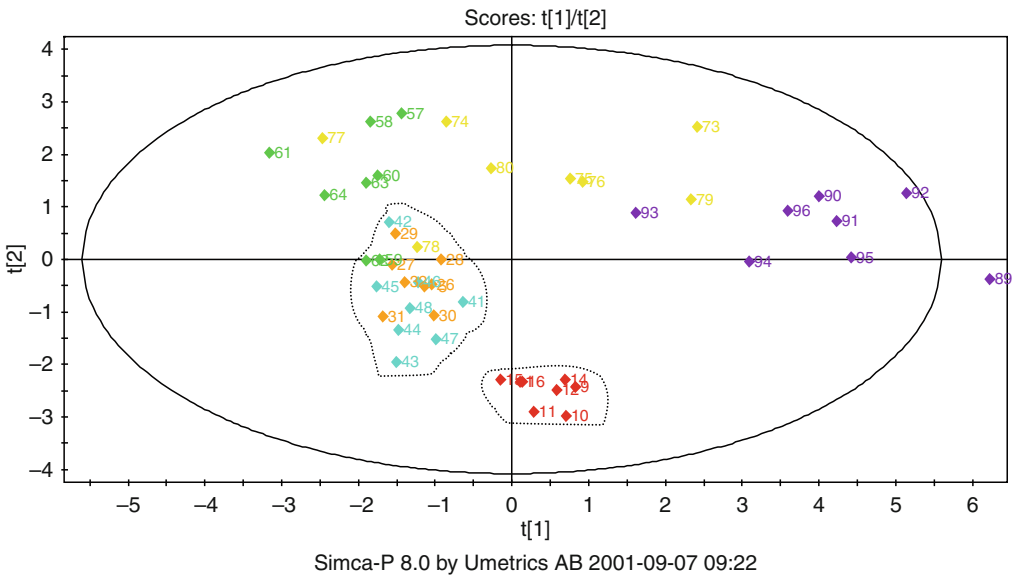

Figure B.19-4

Monitoring disease progression in obese ZDF rats (same animals as illustrated in *Fig. B.19-3*). Principal component analysis based on the defined biomarkers was applied in all animals at different ages. Each animal (characterized by the BM panel) is represented in the plot. Animals of the same age are coded by the same grey grading. There is a distinct cluster in which all 6 weeks old animals are located (animals 9 16; cluster lower image border surrounded by a *dotted line*). During ageing the PCA results move to different positions. Samples for analysis were taken at different ages: 6 weeks (animals 9 16), 8 weeks (numbers 23 30), 10 weeks (numbers 42 49), 13 weeks (numbers 57 64), 16 weeks (numbers 73 80), and 19 weeks (numbers 89 96, all located in the right zone)

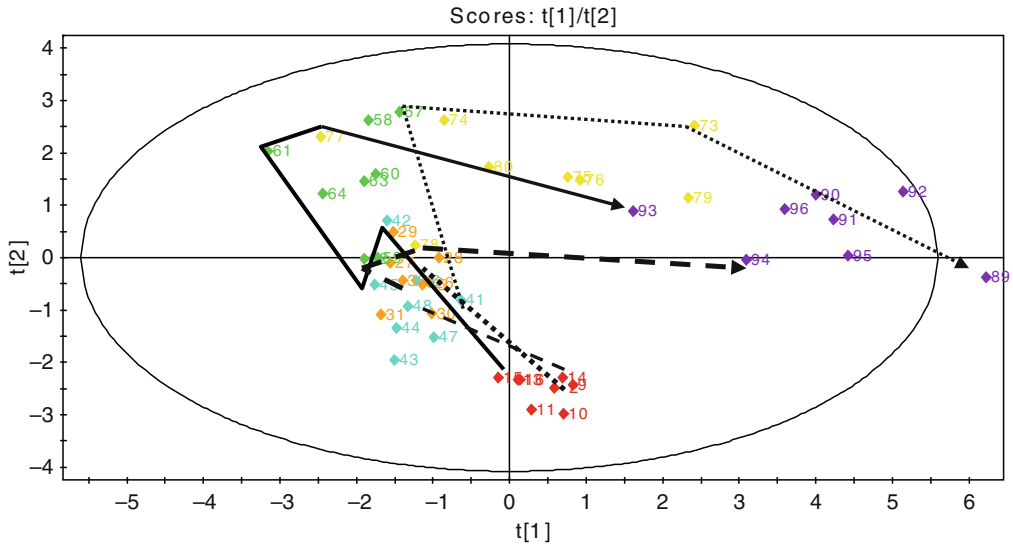

Figure B.19-5

Monitoring disease progression in obese ZDF rats (same date as shown in *Fig. B.19-4*). The different time courses of disease progression is highlighted for three selected rats using different solid lines. Rat 9: following samples of this animal 25, 41, 57, 73, 89 (sample numbers for time points 8w, 10w, 13w, 16w, 19w) Rat 13: following samples of this animal 29, 45, 61, 77, 93 (sample numbers for time points 8w, 10w, 13w, 16w, 19w) Rat 14: following samples of this animal 30, 46, 62, 78, 94 (sample numbers for time points 8w, 10w, 13w, 16w, 19w)

B.19.5.2 Use of Biomarkers for Preclinical Profiling of New Drugs

Preclinical profiling of drug candidates using biomarkers with a proven rational from human pathophysiology is a possible strategy to prioritize and select new drug can didates along the value chain, and finally enhance go or no go decisions. Despite this, there is an additional chance associated with this strategy, because the broad applica tion of biomarkers in validated animal models may reveal new and unexpected effects of new drugs. The advantage of using animal models is based on the parallel investiga tion of healthy control groups, disease control group, and in most cases groups of animals treated with different drug doses. The animal model per se thus is very well controlled and provides controls for the biomarker analysis. In most cases, the applied biomarker assays are not validated for the animal sample matrix in discovery studies and may not be absolutely accurate (see the previous section). This deficit is compensated by the control groups, because a biomarker in which the (semi) quantitative level is different between the diseased control group and the healthy control group is partially validated by this given and a drug related reversal of the "disease value" to the normal level of the BM in each animal or the whole dose group. An example for the identification of an unexpected drug effect is the influence of cariporide, a selective Na^+ H^+ exchange inhibitor, on plasma C reactive protein (CRP) levels in rabbits with myocardial infarction (MI) (Rungwerth et al. 2004). Induction of MI resulted in a significant increase in CRP together with aldosterone levels. Treatment with cariporide significantly decreased both biomarkers in the animal groups with MI, but had no effect on both biomarkers in the sham operated "healthy" control group. In this animal study, cariporide attenu ated left ventricular hypertrophy and improved left ventricular function. But in addition the drug prevented the inflammatory response, as indicated by the reduction of the elevated plasma levels of CRP, which were caused by MI. An increase of the CRP level in an animal model of heart failure could be shown the first time, emphasiz ing an ongoing inflammatory process in chronic heart failure comparable to the human situation (Alonso Martinez et al. 2002). In addition, a positive drug effect could be demonstrated on the level of the unspecific marker of systemic inflammation, CRP, indicating anti inflammatory properties of the drug offering new possible options for profiling, labeling, and drug application.

B.19.5.3 Use of Biomarkers for Demonstrating Proof of Concept, Regimen and Dose Selection

B.19.5.3.1 Adiponectin: A Translational Biomarker

The fast transition of projects from early stages, when screening and in vitro profiling assays are used for proof of concept (POC) studies to animal studies and later to first in human (FIH) studies is very complex and very often results obtained in in vitro assays cannot be directly translated into in vivo models. For the development of peroxisome proliferator activated receptor (PPAR) agonists in diabetes, quantification of the biomarker adiponectin (Acrp30; AdipoQ) (Scherer et al. 1995; Hu et al. 1996; Berg et al. 2001), a 30 kDa secretory protein, predominantly expressed by adipocytes and circulating at high concentrations (0.5 30 μg/mL) in the blood, has been proven as an excellent tool to guide the decision making process.

Adiponectin, which is present in the circulation in complex oligomeric forms, is a very robust marker that is not prone to degradation and is only minimally affected by diurnal variations, pre , or postprandial status, and acute infections (Shetty et al. 2009). Renal dysfunctions lead to an at least twofold increase in serum adiponectin, most likely due to a slower adiponectin clearance rate (Zoccali et al. 2003).

An inverse relationship with body fat mass, insulin resistance, and type 2 diabetes mellitus (T2DM) was demonstrated in humans because a gradual decrease in adiponectin concentration in serum was observed as individuals progressed from lean, to obese, to type 2 diabetics (Hsueh and Law 2003; Trujillo and Scherer 2005). Adiponectin, together with five other biomarkers (C reactive protein, ferritin, interleukin 2 receptor A, glucose, and insulin), was successfully used to assess an individual's 5 year risk of developing type 2 diabetes. The study used subjects from the "Inter99 cohort," a longitudinal population based study of 6,600 Danes (Kolberg et al. 2009).

Adiponectin has been shown to lower plasma glucose concentrations through inhibition of gluconeogenesis and improvement of insulin sensitivity. High plasma levels are correlated with a lower risk of cardiovascular disease, implicating adiponectin levels as a key link between the metabolic syndrome and cardiovascular disorders (Shetty et al. 2009; Szmitko et al. 2007). The ability of adiponectin to improve metabolic function seems to depend, at least in part, on its anti inflammatory function by alleviating pro inflammatory cytokines (i.e., tumor necrosis factor alpha [TNF alpha] or C reactive protein [CRP]) (Matsushita et al. 2006).

Administration of insulin sensitizing agents such as thiazolidinediones (TZDs), also called glitazones, and other PPAR gamma agonists resulted in a robust increase in adiponectin level in the blood of diabetic rodents (Banga et al. 2009; Combs et al. 2002; Yang et al. 2004). This effect is most likely due to upregulating adiponectin through a post transcriptional mechanism, possibly by increasing its rate of secretion (Bodles et al. 2006). Experiences from animal studies could be directly transferred to human "models," showing that the PPAR gamma agonist rosiglitazone increased plasma levels of adiponectin in healthy subjects and type 2 diabetic patients, or demonstrating an association between plasma adiponectin and insulin sensitivity and serum lipid parameters in nondiabetic subjects (Yang et al. 2002; Tschritter et al. 2003).

Recently, Wagner et al. (2009) assembled large datasets of human studies by pooling results from a variety of private sector sources and performing a meta analysis by independent third party statisticians. The study was conducted under the Metabolic Disorders Steering Committee of the Biomarker Consortium (see Sect. B.19.7) and was the first project completed by the consortium. Analysis of blinded data of 2,688 T2DM patients from randomized clinical trials conducted in eight studies by four pharmaceutical companies (Merck, Eli Lilly, GlaxoSmithKline and Roche) demonstrated that adiponectin level increased after PPAR gamma agonist treatment (8 12 weeks) and did not increase with non PPAR drugs. The adiponectin level correlated with reductions in glucose, HBA1C, hematocrit, and triglycerides, and with increases in blood urea nitrogen, creatinine, and high density lipoprotein (HDL). The study not only confirmed previously identified relationships between adiponectin and various metabolic parameters, but also further validated the use of adiponectin as a robust predictor of glycemic control, insulin resistance, cardiovascular risk, and the metabolic status. These observations will accelerate drug development by quantifying adiponectin level in individuals over time and also improve diagnosis of T2DM in individuals who do not yet manifest T2DM.

Additional studies are required to unravel the role of adiponectin in other inflammatory diseases such as rheumathoid arthritis, Crohn's disease, or systemic lupus erythematosus.

Although adiponectin is used as a disease related as well as efficacy biomarker in many clinical investigations in T2DM, the analysis of adiponectin is still "not for diagnostic use." The complex biology of adiponectin and

its involvement in other indications besides metabolic syndrome make interpretations of adiponectin plasma levels in disease state very difficult, thus complicating the acceptance of adiponectin levels as diagnostic parameter (Sun et al. 2009; Shand et al. 2003; Weiss et al. 2003).

The use of adiponectin in healthy subjects, e.g., first in men (FIH) studies as a biomarker that is strictly corre lated with activation of the PPAR gamma (drug effect) is an appropriate tool in development of PPAR agonists. In healthy subjects with normal renal function, the endoge nous levels of adiponectin are not influenced by disease related confounding variables and other variables which contribute to the plasma levels, like gender or age, can be controlled very tightly. Therefore, adiponectin can be used in phase I clinical studies as a tool to monitor the drug related responses of the target and helps to define the optimum dose levels and dosing regimens for further clinical trials (Rohatagi et al. 2008).

In *Fig. B.19 6*, an example of such use of adiponectin analysis in a clinical phase I study with healthy subjects is demonstrated, showing a dose dependent increase of adiponectin plasma levels after treatment with the PPARgamma agonist SAR XYZ. Due to the inter individual variabilities of endogenous adiponectin levels in the subjects allocated to the dosing groups, it is necessary to normalize the data and express the % change related to the baseline results.

B.19.5.3.2 Dipeptidyl-Peptidase-4 (DPP-4) Activity as BM for Dose Selection and Go/Nogo Decisions

Dipeptidyl peptidase 4 inhibitors represent a therapeutic approach for the treatment of type 2 diabetes, since they inhibit an enzyme which rapidly cleaves and inactivates the incretins GLP 1 and GIP which are responsible for >50% of nutrient stimulated insulin secretion. The use of DPP 4 inhibitors has been proven effective on an acute scale in both animals and humans and ex vivo inhibition analysis of DPP 4 activity has been demonstrated as a good measure of the pharmacodynamic effect of DPP IV inhibitors in healthy subjects (Durnix et al. 2000; Pospisilik et al. 2002; Herman et al. 2005, 2006).

Dipeptidyl peptidase enzymatic activity can be ana lyzed using the specific chromogenic substrate Gly Pro p nitroanilide/HCL at pH 8.3 by monitoring the release of p nitroanilide at 406 nm. The activity of DPP 4 in plasma samples is analyzed by adding the appropriate amount of substrate to a predefined volume of plasma and monitor the p nitroanilid signal in a short time period after its addition. This can be performed in pretreatment samples and in samples taken at different time points after dosing. The comparison of the post dose activities with the signals obtained before drug dosing gives an estimate about drug induced enzyme inhibition (*Fig. B.19 7*).

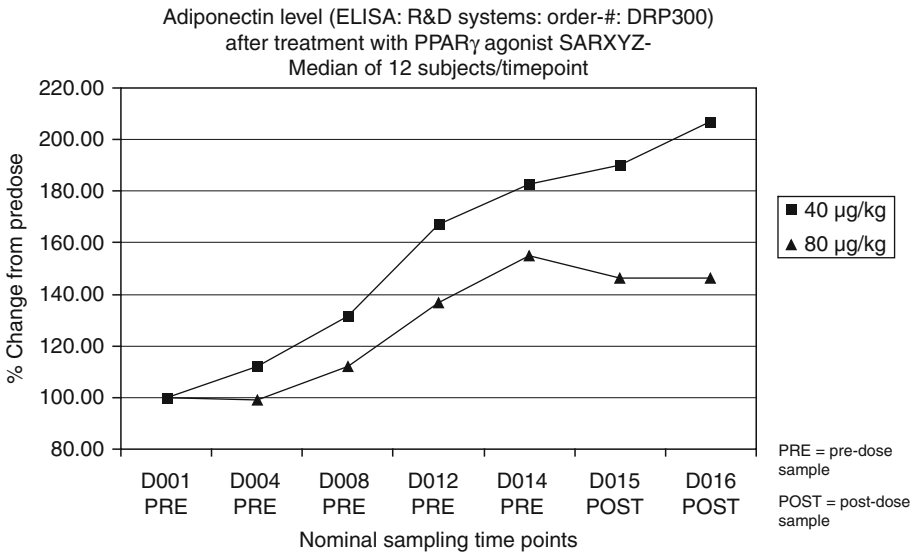

Figure B.19-6

Demonstration of a dose dependent effect of PPARgamma treatment on adiponectin levels in male healthy subjects

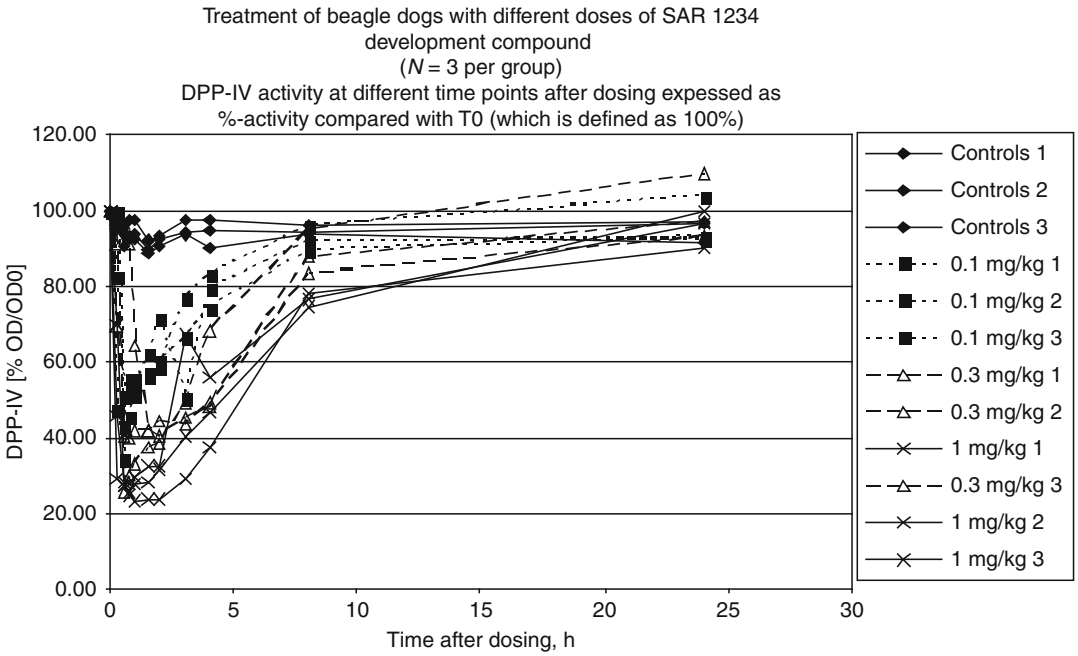

Figure B.19-7

Inhibition of endogenous DPP-4 activity in beagle dogs after treatment with SAR1234 resulting in a dose dependent decrease of enzyme activity

An analytical method for determining DPP 4 activity in human EDTA plasma from samples of clinical studies was established at the bioanalytical laboratory of Sanofi Aventis and applied in phase I studies. The purpose was to proof the inhibitory activity of the drug candidate SAR1234, to define the extent and duration of drug activity and finally to profile the drug against competitors by comparing results with published data for the competitive compound(s). A major challenge for assay development was related to the comparability of the internal assay to published methods, because this comparison is only meaningful when sensitivity and precision of the assays are comparable. In order to proof at least the sensitivity aspect, inhibition experiments with a compound in development and other known inhibitors were conducted and the IC50% results were compared with published data (an example of IC50% determinations is shown in *Fig. B.19 8*). Demonstrating assay sensitivity in this way finally was the basis for applying the assay in the clinical studies to monitor and compare the PD profiles.

Early profiling of internal compounds against competitors or established therapies, e.g., with the help of appropriate biomarkers, is very important, because this application of a PD biomarker enables and contributes to internal go or no go decisions in phases of drug development when clinical trial costs are still moderate. An early, data based no go decision can lead to a reduction of the internal budget and personnel resources and can re allocate liberated resources to other, more promising projects. Thus, rational biomarker application can contribute to portfolio management in pharmaceutical companies.

B.19.5.3.3 Measurement of Target Receptor Occupancy in Order to Define Dosing Regimens (Example of CD33)

SAR 567/HuMy9 6 is an immunoconjugate composed of a humanized IgG1 monoclonal antibody (huMy9 6) that specifically targets the CD33 antigen, conjugated through a disulfide link to the maytansin derivative DM4. The CD33 antigen is expressed on the surface of myeloid cells. After the immunoconjugate is bound to CD33, it is internalized and the cytotoxic is released within the cell.

During clinical trials in acute myeloid leukemia (AML) patients, the expression of CD33 at the cell surface of blasts has been quantified in blood and in bone marrow before drug administration and after treatment together with the occupancy of CD33 by SAR567 by quantitative

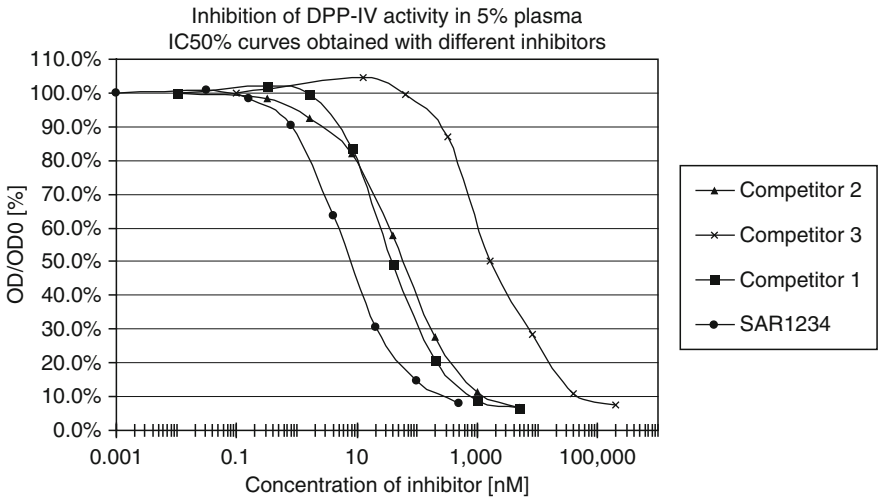

Figure B.19-8

Inhibition of DPP-4 activity in 5% plasma from healthy donors using different DPP-4 inhibitors

flow cytometry (QFCM) after standardized indirect immunofluorescence staining with mouse IgG MAbs used at saturating concentration. The evaluation of CD33 den sity and occupancy was performed using two different mouse monoclonal antibodies (MAbs) of the same IgG1 isotype that recognize different epitopes of the CD33 antigen. MAb1 is competitive with huMy9 6 DM4 immunoconjugate and thus cannot bind in the presence of the immunoconjugate on the cell surface, indicating the number of free, unoccupied CD33 target antigenic sites. MAb2 recognizes a different domain and thus its binding is independent of the binding of the immunoconjugate to the same antigen, indicating the number of total, occupied and unoccupied CD33 antigens, still present at cell surface (*Fig. B.19 9*). Mean fluorescence intensity (MFI) elicited by the MAb bound to the CD33 is compared to a calibration curve prepared to determine the antibody binding capacity (ABC). Correction with the apparent ABC measured using an isotype matched irrelevant MAb provides the specific ABC (sABC) i.e., the number of MAb specifically bound per cell. In addition, the mean number of DM4 per IgG has been measured to evaluate the loading of cytotoxic molecules. In order to describe the evolution of huMy9 6 DM4 conjugation ratio in vivo, irrespective of baseline CD33 density, results were expressed as DM4/CD33 ratio by dividing the number of cell bound anti DM4 MAb molecules (sDM4) by the number of total CD33 molecules at each time point.

Example of patient 007: In patient 007 with elevated counts of peripheral blood blasts, the CD33 receptors reappeared on the surface of peripheral blasts by the third week of the cycle (*Fig. B.19 9c*), concomitantly with increase of hyperleucocytosis and undetectable plasma levels of immunoconjugate. The same dynamic is illustrated in *Fig. B.19 9d* for patient 013 treated with 130 mg/m^2 on day 1 and day 8. These data allow to conclude that the proof of concept of SAR567 has been demonstrated. After SAR567 administration, CD33 is occupied and down modulated in both blood and bone marrow. For patients with hyperleucocytosis, an higher dose of SAR567 could be envisaged to maintain the phar macodynamic effect of SAR567.

With the day 1/day 8 infusion schedule, a rapid decrease of DM4 per cell and of DM4/CD33, in parallel with the decrease of total CD33 was observed on the surface of peripheral blood blasts between day 1 and day 8, with an increase of DM4 par cell and of DM4/CD33 ratio following the second infusion (illustrated in *Fig. B.19 9d* with data from patient 013). In order to increase the loading of DM4 in the cancer cells, repeated administrations at day 1, day 4, and day 7 of SAR567 should enable maintenance of a constant level of DM4 for internalization.

B.19.6 Pharmacogenomic Biomarkers and Personalized Medicine

Francis Collins, when acting as the director of the National Human Genome Research Institute (NHDRI), proclaimed several years ago that by 2007, 90% of all medicines will be prescribed based on the measurement of DNA sequences

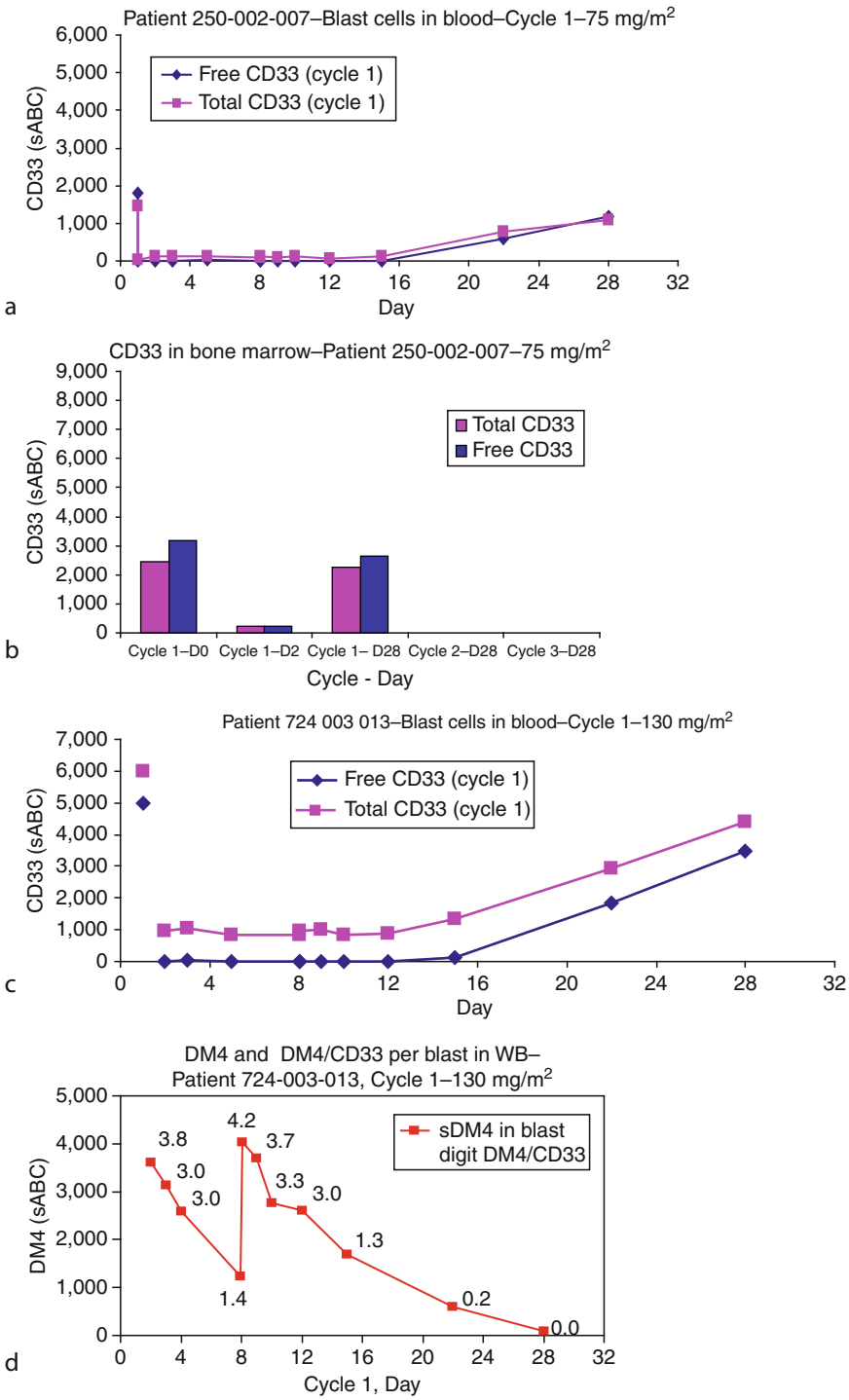

Figure B.19-9

(a-d) SAR567 was administered at day 1 and day 8 every 3 weeks in AML patients (a) CD33 occupancy and down modulation in blood after SAR567 administration. (b) CD33 occupancy and down modulation in bone marrow after SAR567 administration. (c) CD33 occupancy and down modulation in blood after SAR567 administration. (d) Bound DM4 and DM4/CD33 ratio in blood after SAR567 administration

that may be associated with a drug's efficacy, metabolism, and toxicity. This scenario did not occur so far, but identification and use of biomarkers in this area are speeding up. The use of biomarkers to target therapy to likely to benefit groups or to exclude groups or exclude those individuals with a low probability of response is a powerful method of the pharmaceutical industry to demonstrate high value of new, very often expensive (i.e., monoclonal antibodies) novel therapeutics. Pharmacogenomics (PGx) and pharmacogenetics (PGt) are emerging interdisciplinary areas recently identified by the regulatory authorities.

The International Conference on Harmonization (ICH) defined PGx as the "study of variations of DNA and RNA characteristics as related to drug response" (www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm129296.pdf), whereas PGt, a term recognized in pharmacology in the pregenomic area, is a subset of PGx and according to the ICH guideline is "the study of variations in DNA sequence as related to drug response" (Surh et al. 2009). Before the introduction of sophisticated genomic techniques, PGt focused primarily on drug metabolism such as CYP450 iso enzymes.

Overall, PGx has the potential to help better identify target populations for (enriched) further study and to eliminate unpromising drug candidates early in the development process through identification of genomic biomarkers for response.

Scientifically, personalized medicine, also referred to as individualized therapy, is known as PGx (drugs combined with genes), or how genetic differences in individuals affect the way people respond to drugs.

Regulatory agencies such as the European Medicines Agency (EMEA) (http://www.emea.europa.eu/pdfs/human/ich/43798606en.pdf) state that a genomic biomarker reflects (a) the expression of a gene, (b) the function of a gene, and (c) the regulation of a gene. DNA characteristics include, but are not limited to, single nucleotide polymorphisms (SNPs), variability of short sequence repeats, DNA modifications (e.g., methylation), insertions/deletions, copy number variations, and cytogenic rearrangements (e.g., translocations, duplications, deletions, or inversions). RNA characteristics include, but are not limited to, RNA sequences, RNA expression levels, RNA processing (e.g., slicing and editing), and micro RNA levels.

It is estimated that genetics account for 20 95% of variability in drug disposition and effects. Genetic polymorphisms in drug metabolizing enzymes, transporters, receptors, and other drug targets have been linked to individual differences and efficacy and toxicity of many medications. Although interindividual variations in drug response result from effects of age, sex, disease, or drug interactions, genetic factors represent an important influence in drug response and efficacy and remain constant throughout life.

Genetic biomarkers are a major driver for personalized medicine. Genetic tests are potentially useful tools for making therapeutic decisions by identifying patients who should or should not receive a particular drug, as well as for guiding individual drug dosing. Especially in oncology in which the general efficacy is low, biomarker based stratification would benefit the patient with more efficacious treatment options. Pharmacogenetic test information is currently included in more than 200 drug labels in the USA (Ikediobi et al. 2009).

An example of a clinical biomarker that is positively correlated to drug efficacy is the BCR ABL positive tyrosine kinase genotype, which is used to identify patients with chronic myeloid leukemia (CML). Bcr Abl, is the target of imatinib (Gleevec™, Novartis). Clinicians use this mutation as a biomarker that determines the level of receptor expression to better identify those patients who will respond to therapy (Capdeville et al. 2000).

One other example of personalized medicine is a genetic variant of a biomarker that is linked to drug toxicity, the UDP glucuronosyltransferase1A1 enzyme (UGT1A1). When the chemotherapy drug irinotecan (Camptosar™) is given to patients with advanced colorectal cancer, irinotecan is activated to the metabolite SN 38, and then eventually inactivated in the body by the UGT1A1 enzyme. Patients homozygous for the UGT1A1*28 allele (exists in 10% of the US population), associated with decreased UGT1A1 enzyme activity, should be given a reduced dose, since those subsets of patients clear the drug less quickly from their body than the rest of the population. A "normal" drug dose would result in a higher risk of potentially life threatening side effects such as neutropenia, an abnormally low level of neutrophils, and diarrhea. Thus, the FDA had recommended dose adjustments for irinotecan on patients with reduced UGT1A1 activity and mandated that these genetic data be included in the insert of Camptosar™ because these patients are at a five times greater risk of experiencing toxicity than those without the critical allele (Marsh and McLeod 2004; Shin et al. 2009; www.accessdata.fda.gov/drugsatfda_docs/label/2002/20571s16lbl.pdf).

Trastuzumab (Herceptin™, Genentech) and lapatinib (Tykerb™, GlaxoSmithKline) for the treatment of human epidermal growth factor receptor 2 (HER2/neu) overexpressing breast cancer are other drugs where diagnostic tests became a central role to determine whether a patient is eligible to receive these new therapies (Burstein

2005; Hudis 2007; McArthur and Hudis 2009). Getfitinib (Iressa™) and erlotinib (Tarceva™) are linked with testing for the epidermal growth factor receptor (Flockhart et al. 2009).

Further examples of drugs that are affected by pharmacogenetic variation in either drug target and/or metabolism are azathioprine, warfarin, abacavir, tamoxifen, and beta blockers (Flockhart et al. 2009; Tepper and Roubenoff 2009).

A list of valid genetic biomarkers identified in the context of approved drug labels can be found on the FDA website (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm). The Center for Drug Evaluation and Research (CDER) at the FDA has already accepted label changes that add genomic biomarker information for approved drugs. These recommendations generally pertain to dosing (drug metabolizing enzyme polymorphism) and safety (various human leukocyte antigen alleles conferring to an elevated risk).

In December 2008, the US Food and Drug Administration (FDA) held a meeting of the Oncologic Drugs Advisory Committee to discuss the proposals for label changes for the epidermal growth factor receptor (EGFR) targeting cancer drug cetuximab (Erbitux™, ImClone Systems) and panitumumab (Vectibix™, Amgen), both of which were on the market already at the time. By using a variety of assay technologies, the drug's manufacturers presented retrospective data on genetic testing for somatic mutations of the *KRAS* gene in the tumors of patients with colorectal cancer who had participated in the clinical trials. Patients having the mutated *KRAS* genes (in 30–45% of all colorectal tumors) failed to respond to the therapies (Jimeno et al. 2009; Baynes and Gansert 2009). Since *KRAS* mutations in tumors often result in constitutive activation of the pathway and *KRAS* being downstream in the EGFR signaling pathway, this subset of patients could not benefit from the therapy.

The next step in personalized approaches will very likely include whole genome sequencing, which is becoming less expensive and is expected to become widely available within the next few years.

B.19.7 Initiatives and Consortia in Biomarker Discovery and Development

Qualification of biomarkers for regulatory decision making is resource intensive and time consuming. One model for collaboration is consortia with wide stakeholder involvement including regulatory, industrial, academic, and government representation.

The goals of the consortium are to promote the discovery, development, qualification, and regulatory acceptance of biomarkers; to make project research results and data broadly available to the public; and to help speed disease specific research.

In 2004, the US Food and Drug Administration (FDA) published a White Paper on "Innovation or Stagnation: Challenge and Opportunity on the Critical Path to New Medical Products" (http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/CriticalPathOpportunitiesReports/ucm077262.htm). The value of pharmacokinetic/pharmacodynamic relationships was emphasized, proposing the implementation of novel biomarkers and clinical trial methods in drug development to improve success rates and increase the medical value of new therapies. Subsequently, the FDA established the *Critical Path Initiative* (www.c path.org/; Woodcock and Woosley 2008) which is closely aligned with an external partner, the *Critical Path Institute*, a consortium based nonprofit organization created to form partnerships among government, industry, academia, and the nonprofit sector to generate ideas and solutions to these problems.

The *Predictive Safety Testing Consortium (PSTC)* (http://www.c path.org/pstc.cfm), a 15 member pharmaceutical industry collaboration, including Sanofi Aventis, convened by the nonprofit Critical Path Institute, has investigated a group of new biomarkers for early drug induced renal toxicities in animals. Histopathology in preclinical models was correlated with renal biomarkers. In May 2008, the FDA and the European Medicines Agency (EMEA) concluded that seven new urinary biomarkers (i.e., KIM 1, albumin, total protein, beta2 microglobulin, cystatin C, clusterin, trefoil factor 3) are considered qualified for particular uses in regulatory decision making. The preclinical data had demonstrated superiority over the current standards, i.e., serum creatinine and blood urea nitrogen (BUN) used to assess renal injury in drug testing. The full translatability and extrapolation of these rat toxicity markers to extended human studies is still an open question due to marked species, strain, dietary, and sex differences. In addition, there may be differences in dosing levels and regimen and in the absorption, distribution, metabolism, and excretion of potential nephrotoxins. Early clinical trials in humans will have to show the potential for their use in humans.

The 2004 FDA Critical Path Initiative paved the way for numerous policy initiatives. The *Prescription Drug User Fee Act* (*PDUFA IV*) (http://www.ashp.org/DocLibrary/Advocacy/PDUFAIVFDAOversight.aspx) highlights the

role of biomarkers in the "Expediting Drug Development" section by noting that the "FDA will participate in workshops with representatives from the scientific community, including industry, academia, and other interested stakeholders."

To facilitate precompetitive biomarker collaboration, the *Biomarker Consortium* (www.biomarkersconsortium.org) was launched in October 2006. This is a public private biomedical research partnership under the umbrella of the Foundation of the National Institutes of Health (NIH). Among its 60 members are the National Institute of Health (NIH), the FDA, the pharmaceutical and biotechnology industries, diagnostic industries, and advocacy groups (Altar 2008).

Initiatives to better delineate a common framework for biomarker and surrogate end point qualification include the EMEA document "Biomarkers Qualification: Guidance to Applicants" (http://www.emea.europa.eu/pdfs/human/biomarkers/7289408en.pdf), which was issued in April 2008 and finalized by the Committee for Medical Products for Human Use in January 2009, as well as the National Academy of Science Institute of Medicine committee tasked with the "Qualification of Biomarkers as Surrogate Endpoints in Chronic Disease" project (http://www8.nationalacademies.org/cp/projectview.aspx?key=HCSX H 08 06 A).

The *European Medicines Agency (EMEA) Road Map to 2010* (http://www.emea.europa.eu/pdfs/general/direct/directory/3416303enF.pdf) fosters strategies for more efficient drug development, including a central role for safety and efficacy biomarkers which are incorporated in so called innovative medicines initiatives (IMI) (http://imi.europa.eu/index en.html). IMI is a unique public private partnership between the pharmaceutical industry represented by the European Federation of Pharmaceutical Industries and Associations (EFPIA) and the European Union represented by the European Commission (Hughes 2009).

Another biomarker based initiative is provided by the *National Cancer Institute's Cancer Biomarker Project* (http://edrn.nci.nih.gov/about edrn/scicomponents), which includes the following scientific components to detect and develop biomarkers in the field of oncology through the early detection research network: biomarker development laboratories, biomarker reference laboratories, clinical epidemiology and validation centers, data management and coordination centers, and information centers.

The *Kennedy Hutchinson 21th Century Cancer ALERT Act* (http://www.aacr.org/home/public media/science policy government affairs/aacr cancer policy monitor/aacr cancer policy monitor April/senators revitalize the nations war on cancer.aspx), introduced 26 March 2009, includes an extensive discussion of the role of biomarkers in cancer prevention and detection.

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B.20 Toxicokinetics and Safety Ratios

Karl-Heinz Lehr

PURPOSE AND RATIONALE

Toxicokinetics is defined as "the generation of pharmaco kinetic data, either as an integral component in the con duct 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). The ratio of drug expo sure in animals at the no observed adverse effect level (NOAEL) and in humans at the expected therapeutic dose is one of the precautionary principles to determine the risk benefit profile of pharmaceuticals. For this ratio the expressions "safety ratio" and "safety margin" were also used. It is usually based on human plasma AUC exposure (animal/human AUC ratio); however, depend ing on the mode of action or whichever is smaller, the ratio can also be based on the maximum concentration in plasma (animal/human C_{max} ratio).

PROCEDURE

B.20.1 Main Group or Satellite Animals?

Whenever possible, toxicokinetic measurements are per formed on all the animals in the toxicity study. This is the most representative approach and it allows the individual PK data to be directly correlated with the toxicological findings. The second choice is toxicokinetic measurement in representative subgroups or satellite groups. Satellite groups are animals that 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.

B.20.2 Number of Animals and Timepoints

In the ICH Guidance Toxicokinetics (1994), it is stated that "the number of animals to be used should be the minimum consistent with generating adequate toxicokinetic data" and that "the area under the matrix level concentration time curve (AUC) and/or the measurement of matrix concentra tions 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 that are necessary for safety evaluation. The withdrawal of a suffi cient number of blood samples (six to nine) 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 (BVA/FRAME/ RSPCA/UFAW Working Group of Refinement 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. (2001) 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}). For three different compounds, Pai et al. (1996) compared the AUCs from intensive (full) (10 15 time points with four to five rats per time point) sampling schemes with sparse sampling schemes (five time points with two rats per time point). Using Monte Carlo simulation, Pai et al. (1996) could show that the deviation of AUC estimation of the sparse sampling scheme from the full sampling scheme was not larger than 10%. Thus it is seen that a sparse sampling scheme with five to seven time points with two to three animals per time point is well suited for the reliable determination of systemic exposure in small animal toxicity studies.

B.20.3 Analytical Methods

The analytical methods to be used in toxicokinetic studies should be specific for the entity to be measured and of adequate accuracy and precision. The limit of quantifi cation 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).

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B.20.4 Toxicokinetic Evaluation

The following aspects should be considered for toxicoki netic evaluation:

- Pharmacokinetic profile of the compound (exposure)
- Dose dependency of AUC and C_{max}
- Changes of exposure during the course of the toxicity study
- Sex differences

B.20.4.1 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 metabo lites. The area under the matrix level concentration time curve (AUC) and/or the measurement of matrix concen trations at the expected peak concentration time, C_{max}, or at some other selected time (e.g., $C_{(24h)}$ as trough value), $C_{(time)}$, are the most commonly used parameters. Accord ing to the supplementary notes in the ICH Guidance Toxicokinetics (1994), for a profile (e.g., four to eight) 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).

B.20.4.2 Dose Dependency of AUC and C_{max}

According to the ICH Guidance Toxicokinetics (1994), it is one of the primary objectives of toxicokinetics to describe the systemic exposure achieved in animals and its relationship to dose level.

At pharmacological and clinical doses, it can be gen erally assumed that most of the drugs show linear phar macokinetics. Linear pharmacokinetics is given when exposure (AUC) is proportional to dose, and principal pharmacokinetic parameters like bioavailability, elimina tion rate, volume of distribution, and clearance are inde pendent of 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, in addition to the dose proportional increase of exposure (*Fig. B.20 1*), a less than proportional increase

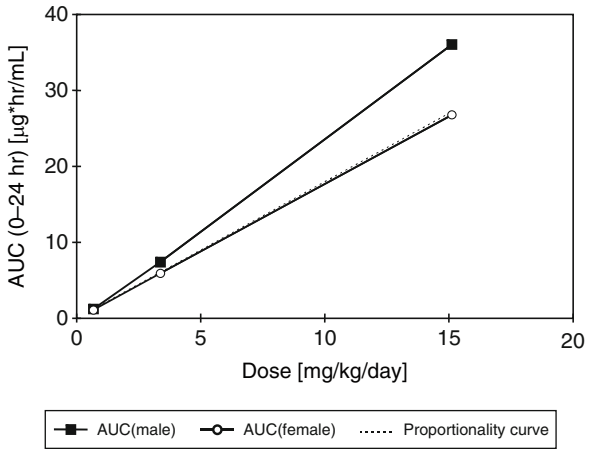

Figure B.20-1
Proportional dose dependency of AUC in an intravenous toxicity study in rat with the test compound A

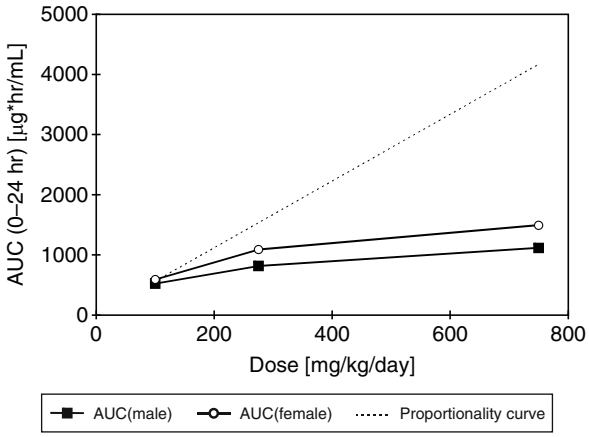

Figure B.20-2
Less than proportional dose dependency of AUC in an oral toxicity study in rat with the test compound B

(*Fig. B.20 2*) and a more than proportional increase (*Fig. B.20 3*) are very frequently 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 or biliary excretion of parent compound or a concentration dependent change of volume of distribution should be considered as potential causes.

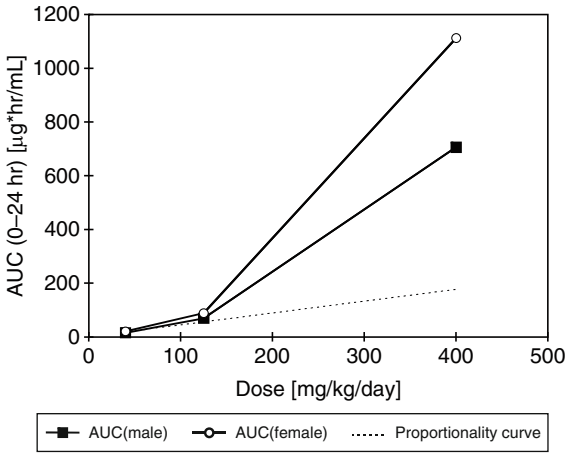

Figure B.20-3

More than proportional dose dependency of AUC in an oral toxicity study in dog with the test compound C

B.20.4.3 Changes 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 deriving pharmacokinetic parameters from measurements made at appropriate time points during the course of the individual studies. In short term studies (1 month or shorter), Day 1 and a day at the end of the toxicity study may be appropriate profiling days. In long term studies, Day 1, 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 that 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 are used at an age at which they are not sexually mature, and during the study, sexual maturation takes place in the first 2 months, with its known impact on the rate and extent of metabolism. 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 change of the administered batch with impact on bioavailability should also be considered.

B.20.4.4 Sex Differences

According to the ICH Guidance Toxicokinetics (1994), it is normal to estimate exposure in animals of both sexes unless some justification can be made for not doing so. For evaluation both sexes should be evaluated separately. The assessment of exposure data of the two sexes can be performed by calculating 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 have to be considered. As a rule of thumb, it can be stated that in rodents sex difference is quite common when CYP metabolism is involved as a major elimination pathway, whereas in nonrodents distinct sex differences are rather rare.

EVALUATION

An important objective of toxicokinetics is to relate the no observed adverse effect dose level with the exposure (expressed as AUC and C_{max}) in the respective animal species at this dose level.

From these data and from the exposure values in humans at the expected therapeutic dose, the multiple of the therapeutic exposure in human versus animal exposure at NOAEL is calculated according to the following formulas:

$$\text{Animal/human AUC ratio} = \frac{\text{AUC}_{\text{animal}} \text{ at NOAEL}}{\text{AUC}_{\text{human}} \text{ at the maximum recommended human dose}}$$

$$\text{Animal/human } C_{max} \text{ ratio} = \frac{C_{\text{max animal}} \text{ at NOAEL}}{C_{\text{max human}} \text{ at the maximum recommended human dose}}$$

Comparison between animal and human exposure is generally based on AUC, but sometimes it may be more appropriate to use C_{max}. The synonyms "safety ratio," "safety margin," or "margin of safety" are frequently used for animal/human exposure ratio.

B.20.5 Most Sensitive Species

The animal/human ratios are always estimated in a conservative way, which means that the lowest exposure data (most sensitive animal species and sex) in animals and the human exposure data at the maximum recommended human dose (MRHD) are taken for calculating the ratio.

B.20.6 Protein Binding

The unbound drug in plasma is thought to be the most relevant indirect measure of tissue concentrations of un bound drug. The rules on how to deal with the protein binding issue are clearly defined (Note 8 of the ICH Topic S1C(R2) 2008). While in vivo determinations of unbound drug might be the best approach, in vitro determinations of protein binding using parent and/or metabolites as appropriate (over the range of concentrations achieved in vivo in rodents and humans) might be used in the estimation of unbound AUC. When protein binding is low in both humans and rodents, or when protein bind ing is high and the unbound fraction of drug is greater in rodents than in humans, the comparison of total plasma concentration of drug is appropriate. When protein bind ing is high and the unbound fraction is greater in humans than in rodents, the ratio of the unbound concentrations should be used.

B.20.7 Steady-state Conditions

The AUC value used for the ratio calculation is generally $AUC_{0–24}$ under steady state condition for animals as well as for humans. Even if the drug is administered more than once daily to either species (e.g., the frequency of admin istration in laboratory animals may be increased com pared to the proposed schedule for the human clinical studies in order to compensate for faster clearance rates or low solubility of the active ingredient.) the exposure per day should be calculated and compared. For the rare cases in which the dosing interval is longer than 24 h, an appropriate calculation has to be performed and men tioned along with the value.

B.20.8 Duration of Treatment

Usually, data of several toxicity studies in the same spe cies, but with different dosing duration, are available. The ratio estimation should be done in the most conservative way, which means that the lowest exposure data in ani mals under steady state conditions should be used when ever the exposure is determined.

It is not recommended that exposure be determined at the end of the life span, and exposure monitoring is not considered essential beyond six months (ICH Guidance Toxicokinetics 1994).

Careful attention should be paid to the interpretation of toxicokinetic results in animals if there are already some toxicological findings. For example, very often it is not clear if the high exposure observed in these animals is the reason for their bad state or rather the consequence of it. Therefore, the direct link between observed exposure in already impaired animals and toxicological finding should be avoided.

B.20.9 Metabolites

Under the following circumstances, the measurement of metabolite concentrations and subsequent evaluation of animal/human exposure ratio is especially important (ICH Guidance Toxicokinetics 1994):

- When the administered compound acts as a “pro drug” and the delivered metabolite is acknowledged to be the primary active entity
- When the compound is metabolized to one or more pharmacologically or toxicologically active metabo lites, which could make a significant contribution to tissue/organ responses
- When the administered compound is very extensively metabolized and the measurement of plasma or tissue concentrations of a major metabolite is the only prac tical means of estimating exposure following admin istration of the compound in toxicity studies

In the recently issued FDA Guidance for Industry Safety Testing of Drug Metabolites (2008), it was additionally emphasized that it is crucial to gather toxicokinetic data from disproportionate metabolites in toxicity studies with direct dosing of the metabolite.

B.20.10 Biotechnology-derived Pharmaceuticals

In the FDA Guidance for Industry S6 Preclinical Safety Evaluation of Biotechnology Derived Pharmaceuticals (1997), it is repeatedly emphasized that systemic exposure should be monitored during the toxicity studies. Thus, there is no difference compared to low molecular weight compounds. However, where a product has a lower affin ity to, or potency in, the cells of the selected species than in human cells, testing of higher doses may be important. The multiples of the human dose that are needed to deter mine adequate safety margins may vary with each class of biotechnology derived pharmaceutical and its clinical indi cation(s). In addition, the effects of antibody formation on pharmacokinetic/pharmacodynamic parameters, incidence and/or severity of adverse effects, complement activation,

or the emergence of new toxic effects should be considered when interpreting the data.

CRITICAL ASSESSMENT OF THE METHOD

B.20.11 Systemic Exposure as Surrogate for Exposure in all Other Tissues

The concept of safety margins based on exposure data is based on the assumption that plasma concentrations of a compound are the surrogate for exposure in all other tissues, including the target organ of toxicity. This approach is justified in the majority of cases. However, in some cases, the systemic exposure in plasma may go in the opposite direction to the specific exposure in the target organ. For example, strong first pass hepatic extraction may increase the exposure in the target organ liver and concomitantly trigger the toxicity, but decrease the systemic exposure in plasma. Another example for systemic exposure going in the opposite direction to target organ exposure was given by Lacy et al. (1998). Probenecid, a competitive inhibitor of organic anion transport in the proximal tubular epithelial cells, was evaluated for its effect on the chronic toxicity and pharmacokinetics of cidofovir in monkeys. Nephrotoxicity was present only in monkeys receiving cidofovir without probenecid. The coadministration of probenecid resulted in an inhibition of the active tubular secretion of cidofovir into the kidneys and concomitantly in a shift from local exposure in the kidney toward higher systemic exposure to cidofovir (as measured by AUC in plasma). The decrease of specific exposure in the kidneys is most likely the reason for the protection against nephrotoxicity.

B.20.12 How to Deal with Small Safety Factors

Generally, an exposure safety margin between the clinical dose and the animal NOAEL of tenfold would be acceptable (EMEA CHMP SWP Reflection Paper on PPARs 2006). However, from the information assessed, this may be unlikely. A retrospective analysis was performed on data from carcinogenicity studies of therapeutics conducted at the maximum tolerated dose (MTD) for which there was sufficient human and rodent pharmacokinetic data for comparison of AUC values (ICH Topic S1C(R2) 2008). In 35 drug carcinogenicity studies carried out at the MTD for which there were adequate pharmacokinetic data available in rats and humans, approximately one third had a relative systemic exposure ratio less than or equal to 1, and another one third had ratios between 1 and 10.

In these cases, the following precautionary principles to determine the risk benefit profile should apply.

- Can reversibility of effects be demonstrated in repeated dose toxicity studies that include a drug free period, which may provide reassurance that the findings will not be irreversible?
- If good mechanistic data for toxic effects are available, it may help in the assessment of relevance to human safety.
- A smaller safety factor might also be used when toxicities produced by the therapeutic are easily monitored by relevant and valid biomarkers, are predictable, and exhibit a moderate to shallow dose response relationship with toxicities that are consistent across the tested species (both qualitatively and with respect to appropriately scaled dose and exposure).

A predicted safety margin close to 1 or even less in a clinical dose escalation study does not necessarily force a stop to the trial, but requires a slower dose progression.

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B.21 In Vitro/In Vivo Correlation for Transporters

Dietmar Weitz

B.21.1 General Introduction

For a long time the role of membrane transporters in the drug development process was underestimated, but over the recent years, their relevance is becoming evident in many aspects. Obviously, transporters can be part of the absorption (*A*), distribution (*D*), and elimination (*E*) process of a drug, but furthermore the metabolism (*M*) rate of drugs can be directly influenced by the uptake of the drug into the metabolizing organ, which most fre quently is the liver. Consequently, all of the so called ADME characteristics of a drug are potentially influenced by transporters.

Drug transporters are integral membrane proteins with multiple transmembrane domains. Two large superfamilies, the SLC (solute carrier family) and the ABC family (ATP binding cassette) have been classified. Presently, 360 different members belong to the SLC family classified in 46 subfamilies (http://www.bioparadigms.org). Most of the SLC members are uptake transporters mediating the transport of nutrients, vitamins, and other endo or xeno biotics into the cell. SLC transporters can function as pure passive facilitators without any energy consumption and the substrate crosses the membrane in the direction of its electrochemical gradient. Alternatively, secondary and tertiary active transporters couple the uptake of a substrate to the symport or antiport of ions or small molecular weight molecules. The cotransported ions or small molecular weight molecules follow their electro chemical gradient thereby providing the driving force for the active transport. Therefore, secondary and tertiary active transporters are able to concentrate their sub strates inside the cell several fold.

The ABC transporter family consists of 49 members divided into seven subfamilies (Dean et al. 2001). All ABC transporters share a conserved catalytic domain for ATP hydrolysis. ABC transporters are primary active and cou ple the unidirectional efflux of a substrate to the hydrolysis of ATP. Therefore, these transporters are very effective and they are able to work against a huge concentration gradi ent of the substrate. By this energy dependent efflux mechanism, the ABC transporter can protect the cell from toxic compounds. As a result, ABC transporters are highly discussed as one resistance mechanism of cancer cells to chemotherapeutics. The first described member of this protein family was the P glycoprotein (P gp, ABCB1) (Juliano and Ling 1976), which was also called MDR1 (multidrug resistance protein) due to its function. P gp and other efflux transporters are upregulated in many cancer cell lines and modulate at least in vitro the sensi tivity of cancer cell lines to toxic compounds.

From a pharmacokinetic point of view, carrier mediated transport is important at four barriers in the body: the gastrointestinal tract, the biliary tract, the renal tubule, and the blood brain barrier. This chapter will focus mainly on the liver and the kidney, which are the most prominent excretion organs of the body. But the methods described can easily be adapted to transporters from other tissues. Figure B.22 1 in Chap. B.22 (Tanja Eisenblätter) shows an overview of the prominent drug transporters expressed in these organs. Numerous publi cations about in vitro and in vivo studies have dem onstrated the relevance of these transporters in drug transport and consequently in drug drug interactions. *Table B.21 1* summarizes the most important drug transporters known so far, their organ expression and localization, a classification of their functional role as uptake (influx) or efflux transporter, and frequently used and well established probe substrates and inhibitors. Many members of the SLC family can principally trans port their substrates in both directions. *Table B.21 1* resembles the transport direction (uptake or efflux), that is currently believed to be the most relevant direction to explain the ADME properties of drugs. Transporters can be very substrate specific like the glucose transporter SGLT1, only glucose and some very similar molecules, for example, galactose are accepted by the binding site of the nutrient transporter. On the other hand, most trans porters relevant for the ADME properties of drugs are characterized by very broad and overlapping substrate specificities. Uptake transporters like OATP1B1, OAT1, OCT2, and especially efflux transporters like P gp and

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.21,

Table B.21-1

Overview of ADME transporters

Transporter	SLC/ABC nomenclature	Organ expression, localization	Transport direction	In vitro probe substrates	In vitro probe inhibitors
OATP1B1	SLCO1B1	Liver, basolateral	Uptake	Estradiol 17β-glucuronide (1 μM)	Rifampicin (10 μM)
OATP1B3	SLC01B3	Liver, basolateral	Uptake	CCK-8 (0.5 μM) Estradiol 17β-glucuronide (1 μM)	Rifampicin (10 μM)
NTCP	SLC10A1	Liver, basolateral	Uptake	Taurocholat (2.5 μM)	Bromosulfophthalein
OCT1	SLC22A1	Liver, basolateral	Uptake	TEA (25 μM)	Quinine (100 μM) Quinidine (100 μM)
OAT1	SLC22A6	Kidney, basolateral	Uptake	PAH (3 μM), 6-CF	Probenecid (100 μM)
OAT3	SLC22A8	Kidney, basolateral	Uptake	Estron-sulfate (0.05 μM)	Probenecid (50 μM)
OATP4c1	SLC04c1	Kidney, basolateral	Uptake	Digoxin	
OCT2	SLC22A2	Kidney, basolateral	Uptake	Creatinin (25 μM) Metformin (25μM)	Cimetidine, Quinidine
OCTN1	SLC22A4	Kidney, apical	Efflux	TEA (20 μM)	Verapamil
OCTN2	SLC22A5	Kidney, apical	Efflux	Carnitine (0.5 μM)	Verapamil
MATE2K	SLC47A2	Kidney, apical	Efflux	TEA, MPP	
OAT4	SLC22A11	Kidney, apical	Efflux	Estron-sulfate (0.05 μM)	Probenecid (500 μM)
MATE1	SLC47A1	Liver, canalicular	Efflux	TEA, MPP	Cimetidine, Quinidine
P-gp	ABCB1	Intestine, liver, kidney, apical	Efflux	Digoxin (5 μM) N-methyl-quinidine (1 μM, vesicles)	Cyclosporine A (10 μM) PSC833
BSEP	ABCB11	Liver, canalicular	Efflux	Taurocholate (2 μM)	Cyclosporine A (10 μM)
MRP2	ABCC2	Intestine, liver, kidney, apical	Efflux	Estradiol 17β-glucuronide	MK-571 (100 μM)
BCRP	ABCG2	intestine, liver, kidney, apical	Efflux	Methotrexat (100 μM)	Ko-134 (1 μM) Fumitremorgin C (10 μM)

ABCG2 can accept very diverse and structurally unrelated compounds as substrates. For efflux transporters like P gp this can be explained in part by different binding site of the protein (Rautio et al. 2006). For SLC transporters this is not known until now.

This chapter describes the analysis of the interaction of a new molecular entity (NME) with transporters, but a few remarks regarding metabolites are stated before that. In drug development, the clearance of a drug is described by the decrease of the compound in a given compartment for example, blood/plasma, which can be by direct excre tion or metabolic functionalization to a pharmacologically non active metabolite. Many marketed drugs, but also many drugs currently in development in the pharmaceuti cal industry are very hydrophobic molecules and their clearance depends basically on metabolism. Most of the drug metabolism takes place inside liver hepatocytes and it is well known that phase I and phase II metabolism lead to more hydrophilic molecules, which are also in most cases less toxic than the parent compound itself. It is still widely unattended, that, unlike the parent drug the apparent per meability of the metabolites is very low and nearly all metabolites have to penetrate the membrane of the hepa tocytes by means of transporters, for instance back into the blood, for renal excretion through the sinusoidal mem brane of the hepatocyte, or into the bile by transporters of

the canalicular membrane. So far, these potential drug drug interactions by metabolites are unpredictable and it should be carefully evaluated in drug development for which metabolites transporter studies should be performed. Pharmacologically active metabolites should be handled like the parent compound. The strategy for other metabolites should be evaluated on case by case decisions taking into account several considerations, like for example, total abundance of the metabolite (% metab olite of drug related material), accumulation phenomena of the metabolite, and multiple excretion pathways of the metabolite.

This chapter will give an overview of the current in vitro techniques used to analyze NMEs as substrates and inhibitors of transporters. Some cases of induction are also described in the literature, especially for P gp and other efflux transporters, but induction phenomena are not discussed as preclinical requirements in the trans porter area presently. Furthermore, this chapter will stress the difficulties that arise, when in vitro findings are used to predict clinical in vivo situations. Thereby, the potential risk of transporter mediated drug interactions might be underestimated if only plasma concentrations are moni tored clinically (what is clinical practice today). The role of drug transporters on tissue distribution, especially with regard to the brain, the heart, and other peripheral tissues seems to be of much higher importance, but the clinical investigation of tissue distribution phenomena requires the development of new techniques like PET (positron emission tomography) and MRT (magnetic resonance tomography).

B.21.2 Characterizing the Potential Interaction of NMEs with Tansporters

Unlike extensive in vitro cytochrome P450 analysis mostly performed late in discovery and during the preclinical phase in development, transporter studies are not rou tinely done up to now. Currently, neither FDA nor EMEA has enforced a guidance on the investigation of trans porters in order to evaluate the potential risks elicited by drug drug interactions. The FDA draft DDI guidance from September 2006 (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf), requests the characterization of NMEs as substrates and inhibitors of P gp. Currently, P gp is the best studied transporter and in their guidance, the FDA suggests detailed methods to analyze a NME as a substrate and inhibitor of P gp, which will be described in Sect. B.21.3.1. Due to inadequate data, the FDA was unable to provide clear advice on all other transporters, but the FDA is certain that the knowledge on the trans porter level will become more relevant in the near future, for the assessment of the safety and the DDI interaction potential of new drugs. An international working group with highly ranked members from industry and academia was formed by the FDA in order to establish the standards for in vitro transporter studies in drug development (Huang et al. 2008). After a first official FDA Critical Path Transporter Meeting in October 2008 in Washington, a White paper of the expert group is eagerly awaited in fall 2009. The first part of this chapter will focus on the highly discussed question: "Which transporters, besides P gp, should be analyzed during drug development?" for a NME.

B.21.2.1 NMEs as an Inhibitor of Transporters

Drug drug interactions mediated by transporters or genetic polymorphisms in transporters markedly affecting the pharmacokinetics of drugs continue to appear in the literature. For example, the distribution and the pharma cokinetics of some statins like rosuvastatin and pravastatin are highly influenced by the activity of the biliary organic anion transporter OATP1B1 (and OATP1B3) (Niemi 2007). Furthermore, it is described for the antidiabetic first line medication metformin, that the pharmacokinetic properties of the drug are influenced by the renal organic cation transporter OCT2 (Kimura et al. 2005a, b; Koepsell et al. 2007), while variations in the pharmacological response to the drug might be determined by the func tional activity of OCT1, expressed in liver hepatocytes (the site of pharmacological action) (Reitman and Schadt 2007; Shu et al. 2007, 2008; Becker et al. 2009). Due to the lack of standardized experimental methods and pre dictive in vitro/in vivo correlations, the investigation of NME as an inhibitor of drug transporters is still a case by case decision, except for P gp. In each project, likely co medications should be evaluated for possible DDI interactions on transporters. Chapter (B.22, Tanja Eisenblätter) lists clinically relevant drug drug interac tions on transporters. As nowadays, metformin and statins are widely prescribed in the population, the analysis of the inhibitory potential of NMEs on OATP1B1 and OCT2 (maybe OCT1?) will most probably become standard dur ing the early phase of drug development. In a later phase of drug development, before submission, in vitro data about the inhibitory potential of the NME on

additional relevant transporters like OAT3 and OATP1B3 will be granted more attention.

B.21.2.2 NMEs as a Substrate of Transporters

The importance of transporters on the pharmacokinetics of a NME itself should also be investigated in drug discovery and development (*Fig. B.21 1*). The biopharmaceutical drug distribution and classification system (BDDCS) is very helpful in predicting the potential role of transporters on the pharmacokinetic profile of a drug (Shugarts and Benet 2009a; Benet et al. 2008; Custodio et al. 2008; Wu and Benet 2005a). BDDCS is a useful extension of the biopharmaceutical classification system (BCS) introduced by Amidon et al. (1995a).

The BCS categorizes each drug into the Classes 1 to 4 due to their solubility and permeability properties (Class 1: high solubility, high permeability; Class 2: low solubility, high permeability; Class 3: high solubility, low permeability; and Class 4: low solubility, low permeability). The BCS was developed to predict the in vivo absorption of a drug by the two parameters of solubility and permeability and should help to minimize in vivo bioavailability and bioequivalence testing (FDA guidance, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070246.pdf). Wu and Benet (2005b) recognized that Class 1 and 2 drugs are, due to their hydrophobicity, mainly eliminated by metabolism while Class 3 and 4 drugs are hydrophilic molecules and excreted mainly unchanged as parent compounds by liver and kidney. So the permeability component of the BCS is replaced in the BDDCS by the degree of metabolism.

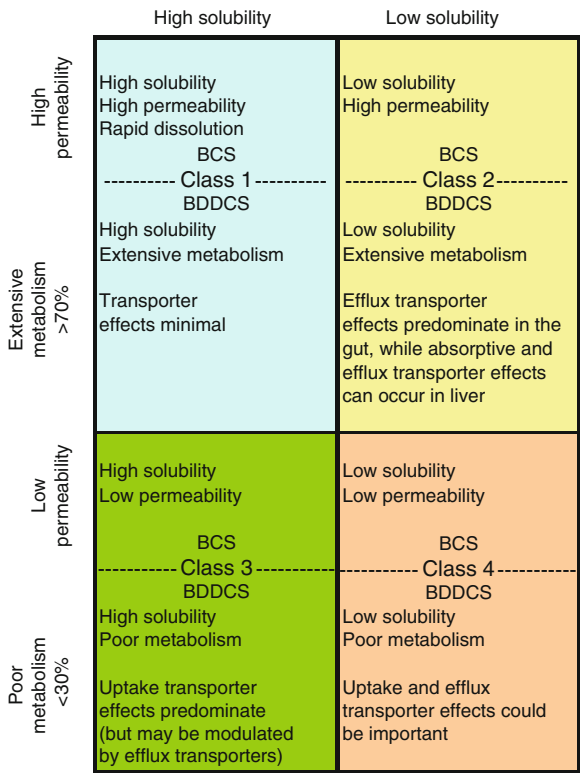

Figure B.21-1

Summary of the BCS and BDDCS and their input for transporter studies. (Adapted and summarized from Amidon et al. [1995b], Wu and Benet [2005c], and Shugarts and Benet [2009b].)

Shugarts and Benet (2009c) classified Class 1 and 2 compounds as extensively metabolized with metabolism rates $\geq$70% and Class 3 and 4 drugs as poorly metabolized with metabolism rates $<$30%. They stated that most marketed drugs fall into those two categories and metabolism rates between 30% and 70% are exceptions. Unlike BCS, BDDCS is not intended to predict in vivo absorption, but BDDCS can help to evaluate for which compounds transporters must be investigated in order to understand intestinal absorption, organ disposition, and potential drug drug interactions of the NME.

Class 1 and Class 2 drugs are hydrophobic compounds with high permeability properties allowing them to cross physiological membranes passively for example, the plasma membrane of enterocytes in the gut. Due to their high solubility Class 1 compounds are able to satisfy efflux transporters in the gut and from the BDDCS system Class 1 drugs are most obviously, minimally affected by uptake or efflux transporters. For Class 2 compounds, the low solubility of the drug in the gut might limit the free concentration and the drug is not able to saturate efflux transporters. Therefore, the active efflux might be able to influence absorption and bioavailability. Another difference between Class 1 and 2 drugs was observed that cannot be explained by the difference in solubility and needs further investigations in the future. All Class 1 drugs can penetrate the sinusoidal membrane of hepatocytes passively, while for some Class 2 drugs, an interaction with liver transporters is described. This observation postulates a qualitative difference in the passive diffusion of the drugs through enterocytes compared to hepatocytes. To summarize, for drugs classified by BDCCS as Class 1, an interaction with transporters is unlikely to interfere with the pharmacokinetics of the drug, while for Class 2 drugs gut absorption might be limited by efflux transporters and hepatic uptake can be partly limited by the interaction with uptake transporters.

BDCCS Class 3 and 4 drugs possess low permeability and need specific uptake transporters not only for absorp tion in the gut, but also for uptake into hepatocytes or kidney tubule cells. Liver uptake transporters have to be evaluated when the compound is excreted by a certain extent by bile. Transporters from the kidney need to be evaluated when active renal excretion exceeds the passive GFR of the compound by about 1.5 fold. The intrinsic GFR rate of the compound is determined by $f_u \times$ GFR (creatinine). In summary with the help of the BDDCS the influence of transporters for the overall PK of a NME can be evaluated.

An alternative tool to evaluate the analysis of drug transporters in development is the decision tree shown in *Fig. B.21 2*. For medium or poorly metabolized drugs that are excreted unchanged by more than 25% of the dose by bile and/or in cases when the active tubular secretion from kidney exceeds 25% of the dose, in vitro studies should be performed in order to evaluate the mechanism of excretion. The excretion of parent compound by bile and active tubular secretion are transporter mediated processes and a deeper knowledge about the excretion mechanisms on the protein level, will help to plan a well directed in vivo studies. By knowing the excretion organ and physiochemical parameters of the drug like the elec trical charge of functional groups and the net charge of the compound, this decision tree guides through the further analysis. For example, for an anionic drug that is actively excreted by the kidney, OAT1 and OAT3 should be ana lyzed as potential uptake transporters into the tubulus cells. As potential efflux transporters MRP2, MRP4, and OAT4 should be considered for further analysis. Today the role of the uptake transporters in liver and kidney for drug drug interactions is by far better understood and should be analyzed as first priority. The lack of knowledge regarding the efflux transporters (except P gp) is mostly due to the lack of clinical probe substrates and inhibitors. This situation might change in the future, but due to the very broad and overlapping substrate specificities of efflux transporters, this will continue to be challenging for the clinicians.

Transporters responsible for the uptake of drugs from the blood into liver or kidney cells are printed in black, transporters responsible for the excretion from liver and kidney cells into bile or urine, respectively, are colored in gray, () transporters in brackets are less relevant due to current knowledge.

B.21.3 Functional Analysis of a NME as a Substrate and Inhibitor of Transporters

B.21.3.1 P-gp

PURPOSE AND RATIONALE

As mentioned earlier the analysis of a new drug as a substrate and inhibitor of P gp was part of the FDA draft DDI Guidance in 2006 and the enforcement of

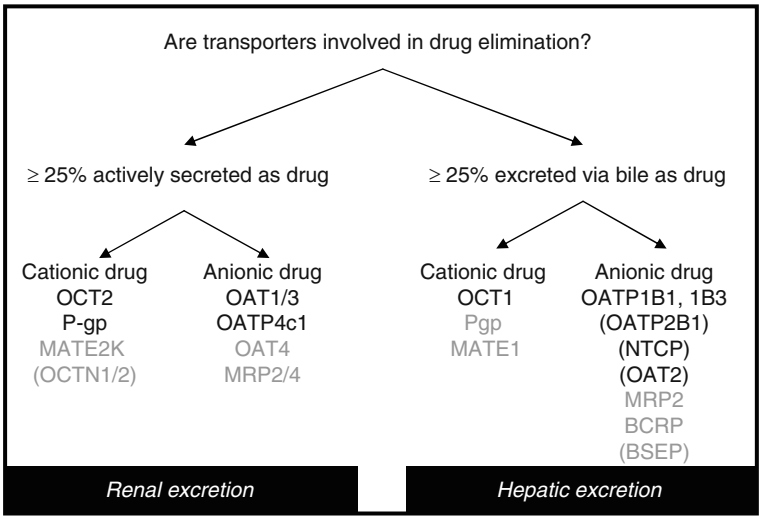

Figure B.21-2
Decision tree on the evaluation of transporters in drug development

the guidance is awaited in the very near future. In the following text, the in vitro models that will be accepted by the authorities for the assessment of new drugs as substrates and inhibitors of P gp, will be described in detail.

PROCEDURE

Currently, there are several methods described in the lit erature to analyze the interaction of drugs with P gp. In 2006, it was pointed out by the FDA that only the bidirec tional transport assay is regarded as the definitive assay for identifying substrates and inhibitors of P gp. Other assays, like the vesicle ATPase assays to identify P gp substrates or inhibition assays using fluorescent probe substrates, are indirect or cannot discriminate between substrates and inhibitors. These assays can be used for screening purposes in discovery projects, but do not fulfill regulatory require ments. Bidirectional transport assays can be performed with the polarized monolayer cell lines Caco 2, MDCK MDR1, and LLCPK1 MDR1. Caco 2 cells are seeded at densities of 0.5 5 $\times$ 10^5 cells/cm^2 on polycarbonate microporous membrane filters and need to grow for 17 21 days to confluency. MDCK MDR1 and LLCPK1 MDR1 should be seeded at a density of 0.05 5 $\times$ 10^6 cells/cm^2 on the same filter type, but require a markedly reduced incubation time of 3 5 days. This advantage of short incubation times is counteracted by the drawback that wild type MDCK and LLCPK1 cells always need to be incubated in parallel for controls. The integrity of the monolayer should be controlled by transepithelial electrical resistance measurements (TEER typical value 100 800 Ω/cm^2) or paracellular markers like [^{14}C] man nitol with permeability values $<0.2 \times 10^{-6}$ cm/s. Fluores cent markers like lucifer yellow added in the apical compartment during the experiments is an alternative method to prove the integrity of the monolayer.

B.21.3.1.1 Experimental Design to Identify P-gp Substrates

Each experiment should include the analysis of a known P gp probe substrate to demonstrate functional P gp expression in the selected cell line. Most laboratories use digoxin (0.01 10 μM), but also loperamide (1 10 μM), quinidine (0.05 μM), vinblastine (0.004 10 μM), and talinolol (30 μM) are accepted. The efflux of investiga tional drugs should be investigated at a range of concen trations (e.g., 1, 10, 100 μM). The investigational drug is added at the appropriate concentration to the apical com partment to determine $A \rightarrow B$ transport or to the basolateral side to determine $B \rightarrow A$ transport. Cells are incubated at 37°C in a humidified incubator with gentle shaking (90 rpm) and through preliminary tests it should be ensured, that during the incubation time, transport from $A \rightarrow B$ and $B \rightarrow A$ is linear or alternatively samples are taken at selected time points (e.g., 15, 30, 60, 120, 180 min), and only time points where transport was linear can be used for calcula tions. If samples are taken, the volume must be replaced by buffer. Each experiment should be performed in triplicates and the FDA also required experiments on different days to assess inter day variability. The test compound in each sample is quantified by liquid scintillation counting or by a bioanalytical method like LC/MS/MS. Recovery of the test compounds should be determined to estimate nonspecific binding or metabolism in the experiments.

The apparent permeability (P_{app}) is calculated with the equation

$$P_{app}[\mathrm{nm/s}] = 1/(S \times C_0) \times (\mathrm{d}Q/\mathrm{d}t),$$

where S = membrane surface area, C_0 = donor concentra tion at time 0, and $\mathrm{d}Q/\mathrm{d}t$ = amount of drug transported per time.

The efflux ratio R_E is determined by

$$R_E = P_{app}B \rightarrow A / P_{app}A \rightarrow B$$

Using Caco 2 cells the ratio can be determined directly. With MDCK MDR1 and LLCPK MDR1 cells the efflux ratio must be normalized by the control cells using the equation

$$R(\mathrm{atio}) = R_T / R_W$$

where R_T represents the efflux ratio of the transfected cell line and R_W represents the efflux ratio of the wild type cell line.

Each cell system should be validated with known P gp substrates and efflux ratios of the described P gp sub strates should be at least >2. An efflux ratio of >2 indicates that efflux transporters are involved in the transport of the investigational drug, but is not a standalone criterion. The efflux ratio experiment is performed with a range of con centrations and the efflux ratio should decrease with increasing concentrations of the drug, reflecting satura tion of the transporter. The lowest concentration of the investigational drug, where efflux ratio was highest, should be selected for further experiments in the presence of P gp inhibitors. Ideally, this would be a specific P gp inhibitor, but until now all inhibitors that are used in vitro do also interact with other uptake or efflux trans porters. Routinely used inhibitors for P gp are cyclospor ine A (10 μM) and PSC833 (1 μM). Cyclosporine A is a very unspecific inhibitor for some uptake and efflux

transporters, but the combination of PSC833 (relative specific for P gp at lower concentrations) and Ko 134 (<1 μM very specific BCRP inhibitor) seems very useful to characterize the efflux characteristics of a drug in cell lines and to better differentiate between transporter types. If the efflux ratio is markedly reduced (by more than 50%) in the presence of a P gp inhibitor, then it is very likely, that the investigational drug is a substrate of P gp. In this case an in vivo drug drug interaction study is recommended, but not mandatory. For example, for a new investigational drug classified as BCS and BDDCS Class 1, it is very unlikely that the pharmacokinetics of the compound is influenced by a transporter like P gp.

CRITICAL ASSESSMENT OF THE METHOD

The bidirectional transport assay is still the best method to identify substrates of P gp, with some weaknesses and real disadvantages. In the cellular assays the intracellular concentration of the test compound is unknown and can depend on uptake transporters expressed in the cell line, which varies not only between cell lines, but also between different laboratories. Furthermore, transwell experiments are always time and labor intensive, which is a disadvantage. Alternative vesicular transport assays are upcoming for many efflux transporters and will be described in Sect. B.21.3.3. These assays are less validated until now and their pros and cons will be discussed later.

B.21.3.1.2 Examples

Numerous examples of efflux studies using Caco 2 and other cell lines overexpressing expressing efflux transporters are given in the studies of Schwab et al. (2003), Polli et al. (2001), Pachot et al. (2003), and others.

B.21.3.1.3 P-gp Inhibitor

PROCEDURE

Experimental Design to Identify P-gp Inhibitors

The inhibitory potential of an investigational drug on P gp protein is evaluated with the same cell lines and with one of the probe substrates mentioned before. Most laboratories have chosen digoxin as the preferred probe substrate, which is also available radioactive labeled. $A \rightarrow B$ and $B \rightarrow A$ transport of the probe substrate is analyzed in the presence of increasing concentrations of the investigational drug for 1 3 h. The linearity of the transport of the probe substrate over time should be verified in preliminary experiments before. The receiver compartment is sampled and substrate concentration is determined. Probe substrate concentration in the donor compartment should also be analyzed to calculate the recovery of the probe substrate. Each experiment should be performed in triplicate on at least two different days to assess intra and inter day variability. IC_{50} values can be determined by different calculations for example, inhibition after nonlinear regression of the data using the Hill equation of the net flux ((probe substrate $B \rightarrow A$) (probe substrate $B \rightarrow A$)) or by the most sensitive method of efflux ratios in the presence of the inhibitor

$$(R_{Ei}/R_{Ea}) = 1 - [(I_{max} \times I^c)/(I^c + IC_{50})]$$

with (R_{Ei}/R_{Ea}) efflux ratio of the probe substrate in the presence of the inhibitor relative to the control in the absence of an inhibitor, I_{max} maximal inhibitory effect, c is the exponent of the hill plot. IC_{50} is the concentration of investigational drug achieving half maximal inhibitory effect.

In Vitro/In Vivo Correlation

Two calculations should be made to determine the risk of P gp mediated drug drug interaction. The calculations are based on the concentration of the investigational drug in relation to the determined IC_{50} value. In the first calculation I_1 equals the mean total plasma C_{max} of the NME at steady state and with the highest therapeutic dose. $I_1/IC_{50} > 0.1$ indicates that the NME is a P gp inhibitor, which may show P gp inhibition also in vivo. The second calculation uses the theoretical gastrointestinal concentration I_2 of the NME, calculated by the ratio of the highest dose to a volume of 250 mL. $I_2/IC_{50} > 10$ describes the risk of a NME to markedly influence the intestinal absorption of a co administered P gp substrate. If one of these two criteria is fulfilled a drug drug interaction study is recommended. In a recent and very comprehensive analysis the predictivity of the two criteria were examined by Fenner et al. (2009). The IC_{50} values for 19 marketed drugs were determined in Caco 2 cells. Calculations of $I_1/IC_{50} > 0.1$ and $I_2/IC_{50} > 10$ were done and compared with the outcome of clinical digoxin interaction studies. Changes in the AUC or $C_{max,ss}$ of digoxin > 25% were defined as clinically relevant. The calculation of $I_1/IC_{50} > 0.1$ was very predictive of positive clinical digoxin drug drug interactions, but also showed a very high false negative rate of 41%. Therefore this calculation alone cannot provide adequate guidance or confidence that digoxin drug drug interactions will not occur in vivo. The $I_2/IC_{50} > 10$ calculation reduced the false

negative rate markedly to 17%, but otherwise increased the false positive rate enormously. This would lead to a lot more unnecessary clinical digoxin DDI studies. In discussions about the publication, a reduction of the I_2/IC_{50} value to >5 was proposed, which will reduce the false negative rate to <10%, but will again increase false positives. There is a strong need for further in vitro and in vivo analysis to better understand and predict digoxin DDIs.

CRITICAL ASSESSMENT OF THE METHOD

The bidirectional transport assay is very well suited to determine the inhibitory potential of NME to P gp. The method is again very time and labor intensive, especially when a complete IC_{50} value has to be determined over a distinct concentration range with about 6–8 test inhibitor concentrations. The regulatory demand to assess for inter day variability increases the efforts additionally. The real disadvantage of the method lies within the fact, that the intracellular concentration of the inhibitory drug is unknown and also depends on the expression of uptake transporters and the passive permeability coefficient of the drug. Furthermore, the expression level of P gp is able to influence IC_{50} values (Bentz et al. 2005). Because of these reasons cell lines must be very well validated, to compare IC_{50} values with very well known and analyzed P gp inhibitors. For analyzing P gp inhibition, vesicular transport assays possess clear advantages (see Sect. B.21.3.3). At the moment a multinational initiative including Big Pharma, Biotech, and Academia is cross validating all available P gp assays (Caco 2, MDCK MDR1, LLCPK1 MDR1, and vesicles) with 19 selected inhibitors. The results will be published in the DDI database of the University of Washington (http://www.druginteractioninfo.org) and will have high impact, on how to validate a P gp inhibitory assay and how to correlate the in vitro results of the assay to the in vivo situation.

B.21.3.2 Uptake Transporters of the SLC Family

PURPOSE AND RATIONALE

Many pharmaceutical drugs (BDDCS Class 3 and 4 and some BDDCS Class 2 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. Total renal secretion is the product of passive glomerular filtration and active tubular secretion, which is facilitated by uptake and efflux transporters. Transporters of the SLC family are responsible for the uptake into liver and kidney and the most relevant ones are summarized in the decision tree (*Fig. B.21.2*).

PROCEDURE

B.21.3.2.1 Cell Culture and Cell Lines

All prominent members of the SLC family can be expressed in cell lines like HEK293 or CHO with state of the art overexpressing systems. HEK293 and CHO cells are cultured in minimum essential medium or HAM's F12 medium, respectively, supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C, 95% humidity, and 5% CO_2. Depending on the transfection method chosen, a further selection antibiotic like geneticin (G418) is necessary. If transporter expression rates are low, induction of the recombinant SLC transporter with 10 mM butyrate, added 24 h before the experiments to cell culture dishes is described (Konig et al. 2000a, b).

B.21.3.2.2 Validation

For every new cell line, the expression of the SLC transporter should be verified by Northern or Western blot analysis. The functionality of the cell line should be verified by determining the K_m value of 1–3 well known substrates of the transporter and by inhibition experiments using a selected probe substrate and 5–15 inhibitors. Results should be carefully compared with published data. Some well established probe substrates and inhibitors are listed in *Table B.21.1*. In principle, the experiments can be performed in 6-, 12-, 24-, or 96-well format and the choice of the format is driven by the quantification limit of the substrates. Radioactive labeled substrates are preferred due to the very sensitive and easy quantification. Bioanalytical methods to quantify the uptake are also possible but time consuming and less sensitive, which often leads to lower throughput.

B.21.3.2.3 Uptake Studies with Radiolabeled Substrates

Using a 96 well plate format for uptake experiments, cells are seeded at 10^5 cells/well/100 µL onto poly-D-lysine coated 96-well plates in cell culture medium. Experiments will be conducted 1 day after cell seeding.

In a 96 well format, 12 different assay conditions can be analyzed as quadruplets in parallel. On every plate the

control substrate of the overexpressed transporter should be incubated in the presence and in the absence of an inhibitor to ascertain functionality of the cell system. This control reduces the overall capacity to ten assay conditions, but increases the confidence in the data.

Cells are washed with 200 µL of an appropriate buffer (i.e., HBSS, 10 mM Hepes adjusted to pH 7.4) and then increasing concentration of the NME (e.g., 0.1, 1, 10, 100 µM) are incubated in quadruplet in a volume of 50 µL/well on the overexpressing cell line and in parallel on wild type control cells. Each or selected concentrations of the NME (i.e., the highest concentration) should be incubated additionally in the presence of an inhibitor, which can be very useful to discriminate between specific uptake and unspecific uptake/binding to the cells. Cells are generally incubated at 37°C, but for experiments with very short incubation times (<5 min), incubations at room temperature are more convenient. The incubation time of an uptake experiment is substrate and not transporter specific. In preliminary experiments, the incubation time of the probe substrate can also be used for the experiments with the NME, for final K_m determinations, it should be verified by additional experiments before, that uptake of the NME is linear over time. The uptake is stopped by adding 150 µL/well ice cold assay buffer. If incubation times are very short, it is useful to add a specific inhibitor of the transporter to the assay buffer. This stops the uptake immediately and improves the quality of the data markedly, by reducing standard deviations. Radioactivity is removed and cells are washed with 200 µL/well ice cold assay buffer for one or two times. After the complete removal of the washing solutions 100 µL/well scintillation liquid (i.e., Microscint^{TM} 40) are added and cells are lysed for 30 min under continuous orbital agitation (500 rpm). Radioactivity in each well is determined by liquid scintillation counting (i.e., TopCount NXT, Perkin Elmer Life Sciences).

Using a calibration curve, the uptake of the NME [pmol/well] is calculated from the radioactivity detected in each well [cpm/well]. Subsequently, the uptake of the cell lines (overexpressing cell line and control cell line) is normalized by the protein amount in each well [mg/well] to give pmol/mg protein. Protein concentration is determined by standard assays like Lowry or BCA. With consideration of the incubation time the uptake into each cell line is given as pmol/min/mg protein. Net uptake is calculated by subtraction of background uptake according to the following equation: uptake by HEK/CHO hTransporter minus uptake by control HEK/CHO cells. Uptake should be at least two times higher in the overexpressing cell line compared to the control, to clearly identify the NME as a substrate. If the NME is identified as substrate of the transporter, a K_m value should be determined with 5 8 concentrations of the NME on log scale.

The K_m and V_{max} values are determined by nonlinear regression fitting of the data to the Michaelis Menten model

$$V_c = V_{max} \times S/(K_m + S)$$

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).

B.21.3.2.4 In Vitro/In Vivo Correlation

Currently, there is no valid quantitative approach to translate K_m and V_{max} values obtained for a NME in cell based transporter in vitro assays to the clinical in vivo situation. If the in vitro study was performed due to the decision tree in *Fig. B.21 2*, transporters are relevant for the overall ADME characteristics of the NME and the qualitatively proven interaction in the in vitro system would result in a targeted clinical interaction studies with specific inhibitors explained in chapter 18 (Tanja).

B.21.3.2.5 Inhibition Studies with Radiolabeled Probe Substrates

Inhibition experiments to obtain IC_{50} values are performed as described before in 96 well plates, in quadruplets and overexpressing cells, and control cells should be investigated in parallel. The IC_{50} value represents the concentration of a drug that is required for 50% inhibition in vitro and can be used to compare the potency of two antagonists. IC_{50} values are dependent on experimental conditions and therefore experimental conditions should be carefully standardized and validated for the probe substrate. The concentration of the probe substrate in the assay should be well below the K_m value to assure that the obtained IC_{50} value for the NME is independent of the concentration of the probe substrate (Cheng Prusoff equation (Cheng and Prusoff 1973): $K_i = IC_{50}/(1 + [S]/[K_m])$, where $[S]$ represents the probe substrate concentration, K_m is the equilibrium dissociation constant of the probe substrate, and IC_{50} is the NME concentration that displays 50% inhibition. If $[S] << [K_m]$ the equation is reduced to $K_i \approx IC_{50}$ and the inhibition constant K_i is independent of the experimental conditions. It is worth mentioning, that this holds only true for IC_{50} values, based

on a pure competitive inhibition mechanism). Furthermore, uptake of the probe substrate should be linear over time during the experiments. The probe substrate is incubated in the absence and in the presence of increasing concentrations of the NME (6 8 concentrations on log scale). A positive control inhibitor (see *Table B.21 1*) should always be analyzed in parallel. The experiment is performed and analyzed as described before. The IC_{50} value is determined by plotting the percentage of residual net uptake (in %) versus inhibitor concentration (in µM). For each inhibitor concentration, the percentage of residual net transport is calculated using the following equation:

$$\textit{Percentage of net residual transport} = \frac{\left[Q_{(hTransporter)i} - Q_{(wt)i}\right]}{\left[Q_{(hTransporter)0} - Q_{(wt)0}\right]} \times 100$$

where Q_i is the net transport Q in the presence of inhibitor in overexpressing (hTransporter) or control (wt) cells

Q_0 is net transport Q in the absence of inhibitor in overexpressing (hTransporter) or control (wt) cells

Results were obtained by nonlinear regression using the 4 parameter logistic model according to Ratkovsky and Reedy (1986) ($y = A + C/(1 + \exp[-B \times \ln(x) - M])$ with A = lower asymptote, C = upper asymptote, B = slope of the curve at EC_{50}, and $M = EC_{50}$). The lower and upper asymptotes were set to 0% and 100%, respectively. The net transport in the absence of an inhibitor is set to 100%. At IC_{50} the net transport is reduced to 50%.

B.21.3.2.6 In Vitro/In Vivo Correlation

The decision tree in *Fig. B.21 3* can be used to decide, if a clinical interaction study is mandatory and reflects the current opinion in the scientific community. The calculation of the potential risk for a DDI is based on the plasma concentration of the NME related to the deter mined IC_{50} value. With $I/IC_{50} > 0.1$ a clinical interaction becomes likely and with $I/IC_{50} > 1$ an interaction is most probably to occur. I/IC_{50} values < 0.1 imply that a clinical interaction is unlikely. This decision tree seems very con vincing and straightforward at first sight and is the best way to decide, if a clinical DDI study is mandatory, but posses some intrinsic weaknesses. First, it is not known and highly discussed which concentration of the com pound in the plasma should be used for the calculation, total C_{max} or unbound C_{max}. Unbound C_{max} is favored by many scientists, because this is the free concentration of the NME in the blood and only the free concentration of NME is able to inhibit the transporter. But how do k_{on} and k_{off} rates of protein binding of the NME influence the inhibitory potential? Will authorities prefer the more con servative total plasma C_{max}? Is only the free drug in plasma responsible for the inhibition of the transporter or is the NME also able to inhibit the transporter from the intra cellular site? Due to the electrochemical gradient across the cellular plasma membrane, in equilibrium, the free concentration of each cation inside the cell is ten times higher compared to plasma and is this relevant here? These questions lead directly to the second major prob lem of the decision tree. The I/IC_{50} calculations are directly adopted from the CYP P450 area and nowadays in vitro and in vivo data for transporters, to prove the validity of the calculations, are missing. A decision tree like the one presented here, is currently the best way to calculate potential DDI risk on uptake transporters in liver and kidney, but the weaknesses should be kept in mind.

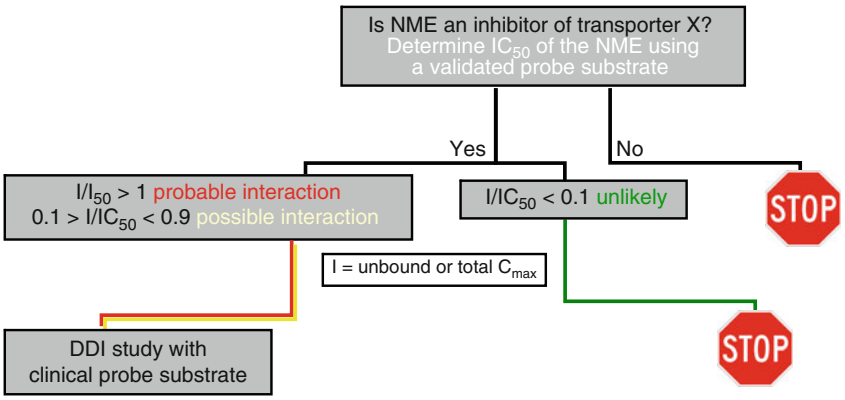

Figure B.21-3
Decision tree for analyzing a NME as an inhibitor of a transporter in vivo

B.21.3.3 Drug Efflux by ABC Transporters

B.21.3.3.1 Drug Transport Mediated by ABC Transporters Using Eukaryotic Membrane Vesicles

PURPOSE AND RATIONALE

ATP binding cassette (ABC) proteins 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 tubulus cells of the kidney, and the canalicular membrane of hepatocytes being involved in intestinal, renal, and hepatobiliary excretion. ABC transporters use the energy of the ATP hydrolysis for transport and therefore they are able to translocate substrates against a huge concentration gradient.

Three different biochemical assay types were developed in the past to analyze the activity of efflux transporters. The ATPase assay is based on the activation of ATP hydrolysis in the presence of substrates and in the nucleotide trapping assay the ATP binding cassette of the efflux transporter is blocked in the transition state after ATP hydrolysis by adding stabilizing agents like vanadate. Both assays are indirect measurements, which is their major disadvantage compared to the vesicular transport assay. In the vesicular transport assay, inside out vesicles are used to directly detect the translocation of a NME by the ABC transporter.

PROCEDURE

B.21.3.3.2 Uptake Assays Using Vesicular Membranes

In principle, membrane vesicles can be prepared from purified membranes of various sources. In the recent years, membranes from the cell line Sf9 from the insect spodoptera frugiperda became very prominent due to the easy culturing conditions and transfection methods. Furthermore, eukaryotic membrane proteins can be functionally overexpressed in these cells with very high yields. The history of the use of membrane vesicles to study ABC transporters and the different techniques are comprehensively described and reviewed in Glavinas et al. (2008).

Inside out vesicles for many different ABC transporters from multiple species are commercially available from SOLVO, Genomembrane, or BD biosciences. A detailed description of the assay is provided by each supplier.

Membrane vesicle suspension should be thawed quickly at 37°C in water bath and stored on ice before use. The radiolabeled test compound in various concentrations and a reference substrate as positive control are added in a test tube together with the respective membrane vesicles and incubated for about 5 min to equilibrate to 37°C (or less if described by the supplier). Each substrate concentration should be incubated at least in duplicates. The uptake is started by adding ATP. A parallel incubation in the presence of AMP is performed as negative control. The tubes are incubated at 37°C for an assay specific time. The assay time depends on the substrate and on the expression rate of the efflux transporter in each batch. Therefore, the correct incubation time of the substrate should be analyzed in preliminary experiments, as it is described before for SLC transporters. The uptake into the vesicles is stopped by adding an excess of ice cold buffer. If data is very scattering, it might be useful again to add a specific inhibitor of the efflux transporter to the stopping buffer. Rapid filtration techniques using glass fiber filters or nitrocellulose membranes are used to separate the membrane vesicles from the incubation solutions. Single filters or 96 well filter plates can be used to increase the throughput. The filters should be washed several times (5 10 times) with ice cold buffer. After drying, the filter bound radioactivity is measured by liquid scintillation counting and the radioactivity on filter plates can be measured by TopCount (Perkin Elmer Life Sciences).

B.21.3.3.3 Inhibition Assays Using Vesicular Membranes

All inhibition experiments to determine IC_{50} values can be performed as described before with a radiolabeled transporter specific probe substrate and the NME in various increasing concentrations. The use of an additional reference inhibitor is recommended.

B.21.3.3.4 Data Analysis of the Assays

Expression of the Results

The radioactivity counting is given in Bq/filter. The drug uptake (pmol/filter) is calculated from the radioactivity detected in each filter [Bq/well] compared to the used radioactivity [Bq/experiment]. Subsequently, the uptake in both ATP stimulated and nonstimulated membrane vesicles is normalized by the amount of membrane proteins per filter to give pmol per mg protein. With consideration of the incubation time the uptake is given as

pmol/min/mg. Net uptake is calculated by the subtraction of background uptake according to the following equation (uptake by ATP stimulated membranes minus uptake by nonstimulated membranes):

$$\text{Net transport} = Q_{(+\text{ATP})} - Q_{(\ \text{ATP})}$$

where Q is the quantity of compound (pmol) transported into a certain amount of membrane vesicles (mg) at the end of an incubation period (min) expressed in pmol/min/mg. Results were expressed as Mean ± SEM (if $n \geq 3$).

B.21.3.3.5 Kinetic Analysis of Uptake Experiments

The K_m and V_{max} values were determined by plotting the net uptake (in pmol/min/mg) versus substrate concentration (in μM). Results were obtained by nonlinear regression analysis using the Michaelis Menten kinetics model (fit = $V_{max} \times [S]/([S] + K_m)$).

B.21.3.3.6 Kinetic Analysis of Inhibition Experiments

The IC_{50} values were determined by plotting the percentage of residual net uptake (in %) versus inhibitor concentration (in μM). For each inhibitor concentration, the percentage of residual net transport was calculated using the following equation:

$$\text{Percentage of net residual transport} = \frac{\left[Q_{(+ATP)i} - Q_{(\ \ ATP)i}\right]}{\left[Q_{(+ATP)0} - Q_{(\ \ ATP)0}\right]} \times 100$$

where Q_i is Q in the presence of inhibitor

Q_0 is Q in the absence of inhibitor (control)

Results were obtained by nonlinear regression using the 4 parameter logistic model according to Ratkovsky and Reedy (1986) ($y = A + C/(1 + \exp[-B \times \ln(x) - M])$ with A = lower asymptote, C = upper asymptote, B = slope of the curve at EC_{50} and $M = EC_{50}$). The lower and upper asymptotes were set to 0% and 100%, respectively. The net transport in the absence of an inhibitor is set to 100%. At IC_{50} the net transport is reduced to 50%.

CRITICAL ASSESSMENT OF THE METHOD

The vesicular transport assay is a very useful tool to identify substrates and inhibitors of efflux transporters with medium throughput. The assay is very straightforward and less time and labour intensive compared to cellular transwell assays.

Vesicles can be made of every membrane source, but due to the advantage of high expression rates most efflux transporter assays are done with membranes from Sf9 cells. Two main differences are described for membrane proteins from Sf9 cells compared to membrane proteins from mammalian cells. Protein glycosylation and the membrane lipid composition differs in Sf9 compared to mammalian cells and especially the reduced cholesterol content of Sf9 membranes can influence the activity of the efflux transporter, nicely shown for ABCG2, which could be overcome by a simple cholesterol loading procedure (Pal et al. 2007; Telbisz et al. 2007). For the different glycosylation pattern of membrane proteins derived from Sf9 cells, no functional consequence has been reported until now.

The major disadvantage of vesicular transport assays in identifying efflux transporter substrates is the observation that compounds with a medium to high passive permeability can penetrate the vesicles in both directions passively much faster, than they are transported by the efflux transporter. This action results in a very high background uptake, which completely hides the specific transport. Therefore, this kind of assay is not sensitive to detect compounds with a medium to high passive permeability as a substrate of efflux transporters.

For inhibition assays passive permeability of the probe inhibitor is not relevant. In inhibition assays the vesicular transport assay possesses a major advantage compared to cell assays, knowing the exact concentration of the inhibitor at the transporter. In the cellular assay the intracellular concentration of the inhibitor is unknown (see above). It is expected that the vesicular transport assay will identify inhibitors of efflux transporters like P gp more precisely and with lower IC_{50} values, compared to a cellular system like Caco 2. As mentioned before a multinational initiative including Big Pharma, Biotechs, and Academia is cross validating all available P gp assays. A P gp vesicle inhibition assay is also part of the validation process and IC_{50} values obtained from 16 selected inhibitors, will be compared with the results obtained from standard cellular transwell assays using Caco 2, MDCK MDR1, and LLCPK1 MDR1. The results are eagerly awaited and will be published in the Washington database. The P gp vesicle inhibition assay could well be a time and labor saving alternative to the conventional cell assays.

B.21.3.3.7 In Vitro/In Vivo Correlation

How to perform the in vitro/in vivo correlation for P gp substrates and inhibitors was described before. For all other efflux transporters such a correlation is impossible

as of today. This is primarily, because in vivo data of drugs specifically interacting with a distinct efflux transporter are missing. There is some evidence in the literature, that the absorption of BCRP substrates like sulfasalazine is limited in the small intestine due to the activity of the efflux pump. Polymorphisms in the ABCG2 lead to the expression of transporters with different functional activ ities and the different isoforms of the protein could be correlated with the variability observed in the absorption of sulfasalazine (Urquhart et al. 2008). Varying absorption values of other BCRP substrates might also be explained by this mechanism. Furthermore, BCRP inhibitors should be able to increase the absorption of BCRP substrate, but this was not detected clinically with sulfasalazine in the presence of the BCRP inhibitor pantoprazole (Adkison et al. 2010).

B.21.4 Outlook and Further Reading

Drug transporters research is a very interesting and dynamic branch of research today. Many labs around the world are working on the improvement of methods to better predict drug drug interactions on the level of trans porters. Despite the well established methods described here, several other and more complex methods are cur rently used to identify or predict clinically relevant drug transporter interactions. Double or multiple transfected MDCK cells are a useful tool to identify a pair of two transporters, functioning as "entrance and exit" gates of the cell (Nies et al. 2008; Bartholome et al. 2007; Liu et al. 2006; Kopplow et al. 2005; Letschert et al. 2005; Cui et al. 2001; Ishiguro et al. 2008; Yamashiro et al. 2006; Matsushima et al. 2005; Sasaki et al. 2002). The use of hepatocytes to study the uptake of drugs is becoming more prominent. Sandwich cultured hepatocytes can even be used to analyze uptake and efflux processes in hepatocytes (Abe et al. 2008, 2009; Ghibellini et al. 2007). Kidney slices have begun to be used for analysis of uptake into tubulus cells (Nozaki et al. 2004, 2007; Deguchi et al. 2004). First attempts of transporter dependent PK/PD modeling are also described (Kusuhara and Sugiyama 2009). These examples show the high interest of researcher to understand and predict the role of transporter in ADME and it will be interesting and exciting to follow the outcome of this development in the subsequent years.

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B.22 Relevance of Transporters in Clinical Studies

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Over the last 10 years, the knowledge of the contribution of transporters to the absorption, distribution, and elimination of drugs has exploded. The pharmacokinetic characteristics of drugs that are substrates for these transporters are expected to be influenced by coadministered drugs that work as inhibitors or enhancers of the transporter function. Therefore, there is an increasing recognition by regulatory agencies also (Zhang et al. 2008; Huang et al. 2008) that clinically significant transporter mediated drug interactions in absorption and secretion from the intestine, secretion from the kidney and liver, and transport across the blood brain barrier (BBB) can occur. As the participation of particular transporters in observed drug–drug interactions is still often difficult to confirm, this chapter focuses on well known and clinically relevant pharmacokinetic interactions only. Methods to predict the potential of a clinically significant interaction from in vitro data are being developed and are summarized in Chap. B.19.

Figure B.22-1 summarizes the localization of clinically relevant transporters in human liver, kidney, intestine, and brain capillary endothelial cells (which form the BBB). These transporters are classified as solute carriers (SLC) and the ATP binding cassette (ABC) transporters. Typical drug transporters of the SLC family are the organic cations (OCTs, OCTNs, MATEs), organic anions (OATs, OATPs), bile salts (ASBT, NTCP), and peptide transporters (PEPTs). Multidrug resistance associated proteins (MRPs, Pgp, BCRP) and bile salt export pump (BSEP) belong to the ABC transporter family.

Of the various transporters, the efflux transporter P-glycoprotein is the most studied. But the uptake transporters for organic anions OATP1B1, OATP1B3, OAT1, and OAT3 and for organic cations OCT1 and OCT2 also appear to be responsible for drug interactions. On the level of absorption, BCRP seems to play a major role in drugs with narrow therapeutic index. *Tables B.22-1* and *B.22-2* summarize transporter inhibitors and substrates, respectively, for which clinically relevant drug–drug interactions have been reported. A PK change of at least 20% (1.2 fold) was considered as clinically relevant. In contrast to drug–drug interactions mediated by cytochrome P 450 enzymes, the maximum observed changes in the plasma pharmacokinetics following a transporter mediated interaction seems moderate. However, animal studies have shown that transporter inhibition has a great impact on the tissue distribution of drugs in addition to the effect on the systemic exposure. Therefore, the potential risk of transporter mediated drug interactions might be underestimated if only plasma concentrations are monitored.

Chapter B.19 gives detailed recommendations when a clinical transporter interaction study is considered in drug development. Physicochemical and pharmacokinetic properties of the drug candidate as well as in vitro transporter studies should guide the decision for a certain clinical interaction study.

Especially drug candidates that are poorly metabolized and mainly excreted unchanged via kidney (e.g., more than 25% of dose excreted as unchanged drug and active renal clearance contributes to more than 25% of total renal clearance) or liver (e.g., more than 25% excretion via bile) should be investigated as substrate of transporters in vitro. Depending on the ionization of the drug candidate, organic cation transporters (e.g., OCT1 or OCT2 for hepatic or renal excretion, respectively) or anion transporters (OATP1B1 and OATP1B3 or OAT1 and OAT3 for hepatic or renal excretion, respectively) are of main interest. In these cases, a clinical interaction study with probe inhibitors are recommended to assess the relevance of the transporter mediated drug–drug interaction on the exposure and thus on the efficacy and toxicity of the drug. Despite the broad overlap of substrate and inhibitor specificities between the different drug transporters and also between drug transporters and drug metabolizing enzymes, the clinical probe inhibitors probenecid and cimetidine can be proposed as inhibitors for organic anion transporters (OAT, OATP) and organic cation transporters (OCTs), respectively. Examples of clinical study designs are documented in Sect. B.22.1.

Besides drug candidates that are excreted unchanged via urine or bile, almost all development candidates are

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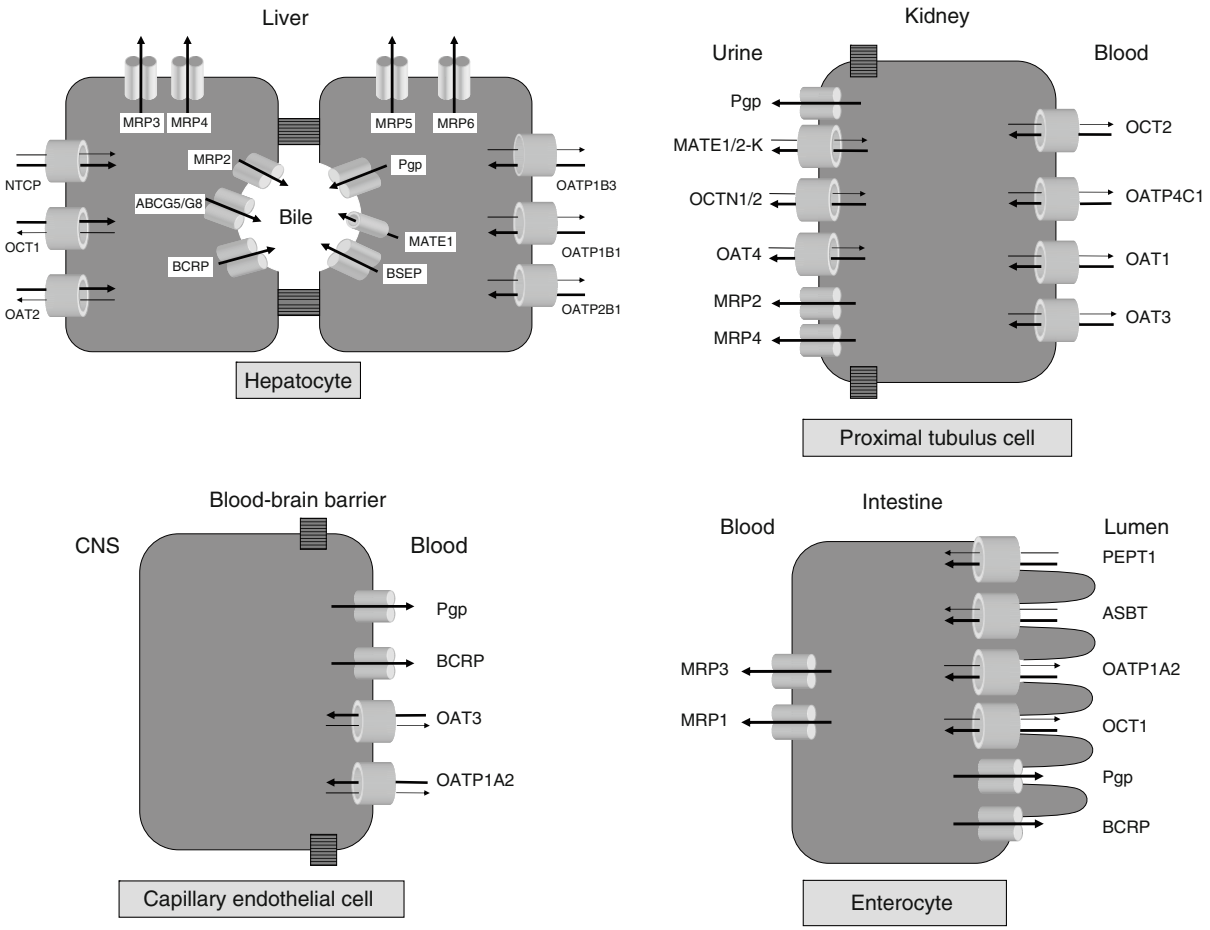

Figure B.22-1

Localization of transporters in liver, kidney, brain, and intestine

nowadays investigated for being inhibitors of certain transporters because of growing numbers of documented reports of transporter mediated drug drug interactions. Liver uptake transporter OATP1B1, kidney uptake trans porter OCT2, and drug efflux pump P glycoprotein belong to the transporters of main interest because of their well known interactions with statins leading to myopathy, with metformin leading to lactic acidosis, and with digoxin leading to cardiac toxicity, respectively. Unless transporter in vitro studies can clearly demonstrate that the drug candidate does not inhibit these transporters, clinical interaction studies with certain probe substrates for organic anion (OATP/OAT), organic cation (OCT), and P glycoprotein transporters are recommended. Sec tion 18.2 summarizes the clinical study design for interac tion studies of the drug candidate with the probe substrates pravastatin, metformin, and digoxin.

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Table B.22-1

List of transporter inhibitors with maximum PK change observed in clinical studies

Inhibitor	Transporter	Inhibitor dose, interval	Maximum PK change of substrate	Substrate for max. PK change	Reference
Amiodarone	Pgp	400 mg, QD	AUC increase: 1.7-fold	Digoxin	Robinson et al. (1989)
Azitromycin	OATP1A2	250 mg, QD	AUC increase: 1.8-fold	Fexofenadine	Gupta et al. (2001)
Cimetidine	OCT2	400 mg, BID	AUC increase: 1.5-fold	Metformin	Somogyi et al. (1987)
Clarithromycin	Pgp	250 mg, QD	AUC increase: 1.6-fold	Digoxin	Rengelshausen et al. (2003)
Co-trimoxazole	OAT1/3	800 mg, QD	CL_r decrease: 50%	Zidovudine	Chatton et al. (1992)
Cyclosporine A	OATP1B1	75 100 mg, BID	AUC increase: 12-fold	Pravastatin	Park et al. (2002)
Cyclosporine A	Pgp	10 mg/kg/day	AUC increase: 1.9-fold	Digoxin	Dorian et al. (1988)
Diclofenac	OAT1/3	50 mg, BID	Ae decrease: 58%	Ceftriaxone	Merle-Melet et al. (1992)
Erythromycin	OATP1A2, Pgp	250 mg, QD	AUC increase: 1.6-fold	Fexofenadine	Petri et al. (2006)
Erythromycin	Pgp	400 mg, QD	CL_r increase: 1.4-fold	Digoxin	Tsutsumi et al. (2002)
Furosemide	OAT1/3	200 mg, QD	CL_r decrease: 34%	Lomefloxacin	Sudoh et al. (1994)
Gemfibrozil	OATP1B1	600 mg, BID	AUC increase: 2.0-fold	Pravastatin	Kyrklund et al. (2003)
Itraconazole	Pgp	200 mg, QD	AUC increase: 1.6-fold	Digoxin	Jalava et al. (1997)
Lopinavir/ ritonavir	OATP1B1	10 40 mg, QD	AUC increase: 2.1-fold	Rosuvastatin	Kiser et al. (2008)
Ofloxacin	OCT2	400 mg, BID	CL_r decrease: 30%	Procainamide	Martin et al. (1996)
Phenazopyridine	OCT2	200 mg, QD	AUC increase: 1.4-fold	Ciprofloxacin	Marcelín-Jiménez et al. (2006)
Piliscainide	OCT2	50 mg, QD	CL_r decrease: 38%	Cetirizine	Tsuruoka et al. (2006)
Probenecid	OAT1/3	1 g, QD	AUC increase: 2.9-fold	Cephradine	Welling et al. (1979)
Probenecid	OAT3	2 g, QD	AUC increase: 1.5-fold	Fexofenadine	Yasui-Furukori et al. (2005)
Quinidine	OCT2	324 mg, tid	CL_r decrease: 41%	Procainamide	Hughes et al. (1987)
Quinidine	Pgp	648 mg, tid	AUC increase: 2.3-fold	Digoxin	Reiffel et al. (1979)
Rifampicin	OATP1B1	600 mg, QD	AUC increase: 6.8-fold	Atorvastatin	Lau et al. (2007)
Ritonavir	Pgp	300 mg, QD	AUC increase: 1.9-fold	Digoxin	Ding et al. (2004)
Ranitidine	OCT2	300 mg, QD	AUC increase: 1.5-fold	Metoprolol	Kirch et al. (1984)
Salicylate	OAT1/3	500 mg, QD	CL_r decrease: 35%	Methotrexate	Kremer and Hamilton (1995)
Sildenafil	OATP1B1	80 mg, tid	AUC increase: 1.5-fold	Bosentan	Burgess et al. (2008)
Trimethoprim (TMP)	OAT1/3	150 mg, QD	CL_r decrease: 48%	Zidovudine	Chatton et al. (1992)
Valspodar (PSC833)	Pgp	200 mg, QD	AUC increase: 3.1-fold	Digoxin	Kovarik et al. (1999)
Verapamil	OATP1A2, Pgp	100 mg, tid	AUC increase: 4.1-fold	Fexofenadine	Tannergren et al. (2003)
Verapamil	Pgp	120 mg, tid	C_{ss} increase: 1.8-fold	Digoxin	Pedersen et al. (1983)

Table B.22-2

List of transporter substrates with maximum PK change observed in clinical studies

Substrate	Transporter	Substrate dose, interval	Maximum PK change of substrate	Inhibitor	Reference
Acyclovir	OAT1/3	1 g, QD	AUC increase: 1.5-fold	Probenecid	Laskin et al. (1982)
Amiloride	OCT2	5 mg, QD	CL_r decrease: 17%	Cimetidine	Somogyi et al. (1989)
Atorvastatin	OATP1B1	40 mg, QD	AUC increase: 15-fold	Cyclosporine A	Lemahieu et al. (2005)
Bosentan	OATP1B1	500 mg, BID	AUC increase: 1.7-fold	Cyclosporine A	Binet et al. (2000)
Cefaclor	OAT3	500 mg, QD	AUC increase: 2.1-fold	Probenecid	Welling et al. (1979)
Cefadroxil	OAT1/3	1 g, QD	CL_r decrease: 46%	Probenecid	Gimeno et al. (1996)
Cefamandole	OAT1/3	1 g, QD	CL_r decrease: 75%	Probenecid	Griffith et al. (1977)
Cefmetazole	OAT1/3	2 g, QD	AUC increase: 1.5-fold	Probenecid	Ko et al. (1989)
Cefonicid	OAT1/3	50 mg	C_{max} increase: 1.5-fold	Probenecid	Pitkin et al. (1981)
Cefoxitin	OAT1/3	2 g, QD	AUC increase: 2.4-fold	Probenecid	Vlasses et al. (1980)
Ceftriaxone	OAT1/3	1 g, QD	CL_r decrease: 32%	Probenecid	Stoeckel et al. (1988)
Cephalexin	OCT2	500 mg, QD	CL_r decrease: 21%	Cimetidine	van Grugten et al. (1986)
Cephradine	OAT1/3	2 g, QD	AUC increase: 2.9-fold	Probenecid	Welling et al. (1979)
Cetirizine	OCT2	20 mg, QD	CL_r decrease: 38%	Piliscainide	Tsuruoka et al. (2006)
Ciprofloxacin	OAT1/3	200 mg, QD	CL_r decrease: 64%	Probenecide	Jaehde et al. (1995)
Ciprofloxacin	OCT2	500 mg, QD	AUC increase: 1.4-fold	Phenazopyridine	Marcelín-Jiménez et al. (2006)
Cyclosporine A	Pgp	4 mg/kg, BID	AUC increase: 1.5-fold	Verapamil	Tortorice et al. (1990)
Dicloxacillin	OAT1/3	500 mg, QD	AUC increase: 1.6-fold	Probenecid	Beringer et al. (2008)
Digoxin	Pgp	0.125 mg	AUC increase: 3.1-fold	Valspodar (PSC833)	Kovarik et al. (1999)
Dofetilide	OCT2	0.5 mg, QD	AUC increase: 1.5-fold	Cimetidine	Abel et al. (2000)
Doxorubicin	Pgp	9 mg/m^2/4d	AUC increase: 2-fold	Valspodar (PSC833)	Sonneveld et al. (1996)
Etoposide	Pgp	100 mg/m^2/day	AUC increase: 1.9-fold	Valspodar (PSC833)	Boote et al. (1996)
Famotidine	OAT3	20 mg, QD	AUC increase: 1.8-fold	Probenecid	Inotsume et al. (1990)
Fexofenadine	OATP1A2, Pgp	20 mg, BID	AUC increase: 4.1-fold	Verapamil	Tannergren et al. (2003)
Fluvastatin	OATP1B1	40 mg, QD	C_{max} increase: 4.1-fold	Cyclosporine A	Park et al. (2001)
Furosemide	OAT3	40 mg, QD	CL_r decrease: 72%	Probenecid	Chennavasin et al. (1979)
Ganciclovir	OAT1	1,000 mg, QD	AUC increase: 1.5-fold	Probenecid	Cimoch et al. (1998)
Indomethacin	OAT1/3	25 mg, tid	AUC increase: 1.6-fold	Probenecid	Baber et al. (1978)
Isofezolac	OAT1	40 mg, QD	AUC increase: 2.2-fold	Probenecid	Bannier et al. (1985)
Metformin	OCT2	250 mg, QD	AUC increase: 1.5-fold	Cimetidine	Somogyi et al. (1987)
Methotrexate	OAT3	200 mg/m^2	$C_{24\ h}$ increase: 4-fold	Probenecid	Aherne et al. (1978)
Metoprolol	OCT2	100 mg, BID	AUC increase: 1.5-fold	Cimetidine	Chellingsworth et al. (1988)
Nafcillin	OAT1/3	500 mg, QD	CL_r decrease: 72%	Probenecid	Waller et al. (1982)
Oseltamivir	OAT1	150 mg, QD	AUC increase: 2.5-fold	Probenecid	Hill et al. (2002)
Paclitaxel	Pgp	60 mg/m^2/day	AUC increase: 8-fold	Cyclosporine A	Meerum Terwogt et al. (1999)
Pilsicainide	OCT2	50 mg, QD	AUC increase: 1.3-fold	Cimetidine	Shiga et al. (2000)

Table B.22-2 (Continued)

Substrate	Transporter	Substrate dose, interval	Maximum PK change of substrate	Inhibitor	Reference
Pindolol	OCT2	15 mg, QD	AUC increase: 1.5-fold	Cimetidine	Somogyi et al. (1992)
Pravastatin	OATP1B1	40 mg, QD	AUC increase: 12-fold	Cyclosporine A	Park et al. (2002)
Procainamide	OCT2	500 mg, QD	AUC increase: 1.4-fold	Cimetidine	Rodvold et al. (1987)
Quinidine	OCTN1/2	400 mg, QD	CL_r decrease: 33%	Cimetidine	Hardy and Shentag (1988)
Ranitidine	OCT2	150 mg, QD	CL_r decrease: 40%	Cimetidine	van Grugten et al. (1986)
Rosuvastatin	OATP1B1	10 mg, QD	AUC increase: 7.1-fold	Cyclosporine A	Simonson et al. (2004)
Saquinavir	Pgp	1,200 mg, tid	AUC increase: 4.3-fold	Cyclosporine A	Brinkman et al. (1998)
Simvastatin	OATP1B1	5 10 mg, QD	AUC increase: 8.0-fold	Cyclosporine A	Ichimaru et al. (2001)
Sulbenicillin	OAT	2 g, QD	CL_r decrease: 67%	Probenecid	Itoh et al. (1998)
Zalcitabine	OAT	1.5 mg, QD	AUC increase: 1.5-fold	Probenecid	Massarella et al. (1996)
Zidovudine	OAT2	200 mg, QD	AUC increase: 2.2-fold	Probenecid	Hedaya et al. (1990)

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B.22.1 Clinical Studies with Probe Inhibitors

B.22.1.1 Probenecid as Probe Inhibitor of OATs

PURPOSE AND RATIONALE

Drug candidate X001 was designed as pro drug, which after oral administration was rapidly and entirely converted in plasma and tissues to the active moiety X001 M. At physiological pH, X001 M was a zwitterion. X001 M was poorly metabolized and predominantly excreted unchanged in urine and feces both in animal models and in humans. In humans, 50% and 35% of the dose were excreted as X001 M in urine and feces, respec tively. Renal clearance of X001 M was approximately 12 L/h. Given that the protein binding was 80% (fraction unbound (f_u): 0.2) and the glomerular filtration rate (GFR) was 7.2 L/h, glomerular filtration contribution of X001 M was approximately 1.44 L/h (f_u * GFR = 0.2 * 7.2 L/h = 1.44 L/h), indicating that secretion pro cesses were likely to be involved in X001 M renal excretion and could contribute to 80 90% of total renal clearance (1 1.44/12 L/h = 0.88; derived from "active renal clear ance contribution" = "total renal clearance (12 L/h) glomerular filtration (1.44 L/h)" divided by "total renal clearance (12 L/h)"). In vitro studies suggested that the hepatic transporter OATP1B1 and the renal transporters OAT3 and OAT4 were involved in the active excretion of X001 M through liver and kidney, respectively. Organic cation transporters OCT1 (liver) and OCT2 (kidney) were not involved in X001 M transport in vitro. Therefore, only clinical interaction study with OATP/OAT inhibitor pro benecid was performed to evaluate the clinical relevance of the transporter mediated excretion pathway of X001 M on X001 M plasma exposure.

PROCEDURE

The design of a pharmacokinetic drug drug interaction study of drug candidate X001 and probenecid as probe inhibitor of organic anion transporters OATP and OAT is presented below.

B.22.1.1.1 Title

Study of pharmacokinetic interaction of 5 day repeated oral doses (2 g/day) of probenecid on X001 (350 mg single dose) pharmacokinetics in healthy young male and female subjects.

B.22.1.1.2 Objectives

Primary objective was to assess the effect of 5 days of repeated oral doses of probenecid (2 g/day) on the phar macokinetic parameters of the active metabolite of X001 (X001 M) after a single oral 350 mg administration of X001.

Secondary objective was to assess the clinical tolerabil ity and laboratory safety of X001 single dose 350 mg when coadministered with probenecid. The results and discus sion of the safety data are not in the scope of this chapter.

B.22.1.1.3 Study Design

It was a single center, open label, randomized, two treatment, two period crossover study with at least a 7 day washout between the two periods.

B.22.1.1.4 Inclusion Criteria

Twenty six healthy male and female subjects aged from 18 and 45 years participated in the study.

B.22.1.1.5 Treatments

Subjects received a single oral dose of 350 mg X001 2.5 h after a standard breakfast on day 1 (X001 alone)

followed by five repeated single daily 2 g doses of proben ecid after a standard breakfast on days 2 6 with an addi tional single dose of 350 mg X001 on day 3 (X001 + probenecid) 2 h after probenecid administration.

EVALUATION
The effect of probenecid on the plasma concentrations and renal clearance of the active metabolite X001 M were studied as primary objective.

B.22.1.1.6 Criteria for PK Evaluation

For pharmacokinetic evaluation, concentrations of X001 M were determined in plasma and urine. At least following pharmacokinetic parameters were assessed using non compartimental analysis.

Plasma: Maximum concentration (C_{max}), time to maximum concentration (t_{max}), area under the concentration time curve from time zero to infinity (AUC), area under the concentration time curve from time zero to the last quantifiable concentration (AUC_{last}), terminal half life ($t_{1/2z}$), and plasma clearance (CL/F) were calculated for X001 M on day 1 after administration of X001 alone and on day 3 after coadministration of X001 and probenecid.

Urine: Renal clearance ($CL_{r,0-T}$) calculated as $CL_{renal} = Ae_{0-T}/AUC_{0-T}$ with Ae_{0-T} being amount of X001 M excreted into the urine within T hours, and fraction of the dose excreted in urine (fe_{0-T}).

B.22.1.1.7 PK Sampling and Bioanalytical Methods

X001 M plasma samples were collected before dosing on day 1 and day 1 (0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 h), day 2 (20, 24, 36 h), day 3 (48 h), and day 4 (72 h) after administration of X001 alone. Predose of X001 + probenecid on day 3, and day 3 (0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 h), day 4 (20, 24, 36 h), day 5 (48 h), and day 6 (72 h) after administration of X001 + probenecid.

Urine samples were collected predose of X001 alone on day 1, and day 1 (0 4, 4 8, 8 12 h), day 2 (12 24 h), day 3 (24 48 h), day 4 (48 72 h). Predose of X001 + probenecid on day 3, and day 3 (0 4, 4 8, 8 12 h), day 4 (12 24 h), day 5 (24 48 h), and day 6 (48 72 h).

X001 M plasma and urine concentrations were assayed by validated liquid chromatography tandem mass spectrometry (LC MS/MS) using a limit of quanti fication (LOQ) of 4 ng/mL in plasma and in urine.

B.22.1.1.8 Statistical Methods

X001 M pharmacokinetic parameters were summarized by the number of observations, arithmetic and geometric means, SD, coefficient of variation (CV%), median, and minimum and maximum for day 1 with X001 alone and for day 3 with X001 + probenecid.

Magnitude effect of probenecid on X001 M pharmaco kinetic parameters was estimated for the log transformed plasma parameters C_{max}, AUC_{last}, AUC, CL/F, and log transformed urine parameters Ae_{0-T}, and $CL_{r,0-T}$ using a linear mixed effects model with a fixed term for sequence, period, treatment, gender, and a random term for subject within sequence by gender,

$$\text{Log (Parameter)} \quad \text{Sequence} + \text{Period} + \text{Gender} + \text{Treatment} + \text{Subject (Sequence} * \text{Gender)} + \text{Error}$$

Estimates and 90% confidence intervals (CIs) for the ratios (X001 + probenecid vs. X001 alone) of geometric means were obtained by computing estimates and 90% CIs for the difference in the means of the log transformed data within the mixed model framework. The antiloga rithm of the confidence limits obtained constituted the 90% CI for the ratio (X001 + probenecid vs. X001 alone) of geometric means.

For $t_{1/2z}$, treatment effect was assessed based on the p value obtained from the above model. For t_{max}, the distribution of values was represented by histogram plots for each treatment. In addition, a histogram was provided of differences in t_{max} between treatments.

B.22.1.1.9 Results

Plasma concentrations of X001 M: Mean X001 M plasma concentrations versus time observed on day 1 (X001 alone) and on day 3 (X001 + probenecid) are presented in *Fig. B.22 2*.

Pharmacokinetic parameters of X001 M: Descriptive statistics on X001 M plasma and urine pharmacokinetic parameters after a 350 mg single oral administration of X001 alone or coadministered with probenecid and geo metric mean ratio estimates (X001 + probenecid vs. X001 alone) and associated 90% CIs are summarized in *Table B.22 3*. After administration of a 350 mg single oral dose of X001 at probenecid steady state, C_{max} and AUC, AUC_{last} were significantly increased by 2.59 and 5.17 , 5.25 fold, respectively, compared to a single admin istration of X001 alone. X001 M t_{max} was not modi fied after coadministration of X001 and probenecid.

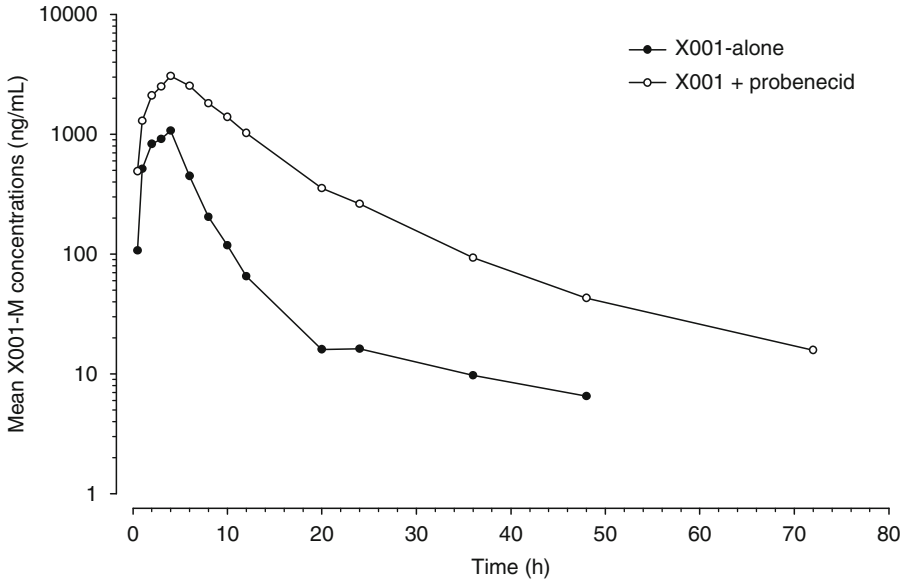

Figure B.22-2

Mean X001-M plasma concentration time profile after 350 mg single administration of X001 (X001 alone) or coadministered with probenecid (X001 + probenecid)

Table B.22-3

Summary of X001-M plasma and urine pharmacokinetic parameters and corresponding ratio estimates [90% CI] after a 350 mg single oral administration of X001 alone or coadministered with probenecid

				Difference between treatments	
		X001 alone	X001 + probenecid	Ratio estimate	90% CI
Plasma	C_{max} (ng/mL)	1,390 ± 723	3,440 ± 1,710	2.59	(2.17, 3.10)
		(52) [1,210]	(50) [3,070]		
	t_{max} (h)	3.0	3.5	na	na
		(1.00, 4.00)	(1.00, 6.00)		
	$t_{1/2z}$ (h)	27.7 ± 10.8	13.1 ± 5.52	na	na
		(39) [25.3]	(42) [12.0]		
	AUC_{last} (ng h/mL)	5,920 ± 2,200	32,300 ± 16,300	5.25	(4.55, 6.05)
		(37) [5,550]	(51) [28,900]		
	AUC (ng h/mL)	5,960 ± 1,960	32,700 ± 16,500	5.17	(4.46, 6.00)
		(33) [5,660]	(50) [29,300]		
	CL/F (L/h)	60.5 ± 20.7	12.4 ± 6.11	0.19	(0.17, 0.22)
		(34) [57.4]	(49) [11.1]		
Urine	Ae_{0-T} (mg)	73.5 ± 25.2	48.4 ± 17.2	0.66	(0.57, 0.77)
		(34) [69.6]	(36) [45.7]		
	fe_{0-T} (%)	22.6 ± 7.74	14.9 ± 5.30	na	na
		(34) [21.4]	(36) [14.1]		
	$CL_{r,0-T}$ (L/h)	13.5 ± 5.21	1.67 ± 0.567	0.12	(0.11, 0.14)
		(39) [12.8]	(34) [1.58]		

Tabulated values are mean ± SD (CV%) [geometric mean] except for t_{max} where values are median (min, max)
na, not applicable

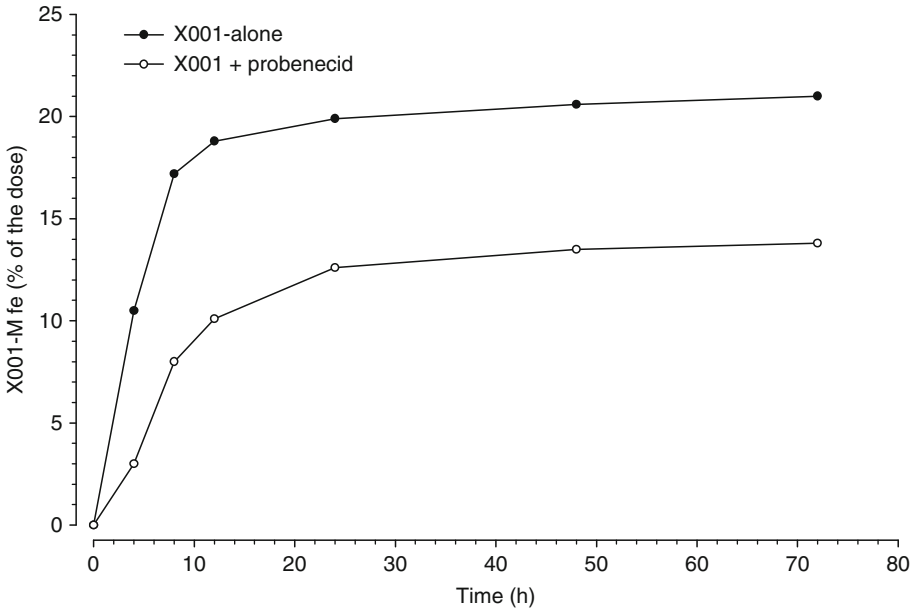

Figure B.22-3

Mean X001-M fraction of the administered does (%) excreted in urine after a 350 mg single oral administration of X001 alone or coadministered with probenecid

This increase in exposure was associated with a 1.50 fold decrease in X001 M amount excreted in urine and an 8.1 fold decrease in renal clearance. Mean X001 M fraction of the dose excreted in urine after administration of X001 alone or coadministered with probenecid are presented in *Fig. B.22 3*.

CRITICAL ASSESSMENT OF THE METHOD

For $t_{1/2z}$, a significant treatment effect was observed ($p < 0.001$). Mean $t_{1/2z}$ was longer after a 350 mg single oral administration of X001 alone than after administration at probenecid steady state (27.7 h vs. 13.1 h, *Table B.22 3*). This statistical difference is inconsistent with the decrease in plasma clearance after coadministration of X001 with probenecid. In addition, $t_{1/2z}$ observed after administration of X001 alone is longer than $t_{1/2z}$ already documented for X001 M (10 12 h) while the one observed after coadministration was within the same range. For these reasons, this statistical difference for $t_{1/2z}$ was considered not to be relevant from a PK perspective.

The increase in exposure (5.17 fold for AUC) was associated with an 8.50 fold decrease in renal clearance meaning that the totality of the renal secretion was inhibited, the renal clearance value (1.67 L/h) being similar to the estimate contribution of the glomerular filtration (1.44 L/h). This decrease in renal clearance was consistent with implication of OAT transporters in renal excretion of X001 M since probenecid was able to inhibit the totality of the renal secretion and OATP1B1 transporter was not expressed at renal level.

The nonrenal clearance was decreased by a maximum of approximately 4.4 fold (for an assumed bioavailability of one, 10.7 vs. 47.0 L/h). As X001 M was only weakly metabolized, it was assumed that the nonrenal clearance was mainly biliary excretion. This finding was consistent with implication of OATP1B1 transporter in X001 M liver uptake as observed in vitro and consequently in X001 M biliary excretion.

MODIFICATION OF THE METHOD

In order to further differentiate between the impact of OATP1B1 and OAT3 on the overall clearance, the influence of OAPT1B1 (SEARCH Collaborative Group 2008) polymorphisms on the pharmacokinetics of such drug candidate like X001 M could additionally be evaluated.

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B.22.1.2 Cimetidine as Probe Inhibitor of OCTs

PURPOSE AND RATIONALE

The cationic drug candidate X002 (pK_{a1} of 9.5 and pK_{a2} of 4.2) was predominantly excreted by the kidneys. The renal clearance of the compound indicated a large secretory component, accounting for approximately 70% of CL_r. Based on in vitro data, among the different renal transporters (P glycoprotein, MRP2, OAT1/3/4, and OCT2), organic cationic transporters are the most probable ones to be involved in X002 excretion. Therefore, a clinical interaction study with OCT probe inhibitor cimetidine was performed in phase III.

PROCEDURE

The design of a pharmacokinetic drug drug interaction study of drug candidate X002 and cimetidine as probe inhibitor for OCTs is presented below.

B.22.1.2.1 Title

Pharmacokinetic interaction of 5 day repeated oral doses of cimetidine (800 mg BID) on a single 40 mg oral dose of X002 in healthy young male and female subjects.

B.22.1.2.2 Objectives

The primary objective of the study was to assess the effect of repeated oral doses (800 mg twice daily (BID)) of cimetidine on the pharmacokinetic profile of a single oral dose of X002 (40 mg) in young, healthy, nonsmoking male and female subjects.

The secondary objective of the study was to assess the clinical and laboratory safety of X002 following a single 40 mg dose coadministered with repeated oral doses of cimetidine, as compared to that of X002 at a single dose of 40 mg, in young, healthy, nonsmoking male and female subjects. The results and discussion of the safety data are not in the scope of this chapter.

B.22.1.2.3 Study Design

It was a single center, randomized, open label, two sequence, two period, two treatment crossover study with a 7 to 10 day washout between treatments.

B.22.1.2.4 Inclusion Criteria

Sixteen healthy male and female nonsmoking subjects aged between 18 and 40 years participated in the study.

B.22.1.2.5 Treatments

There were two treatment periods:

Treatment A: X002, 40 mg single oral dose to a standardized meal (fed conditions).

Treatment B: Cimetidine, 1,600 mg/day, administered as 800 mg twice a day (BID) on days 1 5 to a standardized meal (fed conditions), with a single oral dose of X002, 40 mg, administered on day 3.

Subjects were randomly assigned to 1 of 2 treatment sequences:

Sequence 1: Treatment A, then treatment B.

Sequence 2: Treatment B, then treatment A.

EVALUATION

The effect of cimetidine on the plasma concentrations and renal clearance of drug candidate X002 were studied as primary objective.

B.22.1.2.6 Criteria for PK Evaluation

The following pharmacokinetic parameters were evaluated for X002 using non compartimental analysis.

Plasma: Maximum concentration (C_{max}), time to maximum concentration (t_{max}), area under the concentration time curve from time 0 to the last concentration above the lower LOQ (AUC_{last}), area under the concentration time curve (AUC), total body clearance (CL/F), apparent volume of distribution (Vz/F), and terminal phase apparent half life ($t_{1/2z}$).

Urine: Renal clearance (CL_{renal}), amount of X002 excreted into the urine (Ae), and fraction of the dose excreted in urine (fe).

B.22.1.2.7 PK Sampling and Bioanalytical Methods

Treatment A: Plasma samples for analysis of X002 were collected at the following times: Before administration and 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h after administration X002 on day 1. Urine samples were collected from 0 to 24, 24 to 48, and 48 to 72 h following administration of X002 on day 1.

Treatment B: Plasma samples for analysis of X002 were collected at the following times: Before administration and 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48, and 72 h after administration X002 on day 3. Urine samples were col lected from 0 to 24, 24 to 48, and 48 to 72 h following administration of X002 on day 3.

Plasma and urine samples were analyzed for X002 con centrations using validated liquid chromatography with tandem mass spectrometry methods. The LOQ for X002 was 1 ng/mL in plasma and 100 ng/mL in urine.

B.22.1.2.8 Statistical Methods

Primary analysis: Linear mixed effects model was used on log transformed C_{max}, AUC_{last}, and AUC for X002. Esti mates and the 90% CI for the ratio of adjusted geometric means of X002 + cimetidine versus X002 alone was deter mined within the mixed model framework. These CIs were compared to the bioequivalence reference interval [0.80 1.25]. If the 90% CIs were wholly contained within [0.80 1.25], then lack of interaction was concluded. If any 90% CIs were not wholly contained within [0.80 1.25], then the clinical significance of such mean ratio estimates and confidence limits was interpreted within the context of the therapeutic index.

Secondary analysis: For log transformed X002 plasma parameters ($t_{1/2z}$, CL/F, Vz/F) and urinary parameters (Ae, CL_r), linear mixed effects model was used to determine the *p* value for testing the treatment effect. In the situation where an interaction is detected in the primary analysis, estimates and 90% CI of ratio of adjusted geometric means of X002 + cimetidine versus X002 alone were calcu lated for Ae and CL_r within the mixed model framework.

B.22.1.2.9 Results

Plasma concentrations of X002: Mean X002 plasma con centrations versus time when administered alone or with cimetidine are presented in *Fig. B.22 4*. The max imum plasma concentrations of X002 were found at approximately 2 h after dosing when subjects were dosed with X002 alone and together with cimetidine.

Pharmacokinetic parameters of X002: The mean phar macokinetic parameters of X002 were calculated for subjects receiving X002 alone or with cimetidine and are presented together with treatment ratio estimates with 90% CIs for X002 + cimetidine versus X002 alone in *Table B.22 4*.

Cimetidine showed a limited potential to inhibit the clearance of X002 as demonstrated by a 1.65 and 1.20 fold increase in AUC and C_{max}, respectively, and a decrease in renal clearance from 28.7 to 15.2 L/h when

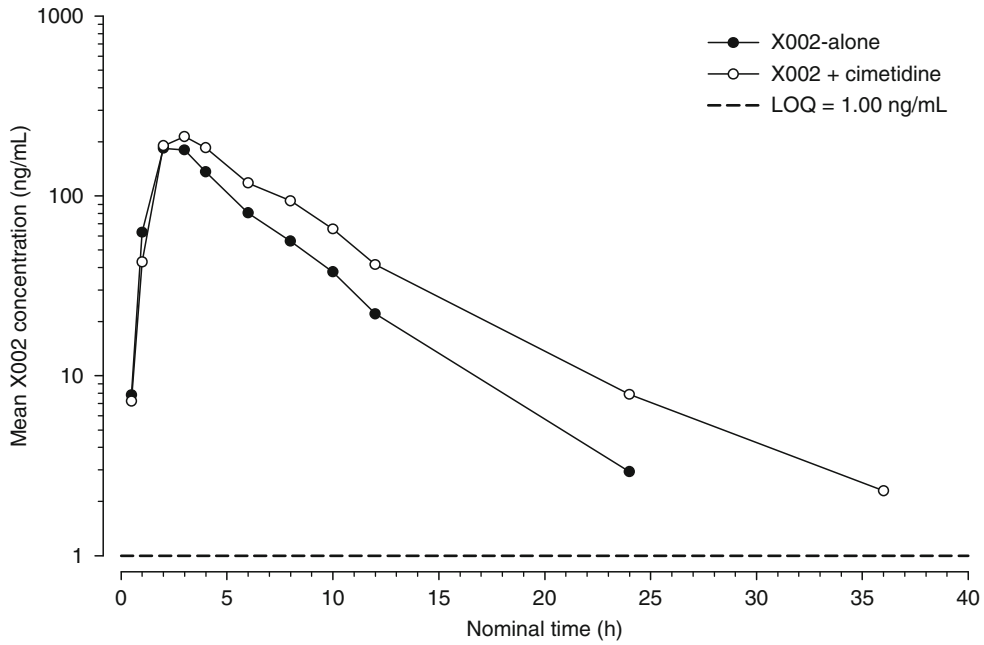

Figure B.22-4
Mean X002 plasma concentrations of X002 administered alone and with cimetidine

Table B.22-4

Summary of X002 plasma and urine pharmacokinetic parameters and corresponding ratio estimates [90% CI]

		X002 alone	X002 + cimetidine	Difference between treatments	
				Ratio estimate	90% CI
Plasma	C_{max} (ng/mL)	220 ± 40	258 ± 40.2	1.20	(1.09, 1.32)
		(18) [216]	(16) [254]		
	t_{max} (h)	2.0	2.5	na	na
		(1.00, 3.00)	2.00, 4.00		
	$t_{1/2z}$ (h)	4.35 ± 1.98	6.73 ± 1.84	na	na
		(46) [4.09]	(27) [6.51]		
	AUC_{last} (ng h/mL)	1,140 ± 295	1,800 ± 313	1.66	(1.52, 1.81)
		(26) [1,100]	(17) [1,770]		
	AUC (ng h/mL)	1,160 ± 298	1,820 ± 316	1.65	(1.52, 1.79)
		(26) [1,120]	(17) [1,790]		
	Vz/F (L)	226 ± 91.5	220 ± 67.6	na	na
		(41) [211]	(31) [210]		
	CL/F (L/h)	37.2 ± 11.1	22.8 ± 5.00	na	na
		(30) [35.8]	(22) [22.4]		
Urine	Ae (mg)	30.9 ± 6.31	27.4 ± 6.52	na	na
		(20) [30.2]	(24) [26.5]		
	fe (%)	77.2 ± 15.8	68.5 ± 16.3	na	na
		(20) [75.5]	(24) [66.2]		
	CL_r (L/h)	28.7 ± 9.11	15.2 ± 2.98	na	na
		(32) [27.4]	(20) [14.9]		

Tabulated values are mean ± SD (CV%) [geometric mean] except for t_{max} where values are median (min, max)
na, not applicable

X002 was coadministered with cimetidine. The X002 amount excreted in urine was reduced from 30.9 to 27.3 mg as illustrated for individual and mean changes in *Fig. B.22 5*.

CRITICAL ASSESSMENT OF THE METHOD

Overall, cimetidine has reduced the renal clearance of X002 by approximately 50%. However, in vitro studies using OCT2 transfected cell lines could not identify OCT2 as the responsible transporter for X002 excretion. A new transporter family called Multidrug And Toxin Extrusion (MATE) has been identified at the brush border membrane of kidney proximal tubules, responsible for the extrusion of small organic cations (Masuda et al. 2006; Terada and Inui 2008). In humans, two isoforms are expressed: MATE1 and MATE2 K both in the apical mem brane (urine side; see *Fig. B.22 1*). As cimitidine is an inhibitor of both OCT2 (IC_{50} 14 μM) and MATE1 (IC_{50} 10 μM) it is difficult to differentiate between the involvement of both transporters.

MODIFICATION OF THE METHOD

Similar as described for OATP1B1 (Sect. B.22.1.1) the investigator could also evaluate the influence of OCT2 polymorphisms on the pharmacokinetics of a drug candi date like X002. The effect of genetic variation in the OCT2 was recently shown for the renal elimination of metformin (Chen et al. 2009).

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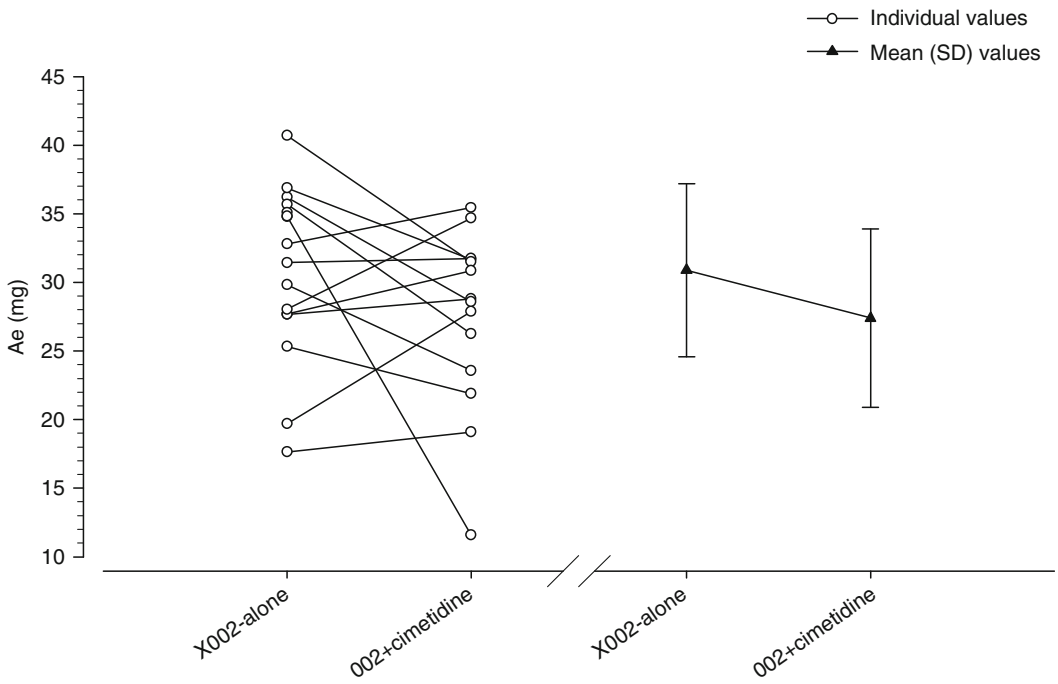

Figure B.22-5

Individual and mean (SD) X002 amount excreted in urine following administration of X002 alone and with cimetidine

B.22.2 Clinical Studies with Probe Substrates

B.22.2.1 Pravastatin as Probe Substrate of OATs

PURPOSE AND RATIONALE

Among the 3 hydroxy 3 methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) pravastatin is a more hydrophilic one, is metabolized only to a minor extent and mainly by enzymes other than cytochrome P 450 (CYP) enzymes (Igel et al. 2002). The pharmacoki netic profile of hydrophilic pravastatin differs from that of the other statins. For example, the potent inhibitor of CYP3A4, itraconazole, greatly increases the plasma con centrations of lovastatin, simvastatin, and atorvastatin but has practically no effect on pravastatin (Neuvonen and Jalava 1996, 1998; Kantola et al. 1998). Pravastatin has an oral bioavailability of about 18% (Singhvi et al. 1990). Of the bioavailable dose, 47% is excreted by the kidneys, mainly in unchanged form (Quion and Jones 1994). Prav astatin is known to be a substrate of organic anion trans porters OATP1B1 and OAT3, expressed exclusively in liver and kidney, respectively. Due to its limited metabolism, pravastatin was used as probe substrate for organic anion transporters (OATP1B1 and OAT3) in a clinical interac tion study of development candidate X003. Similar to pravastatin X003 was eliminated without being metabo lized via urine (approximately 60%) and feces (approxi mately 40%). Therefore, it was investigated whether X003 would compete with the elimination pathway of pravastatin.

PROCEDURE

The design of a pharmacokinetic drug drug interaction study with pravastatin as probe substrate is presented below. In this study, the interaction between development candidate X003 and pravastatin was investigated. The purpose was to study the effects of X003 on the plasma concentrations and renal excretion of pravastatin in healthy subjects.

B.22.2.1.1 Title

An open label, randomized, two treatment crossover pharmacokinetic interaction study of 6 day repeated oral 350 mg BID doses of X003 on a single oral 20 mg dose of pravastatin in healthy subjects.

B.22.2.1.2 Objectives

Primary objective was to assess the effect of 6 day repeated twice a day (BID) oral doses of X003 350 mg on the pharmacokinetics of a single oral dose of 20 mg prava statin, used as a probe substrate of OATs, in healthy subjects. The clinical and laboratory safety of pra vastatin administered alone and coadministered with X003 was also assessed but is out of the scope of this chapter.

B.22.2.1.3 Study Design

It was a single center, open label, randomized, two treatment, two sequence, two period crossover study with a washout of at least 7 days between periods. Twenty three healthy subjects took X003 (BID) for 5 days followed by X003 and pravastatin on day 6 during one treatment period (pravastatin + X003) and a single pravastatin (20 mg Vasten®) dose at day 1 during the other treatment period (pravastatin alone). Blood and urine samples were collected on day 6 in period pravastatin + X003 and on day 1 in period pravastatin alone.

B.22.2.1.4 Inclusion Criteria

Twenty three healthy young male and female subjects, aged between 18 and 45 years.

B.22.2.1.5 Treatments

Subjects took 350 mg X003 (BID) for 5 days followed by X003 (350 mg BID) and a single dose of pravastatin (20 mg Vasten®) on day 6 during one treatment period (pravastatin + X003) and a single pravastatin dose at day 1 during the other treatment period (pravastatin alone). Pravastatin and X003 were administered orally with 150 mL of water under fed conditions.

EVALUATION

The effect of X003 on the plasma concentrations and renal clearance of pravastatin was studied as primary objective. The sample size of 22 subjects (11 per sequence) was chosen so that a possible clinically significant pharmaco kinetic drug interaction could be statistically verified. The statistical power was sufficient to estimate the ratio of treatment means with a maximum imprecision of 17% (i.e., the 90% CI will be 0.83 and 1/0.83 times the observed ratio) and with 90% assurance. The sample size was based on the pooled pravastatin SD_{within} estimate for log transformed AUC, that is 0.30, obtained from own and bibliographic references.

B.22.2.1.6 Criteria for PK Evaluation

For pharmacokinetic evaluation, concentrations of prav astatin were determined in plasma and urine. At least following pharmacokinetic parameters were assessed using non compartimental analysis.

Plasma: Maximum concentration (C_{max}), time to maximum concentration (t_{max}), area under the concentration time curve from time of drug administra tion to the last quantifiable time (AUC_{last}), area under the concentration time curve from time of drug administra tion extrapolated to infinity (AUC), terminal elimination half life ($t_{1/2z}$).

Urine: Renal clearance (CL_{renal}) calculated as $CL_{renal} = Ae/AUC_{0-24h}$ with Ae being amount of pravastatin ex creted into the urine within 24 h were to be evaluated only if a significant effect was observed on plasma prava statin concentration.

To demonstrate steady state conditions of X003 plasma concentrations of X003 were measured and phar macokinetic parameters C_{max}, t_{max}, C_{trough}, and $AUC_{0-12\ h}$ were calculated.

B.22.2.1.7 PK Sampling and Bioanalytical Methods

Plasma samples to determine pravastatin were collected before dosing and at 0 5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, and 24 h after dosing on day 1 of the treatment period with pravastatin alone and on day 6 of the coadministration period. Urine samples for pravastatin assessment were collected cumulatively in fractions of 0 12 and 12 24 h after the administration of pravastatin (day 1 or 6, respectively). Concentrations of pravastatin in plasma and urine were determined by liquid chromatography ion spray tandem mass spectrometry with a LOQ of 0.25 ng/mL for pravastatin.

Plasma samples for X003 assessments were collected just prior to dosing from days 1 to 6, and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, and 12 h post dose on day 6 of the coadministration period.

B.22.2.1.8 Statistical Methods

All pravastatin and X003 PK parameters were summarized by descriptive statistics for each treatment period (prava statin + X003 and pravastatin alone). Pravastatin PK

plasma exposure parameters, that is, C_{max} and AUCs as primary parameters and $t_{1/2z}$ as secondary parameter, were analyzed after log transformation by a linear mixed effect model with fixed terms for sequence, period, treatment, and gender, and a random term for subject within sequence by gender. For C_{max} and AUCs, treatment ratios (pravastatin + X003 vs. pravastatin) of geometric means were estimated with 90% CIs within the mixed model framework after antilog transformation. For pravastatin half life, treatment effect was evaluated based on the *p* value calculated within the linear mixed effect model framework.

B.22.2.1.9 Results

X003 pharmacokinetics: As graphically determined in Fig. B.22 6, steady state conditions for X003 were reached on day 2, and the X003 plasma PK parameters obtained in this study were of the same order of magnitude as obtained in other studies after the administration of X003 350 mg BID under fed conditions.

Pravastatin plasma concentrations: Mean pravastatin plasma concentrations versus time observed on day 1 (pravastatin alone) and on day 6 (pravastatin + X003) are presented in Fig. B.22 7.

Pravastatin pharmacokinetics: Table B.22 5 summarizes pravastatin plasma PK parameters when administered alone and when administered with repeated oral doses of 350 mg BID X003, with corresponding ratio estimates and 90% CI (pravastatin + X003 vs. pravastatin). Pravastatin C_{max}, AUC_{last}, and AUC remained similar when pravastatin was administered alone or was coadministered with X003 (ratios of 1.06, 0.94, and 0.93, respectively). These ratio estimates are close to 1, and the 90% CIs contain 1. Due to the absence of any effect on the pravastatin plasma pharmacokinetics urine samples were not analyzed.

In conclusion, 6 day repeated administrations of X003 350 mg BID up to steady state and under fed conditions did not significantly modify the PK of a single dose of pravastatin. It can therefore be concluded that X003 does not act as an inhibitor of organic anion transporters OATP1B1 and OAT3.

CRITICAL ASSESSMENT OF THE METHOD

The described evaluation provides the study design to assess the effect of a drug candidate as a potential anion transporter inhibitor on pravastatin pharmacokinetics as substrate of OATP1B1 and OAT3 transporters in liver and kidney. In the example of X003, the pharmacokinetics of

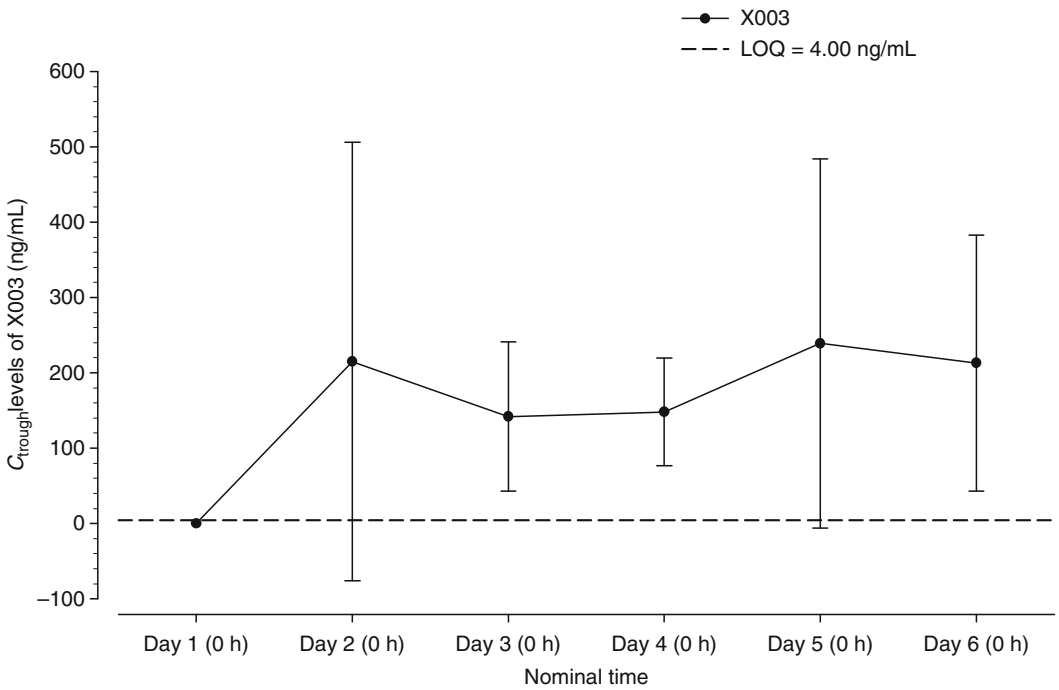

Figure B.22-6

Mean (± SD) X003 trough plasma concentrations (C_{trough}) prior 350 mg BID dosing on days 1 6

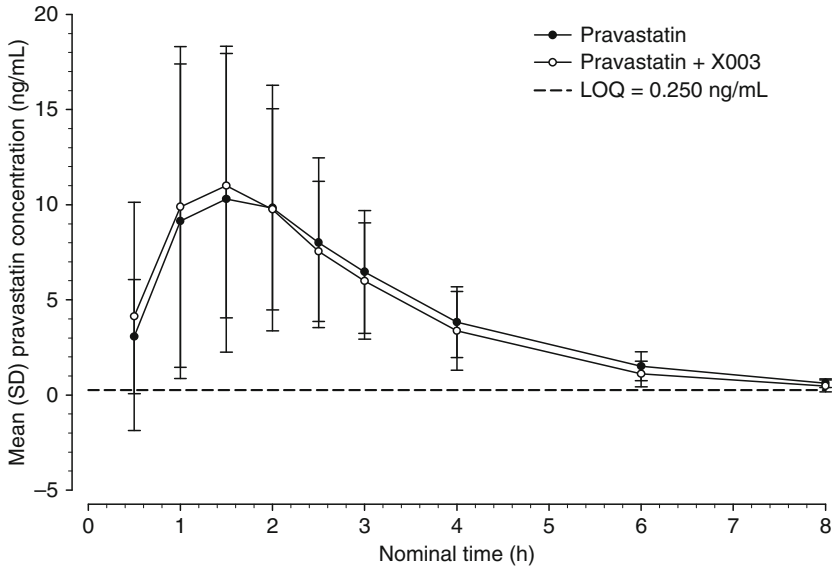

Figure B.22-7

Mean (± SD) pravastatin plasma concentration time profile after 20 mg single administration of pravastatin alone (pravastatin) or coadministered with X003 (pravastatin + X003)

Table B.22-5

Summary of pravastatin plasma pharmacokinetic parameters and corresponding ratio estimates [90% CI] after 20 mg single administration of pravastatin alone (pravastatin) or coadministered with X003 (pravastatin + X003)

				Difference between treatments	
		Pravastatin	Pravastatin + X003	Ratio estimate	90% CI
Plasma	C_{max} (ng/mL)	12.3 ± 8.25	13.0 ± 7.9	1.06	(0.80, 1.40)
		(67) [10.0]	(61) [10.7]		
	t_{max} (h)	1.5	1.5	na	
		(1.00, 4.00)	(1.00, 2.50)		
	AUC_{last} (ng h/mL)	34.6 ± 18.5	33.9 ± 18.9	0.94	(0.76, 1.16)
		(53) [30.3]	(56) [28.9]		
	AUC (ng h/mL)	35.8 ± 18.6	34.8 ± 19.7	0.93	(0.76, 1.15)
		(52) [31.6]	(57) [29.6]		
	$t_{1/2z}$ (h)	2.11 ± 0.887	3.14 ± 1.88	$p = 0.068 > 0.05$	
		(42) [1.95]	(60) [2.61]		

Tabulated values are mean ± SD (CV%) [geometric mean] except for t_{max} where values are median (min, max)
na, not applicable

pravastatin was not influenced. This result could be explained by in vitro transporter studies that were performed in parallel to the clinical study. X003 was no inhibitor of OAT3 (IC_{50} > 100 µM), it was an inhibitor of OATP1B1 with an IC_{50} of 19 µM. At therapeutic doses (350 mg BID) the total maximum plasma concentration C_{max} (= I) of X003 was 4 µM and the unbound concentration $C_{max,u}$ (I_u) was 0.8 µM. With I/IC_{50} of 0.2 and 0.04 for I and I_u, respectively, an interaction between X003 and OATP1B1 substrates was expected to be less likely. Thus, the clinical results were in line with the in vitro data.

Kyrklund et al. (2003) investigated the effect of gem fibrozil on pravastatin exposure in a single center, randomized, placebo controlled, two treatment, two period crossover study with ten healthy subjects receiving either gemfibrozil (600 mg BID) or placebo for 3 days and one

single oral dose of 40 mg pravastatin on day 3. They observed an increase in pravastatin AUC by twofold. Both gemfibrozil and gemfibrozil glucuronide were later on described as potent inhibitors of OATs in vitro (Shitara et al. 2004). Kyrklund et al.'s (2003) interpretation of the results was that in addition to the interaction on the level of renal and hepatic pravastatin elimination the interac tion probably also occurred on the level of pravastatin absorption. Because less than half of the bioavailable prav astatin was excreted renally (47%; Quion and Jones 1994) and because gemfibrozil decreased the renal clearance by 43% on average, the effect of gemfibrozil on pravastatin renal clearance accounted for only 20% increase in prav astatin AUC. In other words, the change in renal clearance was only a minor contributor to the increase in pravastatin AUC. They calculated that on average, about 7 mg (18% of 40 mg) of pravastatin reached the systemic circulation during the control phase. The amount of pravastatin (Ae_{0-24h}) excreted into the urine during a 24 h period during the placebo phase was 3.3 mg (i.e., about 46% of the bioavailable pravastatin). The Ae_{0-24h} remained unchanged in presence of gemfibrozil. This suggests that gemfibrozil slowed down the renal clearance of pravastatin and also increased the bioavailability. Interestingly, gem fibrozil did not influence the $t_{1/2z}$ values of pravastatin in this study. This finding and the finding that gemfibrozil increased the C_{max} values of pravastatin on average by 81% further support the conclusion by Kyrklund et al. (2003) that gemfibrozil may also interact with pravastatin during the absorption phase, increasing its bioavailability. Beside pravastatin being a substrate of hepatic and renal organic anion transporters OATP1B1 and OAT3 it is also described as substrate of MRP2 and P glycoprotein (Neuvonen et al. 2006), both efflux transporters expressed in liver and kidney but also in intestine. Therefore, the interaction between gemfibrozil or any development drug candidate and pravastatin as probe substrate can also be on the level of transporters other than organic anion transporters OATP1B1 and OAT3. However, to date pravastatin is the probe substrate to study drug drug interactions of a new drug candidate on the level of OATs.

MODIFICATION OF THE METHOD

Instead of pravastatin, one might also use the novel statin rosuvastatin (Schneck et al. 2004). The pharmacokinetics of rosuvastatin resembles that of pravastatin. Both prava statin and rosuvastatin are hydrophilic compounds that are not metabolized by CYP, and both are substrates of organic anion transporters (OATP1B1 and OAT3) and share the same uptake mechanisms in liver and kidney. Whereas pravastatin is known to be also a P glycoprotein substrate, rosuvastatin is described in addition as breast cancer resistance protein (BCRP) substrate (Neuvonen et al. 2006).

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B.22.2.2 Metformin as Probe Substrate of OCTs

PURPOSE AND RATIONALE

Interactions occurring in the kidney have not been sys tematically studied for a long time but are now of increas ing interest with the major mechanisms focusing on tubular secretion. Interactions in the proximal tubule involving both organic anions and cations have been stud ied using inhibitors like probenecid (Sect. B.22.1.1) and cimetidine (Sect. B.22.1.2), respectively. In this section, metformin will be presented as probe substrate to test drug drug interactions on the level of OCTs. Metformin has the propensity for causing adverse effects, especially

lactic acidosis, which may be related to high circulating concentrations of the drug (Holstein and Beil 2009; Phil lips et al. 1978). Therefore, testing new drug candidates as potential inhibitors of metformin excretion is of increas ing interest and often requested by regulatory agencies. Metformin (half life 1.5 5 h) undergoes negligible bind ing to plasma proteins and is eliminated unchanged by active tubular secretion and glomerular filtration (Hol stein and Beil 2009). No oxidative or conjugated metabo lites of metformin have been observed in plasma, urine, or feces. Metformin is a substrate of liver transporter OCT1 and kidney transporter OCT2. Whereas OCT1 is required for the antidiabetic efficacy of metformin, OCT2 facilitates the active secretion of metformin into urine.

Development candidate X004 was planned to be marketed as fixed dose combination (FDC) of X004 and metformin. Therefore, an interaction study between met formin and X004 was performed in order to confirm the lack of a pharmacokinetic interaction between both drugs.

PROCEDURE

The design of a pharmacokinetic drug drug interaction study with metformin as probe substrate for OCTs is presented below. In this study, the interaction between X004 and metformin was investigated in both directions because the purpose of this study was to confirm the absence of any effect of X004 on the disposition of met formin and of metformin on the pharmacokinetics of X004. However, the second part (effect of metformin on X004 exposure) was only assessed because of the purpose to develop a FDC and was not part of the evaluation of the effect of X004 on cation transporters OCT1 and OCT2. In this chapter, the focus is on the effect of X004 on metfor min as probe substrate for OCTs.

B.22.2.2.1 Title

An open label, three treatment, single sequence crossover pharmacokinetic interaction between 1,000 mg BID repeated doses of metformin and 10 mg BID repeated doses of X004 in healthy male and female subjects.

B.22.2.2.2 Objectives

Primary objectives of this study were to assess the effect of repeated oral doses of X004 on the steady state pharma cokinetics of metformin and the effect of repeated oral doses of metformin on the steady state pharmacokinetics of X004. As explained earlier, the effect of metformin on X004 is out of the scope of this chapter.

B.22.2.2.3 Study Design

It was a single center, open label, three treatment, three period, single sequence crossover study with no washout period between treatment periods.

B.22.2.2.4 Inclusion Criteria

Fifty healthy subjects aged between 18 and 45 years with a body mass index between 18.0 and 25.0 kg/m^2 partici pated in the study. The number of subjects was given by the purpose to show bioequivalence between the different treat ments as described in detail in statistical methods.

B.22.2.2.5 Treatments

Subjects received 7 days of 1,000 mg metformin alone BID (period 1), then 28 days of 10 mg X004 alone BID (period 2), followed by the coadministration of 1,000 mg BID metformin BID and 10 mg X004 BID for 7 days (period 3). The subjects took their medication under fed condi tions together with a breakfast or a dinner.

EVALUATION

The effect of X004 on the plasma concentrations of met formin was assessed and is summarized below.

B.22.2.2.6 Criteria for PK evaluation

For pharmacokinetic evaluation, concentrations of met formin were determined in plasma. At least following pharmacokinetic parameters were assessed using non compartimental analysis.

Maximum concentration (C_{max}), plasma concentra tion observed before administration during repeated dos ing (C_{trough}), time to maximum concentration (t_{max}), area under the concentration time curve from time of drug administration to time 12 h ($AUC_{0–12\ h}$).

B.22.2.2.7 PK Sampling and Bioanalytical Methods

Plasma samples were collected at predose on days 1, 5, 6, and 7 for the metformin alone (period 1) and the coadministration groups (period 3) and on days 1, 26, 27, and 28 for the X004 alone group (period 2), and at 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 h following last investigation

product administration in each period. Metformin and X004 plasma concentrations were determined using a validated liquid chromatography with tandem mass spectrometry method with lower limits of quantification of 2.03 and 1.00 ng/mL, respectively.

B.22.2.2.8 Statistical Methods

Sample size calculations for the comparison between met formin alone and metformin + X004 was based on the within subject standard deviations (SD_{within}) for log transformed parameters obtained from four bibliographic references (Di Cicco et al. 2000; Timmins et al. 2005; Herman et al. 2006; Atanasova et al. 2003). Pooled esti mates of SD_{within} for metformin were 0.157 and 0.174 for $\log(C_{max})$ and $\log(AUC_{0-12\ h})$, respectively. *Table B.22 6* presents the total number of subjects required to demon strate bioequivalence (using the reference interval [0.80, 1.25] between metformin alone and metformin + X004), for varying true relative ratios (0.9 1.1), true SD_{within} (0.150 0.225) with 5% error risk and 90% power. Thus, if the true SD_{within} for metformin $\log(C_{max})$ or $\log(AUC_{0-12h})$ is as much as 0.175, 19 subjects would have been required to demonstrate equivalence between treatments with 90% power and 5% error risk, allowing for a true difference in treatment means of at most 5%. As in this study design also the effect of metformin on X004 exposure had to be assessed and the SD_{within} of log of PK parameters of X004 was 0.275, at least 45 subjects were needed to demonstrate bioequivalence between X004 and X004 + metformin, and bioequivalence between metfor min and metformin + X004. Looking only on the effect of X004 on metformin (*Table B.22 6*), the power raises to 99.8% with 45 subjects in this study.

Metformin and PK parameters were summarized by treatment period using descriptive statistics. For metformin C_{max} and $AUC_{0-12\ h}$, the effect of repeated BID doses of 10 mg X004 on PK parameters of repeated BID doses of 1,000 mg metformin was analyzed using estimates and 90% CIs for the ratios of geometric means of metformin when coadministered with X004 versus metformin alone, using a linear mixed effects model on log transformed parameters. If the 90% CIS were wholly contained within (0.80, 1.25), then lack of interaction was concluded. If any 90% CIs were not wholly within (0.80, 1.25), then the clinical significance of such mean ratio estimates and confidence limits were interpreted within the context of the therapeutic index.

B.22.2.2.9 Results

Plasma concentrations of metformin: Mean (± SD) plasma concentrations of metformin administered alone and with X004 are presented in *Fig. B.22 8*. The maximum plasma concentrations of metformin were found at approximately 4 h after dosing when subjects were dosed with metformin alone and together with X004.

Pharmacokinetic parameters of metformin: The mean pharmacokinetic parameters of metformin were calcu lated for subjects receiving metformin alone or with X004 and are presented together with treatment ratio estimates with 90% CIs for metformin + X004 versus metformin alone in *Table B.22 7*.

The 90% CIs for C_{max} and $AUC_{0-12\ h}$ geometric mean ratio of metformin were entirely within the protocol spec ified boundaries (0.80, 1.25), thus demonstrating the lack of interaction of repeated doses of X004 at 10 mg BID on the repeated dose PK of metformin at 1,000 mg BID.

CRITICAL ASSESSMENT OF THE METHOD

The design of this study was chosen with the primary objective to show bioequivalence between administration

Table B.22-6

Total number of subjects for varying ratios between treatments and SD_{within}

True relative ratio	90% Power			
	SD = 0.150	SD = 0.175	SD = 0.200	SD = 0.225
0.9	29	39	51	64
0.95	14	19	25	31
1	11	15	19	23
1.05	14	19	24	30
1.1	25	33	43	54

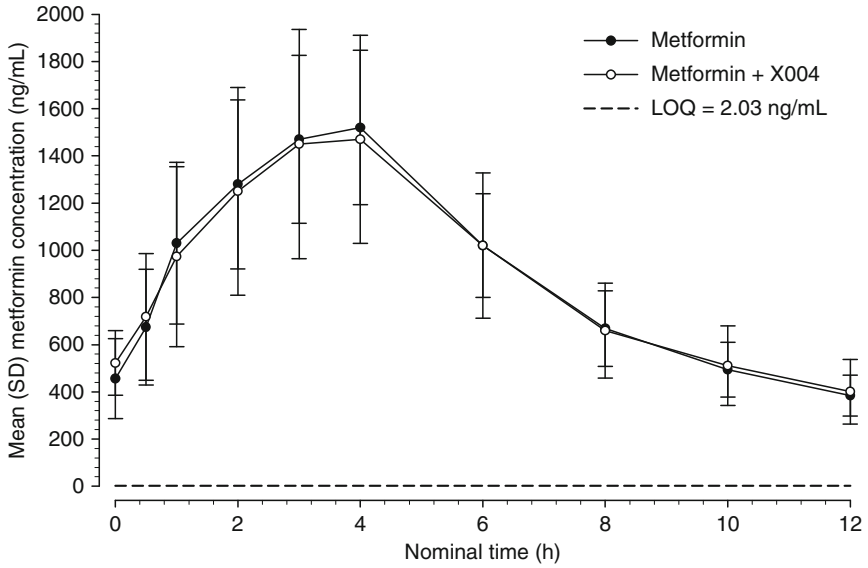

Figure B.22-8

Mean (± SD) plasma concentrations of metformin administered alone and with X004

Table B.22-7

Comparison of pharmacokinetic data for metformin when given alone (1,000 mg BID) and when combined with X004 (10 mg BID)

		Metformin	Metformin + X004	Difference between treatments	
				Ratio estimate	90% CI
Plasma	C_{max} (ng/mL)	1,560 ± 330	1,570 ± 454	1.00	(0.88, 1.14)
		(21) [1,530]	(29) [1,490]		
	t_{max} (h)	4.00	4.00	na	
		(1.00, 4.07)	(0.00, 4.00)		
	$AUC_{0-12\ h}$ (ng h/mL)	11,000 ± 2,270	10,900 ± 3,030	0.97	(0.82, 1.14)
		(21) [10,700]	(28) [10,200]		

Tabulated values are mean ± SD (CV%) [geometric mean] except for t_{max} where values are median (min, max)
na, not applicable

of drugs alone and coadministration of X004 and metformin in order to support the development of a FDC. A typical two treatment, two period, two sequence crossover study design with less subjects (typically between 12 and 24) as described in Sect. B.22.2.1 is normally adequate to investigate the effect of the development candidate on metformin pharmacokinetics. The most prominent pharmacokinetic interaction on the level of OCTs has been described so far for the effect of cimetidine on metformin exposure (Somogyi et al. 1987). In this single center, non placebo controlled, two treatment, one period sequential study, seven healthy subjects received a single daily oral dose of 250 mg metformin each day for 10 days, and between days 6 and 10 they received in addition 200 mg cimetidine twice daily. Cimetidine increased the plasma concentrations of metformin by an average of 81% and the area under the plasma metformin concentration time curve by an average of 50% (Somogyi et al. 1987). This was probably not due to an increase in the absorption of metformin, as the total urinary recovery was the same in both groups (fractional excretion of metformin during the first 12 h was 0.47 ± 0.12 and 0.49 ± 0.11 when combined

with cimetidine). Cimetidine reduced the overall renal clearance of metformin by almost 150 mL/min, indicating that the interaction involved tubular secretion. The mecha nism is considered as its competitor competition by OCT2.

MODIFICATION OF THE METHOD

Subjects with the 808G > T polymorphism in the OCT2 gene (Takane et al. 2008), which was associated with a reduced metformin clearance, should be excluded from the study population in order not to further increase the risk for lactic acidosis when metformin is given with potential OCT inhibitors. Thus, candidates for study par ticipation should be studied for this polymorphism.

It might be of interest also to measure the creatinine clearance in parallel to metformin clearance. Creatinine is reported as OCT2 and OCTN1/N2 substrate and could give additional information about the effect of new drug candidates on renal transporter pathways.

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B.22.2.3 Digoxin as Probe Substrate of P-glycoprotein

PURPOSE AND RATIONALE

Cardiovascular drug digoxin is one of the most extensively studied cardiac drugs for possible interactions. After oral administration, digoxin is well absorbed, with an average bioavailability between 70 and 80% (Magnani and Malini 1995). Hepatic metabolism is limited, and digoxin metab olites represent less than 15% of total urinary recovery. Digoxin is mainly eliminated by kidney as unchanged drug (Hinderling and Hartmann 1991). It is known to interact with a large and diverse group of coadministered drugs wherever they act as modulators of the P glycoprotein mediated transport of digoxin (Fenner et al. 2009). Because absorption and disposition of digoxin are influenced by P glycoprotein, whereas its metabolism is less important for its pharmacokinetics, the compound is frequently used as probe substrate of P glycoprotein. Digoxin has a narrow therapeutic window and even slight exposure changes have been associated with adverse reac tions; this has resulted in close monitoring of digoxin serum levels. As a consequence of the relative safety con cerns, digoxin is the recommended probe substrate for assessing P glycoprotein inhibitors (Zhang et al. 2008; Huang et al. 2008). A clinical interaction study should be considered when the development candidate meets either criterion: $[I_1]/IC_{50} > 0.1$ with $[I_1]$ being the total (bound and unbound) drug candidate concentration at C_{max} or $[I_2]/IC_{50} > 10$ with $[I_2]$ being the gastrointestinal drug concentration estimated by the ratio of the highest clinical dose to a volume of 250 mL. For the drug candidate X005 the IC_{50} was 2 μM. Given the plasma concentration $C_{max} = [I_1] = 0.4$ μM and the intestinal concentration $[I_2] = 3$ mM both criteria were fulfilled and an interaction study with digoxin was planned.

PROCEDURE

The design of a pharmacokinetic drug drug interaction study of drug candidate X005 and digoxin as probe sub strate for P glycoprotein is presented below.

B.22.2.3.1 Title

Pharmacokinetic interaction of repeated oral doses of 400 mg (BID) X005 for 10 days on repeated oral 0.25 mg (QD) digoxin in healthy young male subjects.

B.22.2.3.2 Objectives

Primary objective was to assess the effect of repeated oral doses of 400 mg twice daily (BID) X005 on the pharma cokinetic profile of digoxin after repeated oral doses of 0.25 mg once daily (QD) digoxin.

Secondary objective was to assess the clinical and lab oratory safety of X005 coadministered with digoxin as compared to that of digoxin coadministered with placebo in healthy young male subjects. Therefore, a double blind, placebo controlled study design was chosen. However, the results and discussion of the safety data are not in the scope of this chapter.

B.22.2.3.3 Study Design

It was a single center, randomized, double blind, placebo controlled, 10 day repeated dose, two sequence, two treatment, two period crossover study with a mini mum 10 day washout between periods.

B.22.2.3.4 Inclusion Criteria

Twenty healthy male subjects aged between 18 and 40 years with a body weight participated in the study.

B.22.2.3.5 Treatments

Subjects received a single daily oral dose of 0.25 mg digoxin (except on day 1, 0.50 mg AM and 0.25 mg PM) in the morning and 400 mg X005 in the morning and evening with 240 mL of non carbonated water in fed conditions.

EVALUATION

The effect of X005 on the plasma concentrations and renal clearance of digoxin was studied as primary objective. X005 plasma concentrations were monitored to demon strate steady state conditions for X005.

B.22.2.3.6 Criteria for PK Evaluation

For pharmacokinetic evaluation, concentrations of digoxin were determined in plasma and urine. At least following pharmacokinetic parameters were assessed using non compartimental analysis:

Plasma: Trough plasma concentration (C_{trough}) observed before morning dose from days 1 to 10.

Maximum concentration (C_{max}), time to maximum concentration (t_{max}), area under the concentration time curve from time of drug administration to time 24 h ($AUC_{0–24h}$) on day 10.

Urine: Renal clearance (CL_{renal}) calculated as $CL_{renal} = Ae_{0–24h}/AUC_{0–24h}$ with $Ae_{0–24h}$ being amount of digoxin excreted into the urine within 24 h, and fraction of the dose excreted in urine ($fe_{0–24h}$).

For steady state assessment of X005, trough plasma concentration (C_{trough}) from days 1 to 10 were determined.

B.22.2.3.7 PK Sampling and Bioanalytical Methods

Digoxin plasma samples were collected before dosing on days 1, 2, 3, 5, 7, 9, and 10, and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, and 24 h after last digoxin administration on day 10. Urine samples were collected before first digoxin admin istration on day 1, and in the 0 24 h interval on day 10. Digoxin plasma and urine concentrations were deter mined by validated radio immunoassay methods with a LOQ of 0.2 ng/mL in plasma and 0.5 ng/mL in urine.

X005 plasma samples were collected before morning administration of days 1, 2, 3, 5, 7, 9, and 10. X005 plasma concentrations were determined by a validated liquid chromatography tandem mass spectrometry (LC MS/ MS) with an LOQ of 0.5 ng/mL.

B.22.2.3.8 Statistical Methods

Digoxin pharmacokinetic parameters were summarized by number of observations, arithmetic, and geometric means, SD, coefficient of variation (CV%), median, min imum, and maximum for day 10 of the period with digoxin + placebo and digoxin + X005. Log transformed C_{max}, $AUC_{0–24\ h}$, $Ae_{0–24\ h}$, and $CL_{r,0–24\ h}$ values and rank transformed t_{max} values were analyzed using a linear mixed effect model with fixed terms for sequence, period, treatment, and a random term for subject within sequence

$$\text{Parameter} = \text{Sequence} + \text{Period} + \text{Treatment} + \text{Subject (Sequence)} + \text{Error}$$

Potential effect of X005 on digoxin for C_{max}, $AUC_{0–24h}$, $Ae_{0–24h}$, and $CL_{r,0–24h}$ parameters was evaluated by esti mating digoxin + X005 versus digoxin + placebo coadministration mean differences with 90% CIs, within the mixed model framework, and converting to ratios by antilog transformation. If 90% CIs were entirely within 0.80 1.25, lack of interaction was demonstrated. Other wise, the magnitude of the effect of X005 on digoxin pharmacokinetics was based on the estimated ratios and

90% CIs. For t_{max}, differences between treatments were tested for significance with p values from the mixed model analysis of rant t_{max}. Moreover, an estimate and 90% CI for the difference between treatment medians were obtained with the Hodges Lehman method.

For digoxin and X005, times for reaching steady state were assessed graphically from plots of C_{trough}, and then fitting C_{trough} values with a nonlinear mixed effect model.

B.22.2.3.9 Results

Steady state of X005: When coadministered with digoxin, average steady state of X005, as expressed by 50th percen tile, was reached after four treatment days. Individual steady state, as expressed by 90th percentile, was reached after five treatment days of repeated BID administration of X005.

Steady state of digoxin: Visual inspection of *Fig. B.22 9* showed that coadministration of X005 did not modify the reach of steady state conditions for digoxin, which was reached within two treatment days using a 0.75 mg loading dose. Trough levels were measured on days 1, 2, 3, 5, 7, 9, and 10.

Plasma concentrations of digoxin on day 10: Mean (SD) digoxin plasma concentrations versus time observed on day 10 are presented in *Fig. B.22 10*.

Pharmacokinetic parameters of digoxin on day 10: A summary of digoxin plasma and urine pharmacokinetic parameters observed on day 10 after digoxin administered for 10 days alone or with X005 is presented in *Table B.22 8*. A 10 day concomitant administration of X005 400 mg BID and digoxin 0.25 mg OD led to a significant increase in digoxin C_{max} and AUC_{0-24h} by 1.75 and 2.57 fold, respectively, and no relevant change in digoxin t_{max}. Digoxin renal clearance was significantly decreased by 43% and the amount of digoxin dose excreted in urine was increased by 1.45 fold when digoxin was coadministered with X005.

CRITICAL ASSESSMENT OF THE METHOD

Since plasma concentration was not quantifiable up to 24 h in nine subjects among the 19 who received digoxin alone, AUC_{0-24h} and $CL_{r,0-24h}$ ratio estimates were overestimated and underestimated, respectively, for these nine subjects. Ratio [90% CI] was 2.01 [1.69, 2.39] for AUC and 0.82 [0.64, 1.04] for CL after exclusion of these subjects. In addition, the analysis performed on individual AUCs calculated for the two groups from time 0 to last time for which plasma concentration was above LOQ in the digoxin alone group showed that a 10 day conco mitant administration of X005 400 mg BID and digoxin 0.25 mg OD led to a significant increase in digoxin AUC_{last} by 2.03 fold [1.85, 2.23].

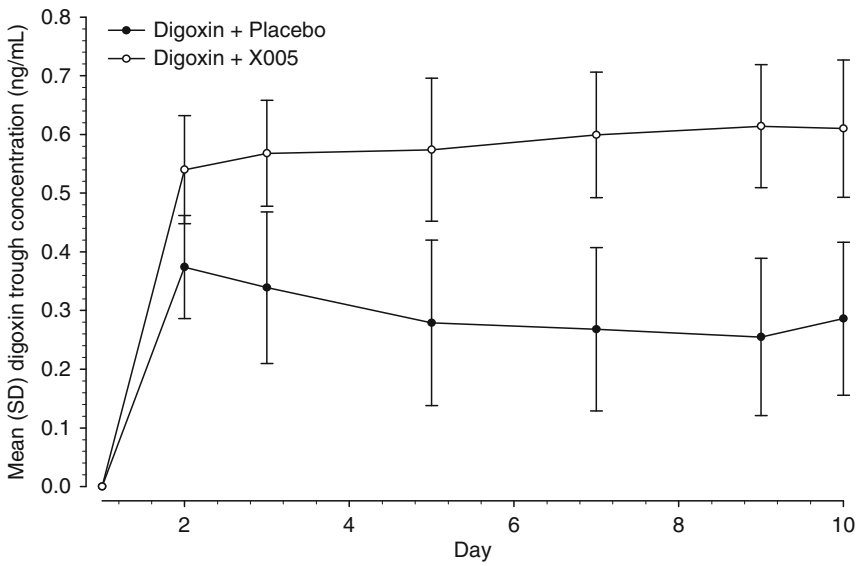

Figure B.22-9

Mean(SD) digoxin trough plasma concentrations from days 1 to 10 after digoxin 0.25 mg OD administered alone or with X005 400 mg BID

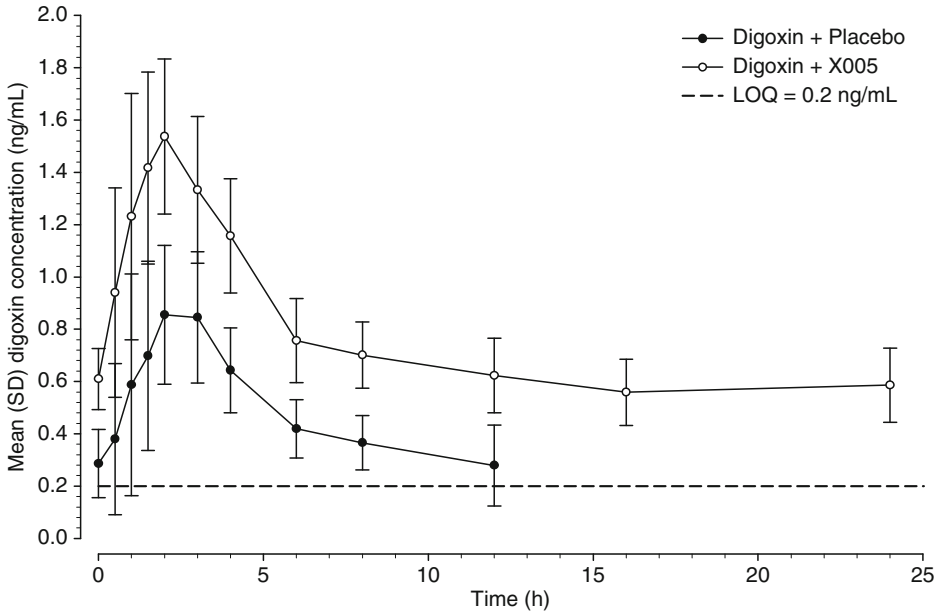

Figure B.22-10

Mean (SD) digoxin plasma concentrations on day 10 after digoxin 0.25 mg OD administered alone or with X005 400 mg BID

Table B.22-8

Summary of digoxin plasma and urine pharmacokinetic parameters and corresponding ratio estimates [90% CI] on day 10 after digoxin 0.25 mg OD administered alone or with X005 400 mg BID

		Digoxin + placebo	Digoxin + X005	Difference between treatments	
		Mean (CV%)	Mean (CV%)	Ratio estimate	90% CI
Plasma	C_{max} (ng/mL)	1.02 (33)	1.71 (17)	1.75	[1.58, 1.93][b]
	t_{max} [a] (h)	3.0 (1.0 4.0)	2.0 (1.0 3.0)	0.5	[1.00, 0.00][c]
	$AUC_{0-24\ h}$ (ng h/mL)	7.73 (45)	18.0 (18)	2.57	[2.21, 2.98][b]
	$Ae_{0-24\ h}$ (mg)	0.097 (32)	0.139 (28)	1.45	[1.19, 1.78][b]
Urine	$fe_{0-24\ h}$ (%)	38.8 (32)	55.8 (28)	na	na
	$CL_{renal\ (0-24\ h)}$ (L/h)	14.2 (41)	7.80 (27)	0.57	[0.46, 0.69][b]

[a]Median (min max)
[b]Geometric mean ratio: digoxin with X003/digoxin alone
[c]Estimate of median difference [90% CI of median difference]
na, not applicable

MODIFICATION OF THE METHOD

In order to evaluate the clinical relevance of the interac tion with digoxin it is recommended to additionally assess the pharmacodynamic interaction by monitoring cardiac output (e.g., heart rate and QTc prolongation). Therefore, a placebo controlled study design, as chosen in this study, is preferable.

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B.23 Specificities for Oncology Studies

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PURPOSE AND RATIONALE

This chapter examines the clinical study designs that are typically used to look at the pharmacokinetics (PK) of anti cancer drugs in phase I development. US Food and Drug Administration (2007) and European Medicines Agency (2005) regulatory guidances on phase I oncology studies are available.

PK of cancer drugs in phase I exploratory trials are generally evaluated in refractory cancer patients (15 30 patients) presenting a broad spectrum of tumor types, or in some cases a specific tumor type. Because many anti cancer agents are inherently cytotoxic and/or genotoxic, healthy volunteer studies cannot be conducted. However, for some targeted agents, chemomodulators, or noncytotoxic or cytostatic compounds, it may be possible to conduct healthy volunteer studies in phase I on a case by case basis.

PK is generally not a primary objective of oncology trials; however, it is important that the trial design permits the adequate characterization of PK and the determina tion of the relationship between exposure and dose. Trial design may be restricted by current clinical practices in the disease type but ideally should reflect optimal dosing strategies from preclinical efficacy data.

The starting dose in man for cytotoxics is classically defined and accepted by regulators as one tenth the LD_{10} in the rodent or one sixth the highest non severely toxic dose (HNSTD) in non rodents, depending on the appro priateness of the species. This is notably different to noncytotoxics, where the human starting dose is depen dent on a no effect toxicity level in a rodent and non rodent species. Dosing in patients is based on individual body surface area (BSA), but the potential to deliver a flat dose should be investigated in phase I/II for future development.

Cancer drugs in early development ideally should be administered intravenously to avoid variability associated with oral bioavailability. Infusions of various durations and even continuous infusion have been explored and avoid peak related effects from bolus injection. Multiple daily dose escalating trials are typically 3 week cycles with an "off drug" period up to day 28 to allow time for recovery before the next treatment. In multiple dose stud ies, the PK should be assessed extensively on day 1 and on the last day within the first cycle in the first, second, or third week depending on the schedule in order to define any change in the PK with time. Spot sampling is usually adequate for subsequent cycles of treatment.

Dose escalation schemes follow a modification to a Fibonacci scale whereby the percentage increase in dose diminishes as the trial progresses from 100%, down to 33% for the final steps. The criteria for stopping trials is based on predefined patient numbers who experience dose limiting toxicities (DLTs). Conventionally, three or six patient cohorts are escalated chronologically to assess toxicity and define the maximum tolerated dose (MTD). However, accelerated dose escalation schemes have been applied (100% dose increments, pharmacokinetically guided, or titration methods) that may maximize the number of patients potentially receiving active doses. As part of the dose escalation assessment, it is highly recommended that the PK are evaluated at each dose or the penultimate administered dose, prior to proceeding to the next dose level. This may either be done on a patient per patient basis or on a dose cohort basis, dependent on the rate of patient accrual or samples analyzed. If severe adverse events occur, additional PK samples should be taken by the investigator as close to the event as possible.

Although there are clearly limitations to conducting phase I PK in cancer patients, the advantage is that the PK are representative of the target population, and there exists an opportunity to evaluate the relationship between PK and anti tumor response (PK/PD) over a wide dose range.

PROCEDURE

The design of a typical dose escalation phase I cancer trial is given below.

B.23.1 Primary Objectives

To determine tolerability and toxicities of XYZ1234, as a 1 h single intravenous infusion, and to determine the MTD (MTD, equivalent to the recommended phase II dose).

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 B.23,

To assess the PK profile of XYZ1234 after single intra venous doses.

B.23.2 Secondary Objectives

To evaluate clinical responses in those patients with mea surable disease.

B.23.3 Study Design

The study was a phase I, single center, nonrandomized, dose escalation study of a single dose XYZ1234 adminis tered intravenously every 3 weeks to patients with refrac tory solid tumors. An accelerated dose escalation scheme was used. The rationale of the scheme was to rapidly increase the dose when no toxicities were found, in order to speed up the trial and to minimize the number of patients treated at sub therapeutic doses.

B.23.4 Number of Subjects

The PK assessment included 43 patients, with ages ranging from 35 to 78 years. All patients had prior chemotherapy and presented various tumors including cancers of the colon, lung, and pancreas. Only one patient discontinued treatment due to an adverse event. Around 60% of patients discontinued treatment due to disease progres sion or lack of efficacy.

Patients were distributed among 16 dose cohorts (*Table B.23 1*), with a maximum administered dose (MAD) of 1,320 mg/m^2. The total number of cycles given at any dose level varied from 1 to 18 and the max imum number of cycles that any patient received was 11 cycles (at dose level 942 mg/m^2). Two patients were excluded from the PK analysis as outliers due to poor venous access.

B.23.5 Treatments

The starting dose for XYZ1234 was determined based on one tenth of the mouse LD_{10} using the body surface equiv alent. The mouse LD_{10} was 74 mg/kg (57.2 80.7 mg/kg at 95% CI). The lower confidence limit of the measure ment was 57 mg/kg, which is equivalent to 171 mg/m^2 in man. Thus, the clinical starting dose was 17 mg/m^2 given over 1 h intravenously.

XYZ1234 was administered intravenously over 1 h every 3 weeks. Patients' blood pressure and heart rate were measured prior to infusion and every 15 min during the infusion. Patients underwent continuous cardiac monitoring during the infusion and until discharge. ECGs were performed every 15 min during the infusion, 15 min after infusion, 3 h after the expected C_{max} (EOI), and at 5, 11, and 23 h after infusion.

B.23.6 PK Data

Blood samples were obtained for the determination of plasma concentrations of XYZ1234 for every patient dur ing cycle 1 and only during cycle 2 if patients were esca lated to a higher dose. From the start of the infusion, blood was drawn at 15, 30, 45, 60 (end of infusion), 65, 75, and 90 min, and 2, 3, 4, 5, 6, 8, 10, 12, 24, 32, and 48 h.

For each sample, a 5 mL blood sample was drawn into a polypropylene tube containing lithium heparin as the anticoagulant. Within 30 min of collection, plasma was separated by centrifugation of the blood sample at 1,500 × *g* for 10 min at 4°C, and transferred into a 5 mL screw capped polypropylene tube. All samples were kept on ice (4°C) during processing. Plasma samples were stored at −20°C until analysis.

The concentrations of XYZ1234 in plasma samples were determined using a validated high performance liq uid chromatographic method with tandem mass spectro metric detection (LC MS/MS) with a lower limit of quantification (LLOQ) of 2.5 ng/mL. Urine samples were analyzed using an exploratory method based on the assay for plasma.

EVALUATION

XYZ1234 PK parameters for individual patients were esti mated by non compartmental techniques using WinNonlin version 4.0 (Pharsight Corporation, Moun tain View, CA, USA). The following parameters were determined for each subject:

- Plasma concentration at the end of the infusion (C_{end}).
- The maximal observed plasma concentration (C_{max}) and its time of occurrence (t_{max}).
- The area under the plasma concentration versus time curve from time 0 to 24 h, calculated using the trape zoidal rule ($AUC_{0–24}$).
- Area under the plasma concentration versus time curve from time 0 to the last measurable concentra tion, calculated using the trapezoidal rule (AUC_{last}).

Table B.23-1

Pharmacokinetic parameters of XYZ1234 following a single intravenous infusion for 1 h (17 1,320 mg/m^2) (Arithmetic mean, (SD), [geometric mean])

Dose (mg/m^2)	*n*	C_{end} (ng/mL)	C_{max} (ng/mL)	AUC_{0-24} (h ng/mL)	AUC_{last} (h ng/mL)	AUC (h ng/mL)	$t_{1/2z}$ (h)	Cl (L/h)	V_{ss} (L)
17	1	230	251	2,020	2,320	2,360	8.17	7.19	74
24	2	326	341	2,010	2,290	2,340	9.26	12.8	133
		(66.4)	(69.7)	(64.0)	(62.8)	(62.5)	(6.40)	(62.5)	(67.5)
		[228]	[297]	[1,790]	[2,050]	[2,090]	[9.25]	[11.5]	[117]
33	1	2,290	11,200	15,400	16,400	16,500	8.07	2.00	11.4
47	1	524	667	3,490	3,720	3,740	6.59	12.60	90.5
65	1	577	577	3,540	3,560	3,840	6.93	16.9	143
91	2	725	971	4,430	4,880	4,920	7.08	19.7	162
		(45.2)	(49.5)	(37.1)	(35.2)	(35.2)	(3.60)	(35.2)	(43.9)
		[687]	[909]	[4,280]	[4,730]	[4,760]	[7.08]	[19.1]	[154]
128	5	1,330	1,380	8,180	9,470	9,650	7.33	17.2	128
		(13.8)	(8.3)	(45.1)	(51.6)	(52.9)	(31.2)	(58.9)	(22.9)
		[1,320]	[1,370]	[7,470]	[8,400]	[8,510]	[7.08]	[15.0]	[125]
170	4	1,590	1,690	9,270	10,200	10,300	6.91	17.2	139
		(23.9)	(23.2)	(23.3)	(24.7)	(24.9)	(14.5)	(22.0)	(22.4)
		[1,550]	[1,640]	[9,100]	[10,000]	[10,100]	[6.85]	[16.9]	[137]
226	3	2,120	2,190	14,100	15,800	16,000	7.53	14.3	129
		(20.7)	(16.9)	(12.8)	(14.0)	(14.0)	(2.90)	(14.6)	(8.10)
		[2,090]	[2,170]	[14,000]	[15,700]	[15,900]	[7.53]	[14.2]	[128]
301	4	2,580	4,540	16,200	18,800	19,200	7.97	32.2	190
		(55.3)	(65.6)	(65.0)	(72.3)	(73.7)	(36.4)	(113)	(81.3)
		[2,170]	[3,530]	[12,600]	[14,000]	[14,200]	[7.60]	[21.2]	[156]
400	6	7,320	7,320	39,400	43,700	44,200	7.54	10.6	90
		(23.5)	(23.5)	(48.8)	(48.8)	(49.4)	(7.10)	(37.6)	(34.8)
		[7,160]	[7,160]	[36,200]	[40,100]	[40,500]	[7.52]	[9.88]	[84.8]
532	5	7,310	7,690	36,200	39,600	40,100	7.2	14.5	104
		(28.6)	(31.5)	(28.4)	(30.4)	(31.0)	(24.7)	(33.4)	(38.7)
		[7,020]	[7,330]	[34,900]	[38,100]	[38,500]	[7.02]	[13.8]	[97.9]
708	2	7,900	8,400	52,400	57,500	57,800	6.39	12.8	112
		(62.7)	(67.3)	(33.4)	(29.4)	(29.0)	(16.6)	(29.0)	(48.8)
		[7,080]	[7,390]	[50,900]	[56,200]	[56,600]	[6.35]	[12.5]	[105]
942	3	5,840	7,120	42,900	48,200	48,500	7.06	19.5	171
		(21.8)	(24.5)	(4.70)	(6.30)	(6.50)	(2.20)	(6.50)	(8.40)
		[5,740]	[6,980]	[42,900]	[48,100]	[48,500]	[7.06]	[19.4]	[170]
1,320	3	8,700	12,700	83,200	91,900	97,700	9.47	14.2	159
		(25.5)	(28.2)	(37.2)	(27.1)	(26.0)	(104)	(29.1)	(70.3)
		[8,530]	[12,400]	[79,700]	[89,700]	[95,300]	[6.62]	[13.9]	[136]

- Area under the plasma concentration versus time curve from time zero extrapolated to infinity, calculated using the trapezoidal rule (AUC). The extrapolated portion was estimated using the terminal rate constant.
- The terminal half life ($t_{1/2z}$) associated with the terminal slope (λ_z) determined from the slope of a semi logarithmic regression line through the terminal phase of the plasma concentration versus time curve. Half life was calculated using at least three data points.
- Total clearance of drug from plasma (Cl).
- Apparent volume of distribution at steady state (V_{ss}).

PK analysis was conducted using drug concentration data, which was rounded up to three significant figures. Consequently, all data regarding PK parameters were expressed to no more than three significant figures.

Descriptive statistics were determined for each dose level at which more than one subject was dosed. PK were also generated for intra subject dose escalations. In the event that results from sufficient subjects and dose levels were obtained, the relationship between dose and C_{end}, AUC_{0-24}, AUC_{last}, and AUC were estimated.

CRITICAL ASSESSMENT OF THE METHOD

B.23.7 Disease Progression

Analysis of PK in terminally ill patients with progressive disease can be difficult and it is to be expected and anticipated that patients may withdraw or be taken off the study.

B.23.8 Compounding Factors

Other compounding factors include the likelihood of patients experiencing severe adverse events and thus dosing may be terminated or disrupted. In the case of oral chemotherapy, vomiting can affect the reliable measure of drug kinetics. These factors along with disease progression, can lead to incomplete datasets and sub optimal PK assessments in cancer trials.

B.23.9 Interactions

It should be noted that cancer patients will be taking multiple drug combinations, as part of their standard care medication; therefore, careful consideration should be given to the potential for drug drug interactions. An in vitro drug drug interaction screen should be made prior to the in life phase of the trial, as well as during the trial with appropriate CYP "probes" at the MTD to explore specific drug drug interactions that have been flagged in preclinical development. It should be noted that PK variability in phase I could be attributed to these extrinsic factors. Specific PK studies are recommended for combination chemotherapy.

B.23.10 Biomarkers

An important part of oncology drug development is being able to characterize the drug with respect to the biological response; therefore, early identification of suitable biomarkers and validation is highly recommend prior to phase I development. The use of biomarkers in clinical oncology phase I trials are gaining increasing interest for targetted therapies, although they are not generally accepted by regulators as primary end points of activity, they can be used to stratify patients and aid drug development decisions. A PK response evaluation strategy should be implemented as early as possible in clinical development.

MODIFICATIONS OF THE METHOD

B.23.11 General

The main clinical objective is to define the MTD in the first in patient study. If patients tolerate the drug, and/or there is evidence for efficacy, then various phase I/II dosing strategies could be investigated to examine the effects of dosing frequency and cycle dependency. For example, the clinical outcome may be evaluated by extending the infusion duration for safety reasons or to explore effects on the anti tumor response. It is also worth considering how the drug behaves upon multiple cycles of treatment. PK will play an integral part in determining the optimal schedule for chemotherapeutic dosing.

It is also recommended that a preliminary evaluation of metabolism is made in the first in patients trials in order to assess whether any active metabolites are circulating. At least an exploratory determination of the major metabolites in plasma should be investigated.

B.23.12 Blood Sampling

Blood sampling during cancer studies should be kept to a minimum, as patients are often immunocompromised and blood volume can be an issue. Duplicate sampling

(back up samples) should be avoided. Any reduction of blood samples/volumes should be made without compromising the PK objective. Patients, investigators, and internal review boards and their ethics committees will all have issues with large numbers of blood samples for PK or PD assessments if they do not add value. Sim ulations should be used wherever possible in order to derive the best sampling strategy to satisfy the main objectives.

It is recommended that blood samples be taken peri odically for at least 72 h on last day of a daily schedule in the first cycle. In the case of a bolus or short infusion, the sampling regime should be designed to capture the initial distribution phase and later phases. In the case of infusion >2 h, it is recommended to obtain blood samples at least at mid infusion.

B.23.13 Repeated Dose Studies

Daily 5× regimes are frequently administered in cancer chemotherapy and as such it is important to determine the PK on days 1 and 5. Cancer drugs are rarely dosed to steady state and are not lifetime treatments; therefore, a steady state assessment (C_{trough} analysis on intermediate days) is generally not necessary. In addition, it is not always necessary to evaluate the PK upon multiple treatment cycles. Limited sampling (one or two samples at C_{max} or the end of infusion) for approximately five to six treatment cycles could be considered enough to estab lish the long term behavior of the drug depending on the tumor type and prognosis.

EXAMPLE

The results that could be obtained from the study type described above are presented below.

Arithmetic mean (±SD) XYZ1234 plasma concentra tion versus time curves observed after 1 h infusion admin istration of XYZ1234 (17 1,320 mg/m^2) to patients with refractory solid tumors are given in *Fig. B.23 1*. Peak plasma concentrations were generally observed at the infu sion end and then declined in a mono phasic manner with $t_{1/2z}$ of approximately 8 h.

C_{max} was included in the PK analysis as C_{end} was not always representative of the maximal XYZ1234 plasma concentration.

At the top dose level, 1,320 mg/m^2 urine concentra tions of XYZ1234 represented less than 5% of the dose and, therefore, no further analysis was conducted on uri nary parameters.

Table B.23 1 summarizes the PK parameters of XYZ1234 in cancer patients receiving XYZ1234 as a 1 h intravenous infusion. The C_{max} of XYZ1234 was generally

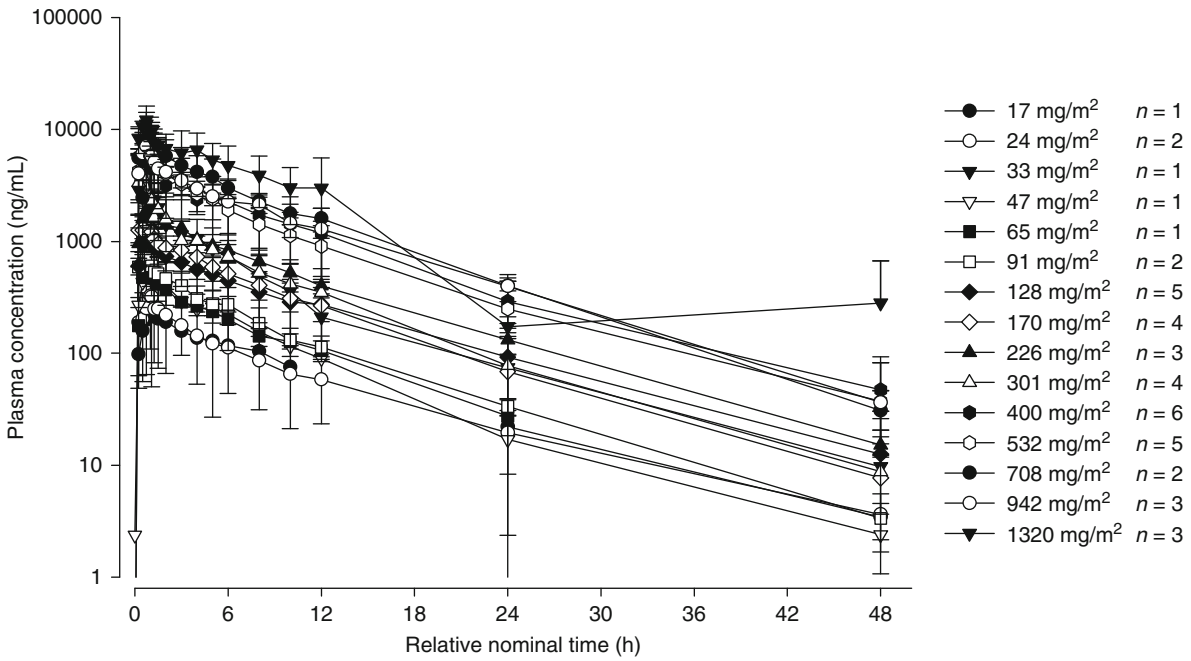

Figure B.23-1

XYZ1234 plasma concentration versus time profile over the dose range 17 1,320 mg/m^2)

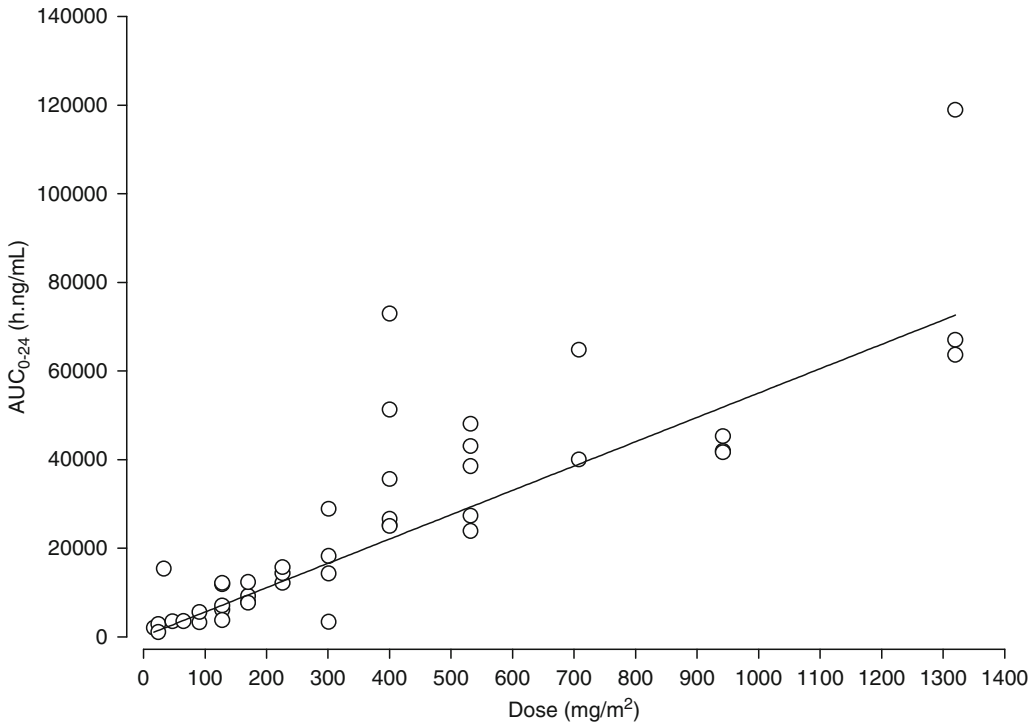

Figure B.23-2
Dose proportionality assessment for $AUC_{0–24}$

observed at the end of the infusion, with mean values ranging from 251 to 12,700 ng/mL over the dose range 17 1,320 mg/m^2 (*Fig. B.23 2*). XYZ1234 had a systemic clearance ranging from 17.0 to 86.0 L/h (mean, 16.1 L/h), a terminal elimination half life ($t_{1/2z}$) in the range 3.1 20.8 h (mean, 7.6 h) and showed a distribution in excess of blood volume (V_{ss} ranging from 11.4 to 421.0 L; mean, 128.6 L). The lower values for Cl and V_{ss} reflect limited data or poor characterization of the PK profile at lower doses rather than a true dose effect. The systemic exposure of the drug (C_{max}, $AUC_{0–24}$, AUC) increased in a close to dose proportional manner over the dose range from 17 to 1,320 mg/m^2 (*Fig. B.23 2*), and there was no evidence for any systematic change in Cl, $t_{1/2}$, or V_{ss} with dose, indicating linear PK of XYZ1234 over a wide dose range.

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B.24 Pharmacogenomics in and its Influence on Pharmacokinetics

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II.T.1	PHASE I ENZYMES
II.T.1.1	CYP1A2
II.T.1.2	CYP2C9
II.T.1.3	CYP2C19
II.T.1.4	CYP2D6
II.T.1.5	CYP3A
II.T.1.6	OTHER CYPS
II.T.1.6.1	CYP2A6
II.T.1.6.2	CYP2B6
II.T.1.6.3	CYP2C8
II.T.1.6.4	CYP2E1
II.T.2	PHASE II ENZYMES
II.T.2.1	*N*-ACETYLTRANSFERASES
II.T.2.2	Uridine Diphosphate Glucuronosyltransferases
II.T.2.3	Methyltransferases
II.T.2.4	Glutathione *S*-transferases and Sulfotransferases

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 metab olizing enzymes (DME) considerably increased in the early 2000s, and continues to increase. The clinical conse quences or at least genotyping phenotyping relation ships of DME polymorphisms have not been demonstrated for all variants. In the text below, only those DME allele variants will be mentioned for which significant changes in enzyme activity have been found using probe drugs. Comprehensive information on the nomenclature of cytochrome P450 (CYP) alleles can be found at www.imm.ki.se/CYPalleles and Phase I and Phase II DMEs at www.pharmgkb.org/index.jsp.

B.24.1 PHASE I ENZYMES

B.24.1.1 CYP1A2

PURPOSE AND RATIONALE

CYP1A2 is involved to a major extent in the metabolism of several drugs (imipramine, clozapine, fluvoxamine, olanzapine, theophylline, acetaminophen, propranolol, and tacrine) as well as of diet components (methylxan thines), endogenous substrates (estrogens), numerous aryl, aromatic and heterocyclic amines, and polycyclic aromatic hydrocarbons. It is inducible, notably by ciga rette smoking, diet habits such as consumption of crucif erous vegetables (e.g., broccoli, watercress, collard greens, Brussels sprouts, and mustard) and of charbroiled meats, some drugs (omeprazole, phenytoin, and rifampicin) and is a target enzyme for the development of some cancers. Up to now, more than 25 CYP1A2 alleles have been detected. 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, and coffee) ranging from 1 to 3 mg/kg is administered. Diet requirements have to be respected (stable xanthine free diet avoiding beverages such as coffee, tea, cola, chocolate, no food component with CYP1A2 inducing properties) during the test period. As smoking is known to induce CYP1A2, control of stable smoking status is mandatory.

There are two commonly used and robust methods for phenotyping. The first one measures caffeine

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(1,3,7 methylxanthine) and its *N* demethylated metabolite 1,7 dimethylxanthine (paraxanthine) in a plasma or saliva sample collected within 5–7 h post caffeine dosing (Fuhr and Rost 1994). The second one uses the assay of the metabolites 1 methylurate (1U), 1 methylxanthine (1X), 5 acetylamino 6 formylamino 3 methyluracil (AFMU), and 1,7 dimethylurate (17U) levels in urine collected at least for 8 h 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 1998; 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*1F alleles in smoking subjects. Induction of CYP1A2 activity has been associated with these alleles, but the effect of CYP1A2*1F mutation on CYP1A2 activity has not been confirmed (Nordmark et al. 2002). In Caucasians, frequency of the CYP1A2*1C and CYP1A2*1F variants is about 1% and 33%, respectively (Sachse et al. 2003).

EVALUATION

Metabolic ratios (MR) used are plasma 17X/137X and urinary (1U + 1X + AFMU)/17U.

In controlled conditions, in nonsmoking young and elderly subjects, intraindividual and interindividual variability in 17X/137X MR was about 17% and 47%, respectively, with no effect of age (Simon et al. 2003). A 70 fold range in MR has been observed in smoking and nonsmoking 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 nonsmokers, 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 (PM, characterized with a MR <0.12) have been identified in Chinese population and represented about 5% of the population tested, whereas PM 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 CYP1A2 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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B.24.1.2 CYP2C9

PURPOSE AND RATIONALE

CYP2C9 is involved in the hydroxylation of about 16% of drugs (Schwarz 2003), including drugs with narrow therapeutic index such as anticoagulants (warfarin, acenocoumarol, and phenprocoumon active *S* enantiomers), and anticonvulsivants (phenytoin and hexobarbital), as well as numerous antidiabetic agents (i.e., tolbutamide, glibenclamide, and glipizide), antihypertensive drugs (losartan, irbesartan), nonsteroidal anti inflammatory agents (i.e., diclofenac, ibuprofen, and celecoxib), diuretic (torsemide), and anti rheumatoid agents (leflunomide).

A couple of CYP2C9 variants mainly CYP2C9*2 and CYP2C9*3 code for in vivo decreased activity, and two CYP2C9*6 and CYP2C9*15 have been reported to be associated with no activity. In Caucasian populations, CYP2C9*2 and CYP2C9*3 are encountered in 20 25% of subjects, while these genotypes have been found in less than 5% of East Asian subjects (Rosemary and Adithan 2007).

Probe drugs regularly used for CYP2C9 phenotyping are tolbutamide, warfarin, phenytoin, and losartan. Diclofenac, flurbiprofen, phenprocoumon, and torsemide 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 hydroxytolbutamide and the subsequent carboxytolbutamide metabolite, the latter formed by dehydrogenase enzymes. The urinary excretion of these two metabolites represented more than 85% dose of administered tolbutamide (Veronese et al. 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 metabolites is usually performed using HPLC and UV or fluorescence detection (Csillag et al. 1989; Veronese et al. 1990; Kirchheiner et al. 2002a, b; Hansen and Brosen 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 et al. 2001; Lee et al. 2002a, b; Schwarz 2003). More than 95% of Afro American subjects express the wild genotype C9*1/*1 (Lee et al. 2002a, b). In Asian populations, CYP2C9*1/*3 is expressed in 2 8% subjects, but CYP2C9*2 is absent or extremely rare (Rosemary and Adithan 2007; Schwarz 2003; Xie et al. 2002). Overall, it has been estimated that 0.2 1% and 2 3% of Caucasian and Asian population could be qualified as PM, respectively (Meyer 2000).

EVALUATION

The urinary MR (MR, hydroxytolbutamide + 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 et al. 2002a, b; Kirchheiner et al. 2002a, b, 2003; Lee et al. 2002a, b; Miners and Birkett 1998; Morin et al. 2004), highlighted that a PM status could be given to subjects who 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 et al. 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 et al. 2004; Versuyft et al. 2003), glipizide, and glyburide (Kirchheiner et al. 2002a, b) or drug side effects (Sevilla Mantilla et al. 2004). Inconstant results were found regarding tolbutamide effects (Kirchheiner et al. 2002a, b; Shong et al. 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 and King 2003). An Afro American subject with only the CYP2C9*6 variant exhibited serious phenytoin side effects associated with a marked impaired elimination of the drug (Kidd et al. 2001).

The variability of CYP2C9 activity observed among ethnic groups cannot be explained with our current knowledge on CYP2C9 variant alleles distribution (Xie et al. 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 h urine or in plasma at 6 h post dosing have been proposed (Yasar et al. 2002a, b; Sekino et al. 2003). However, in a comparative study in 16 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 et al. 2003).

Recently, a 125 mg tolbutamide dose has been validated, with proposal of the use of just one blood sample collected 24 h post dosing. Its safer use needs the drug to be assayed using LC MS/MS methodology (Jetter et al. 2004).

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B.24.1.3 CYP2C19

PURPOSE AND RATIONALE

CYP2C19 contributes to the metabolism of about 8% of drugs (Rogers et al. 2002), including *S* mephenytoin, proton pump inhibitors (omeprazole, lansoprazole, and pantoprazole), tricyclic antidepressants (amitriptyline, imipramine, clomipramine, and citalopram), benzodiazepines (diazepam and flunitrazepam), torsemide, fluvastatin, and proguanil. Two main variants CYP2C19*2 and CYP2C19*3 are coding for in vivo nil activity, as well as CYP2C19*4, *5, *6, *7, and *8 variants. About 15 20% Asians, 4 7% Black Africans, and 3% Caucasians are PM (Scordo et al. 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 and Persson 1984; Ieri 1996; Yim et al. 2001; Tybring et al. 1997) or LC MS/MS assay (Kanazawa et al. 2002).

Genotyping: The two alleles CYP2C19*2 and CYP2C19*3 account for quite all PM 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 et al. 2001). The CYP2C19*3 accounts for about 25% of inactive forms in Orientals, and is extremely rare in Caucasians (Scordo et al. 2004; Rosemary and Adithan 2007).

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 et al. 2004).

Decreased CYP2C19 activity has been observed with oral contraceptives containing ethinylestradiol (Tamminga et al. 1999; Laine et al. 2000).

CRITICAL ASSESSMENT OF THE METHOD

Omeprazole hydroxylation rate correlates with *S* mephenytoin hydroxylation rate, which was initially the CYP2C19 probe drug (Andersson et al. 1990; Chang et al. 1995; Balian et al. 1995). The alternate pathway conversion of omeprazole to its sulfone derivative that is mediated via CYP3A4, does not influence the CYP2C19 pathway of omeprazole (Balian et al. 1995).

Time dependent kinetics of omeprazole limits its use for phenotyping during chronic therapy (Gafni et al. 2001). CYP2C19 phenotyping with omeprazole may be affected by age, liver disease, and omeprazole therapy (Kimura et al. 1999).

Interethnic differences observed with different CYP2C19 substrates for subjects with same genotype have been attributed to differences in substrate specificity or enzyme isoforms (Bertilsson et al. 1992). The clearance of omeprazole is higher in Caucasian extensive metabolizers (EM) than in Oriental EM, due to a higher

proportion of heterozygous EM in this latter population (Ishizaki et al. 1994).

MODIFICATIONS OF THE METHOD

It has been proposed to use omeprazole for both CYP2C19 and CYP3A4 phenotyping (Gonzalez et al. 2003).

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B.24.1.4 CYP2D6

PURPOSE AND RATIONALE

CYP2D6 is involved significantly in the metabolism of drugs mainly used in CNS (antidepressants, i.e., imip ramine, paroxetine, citalopram; neuroleptics, i.e., haloper idol, risperidone), or cardiovascular (β adrenoceptor blockers, i.e., metoprolol; antiarrhythmics, i.e., propafenone and flecainide) disorders. Significant interethnic and interindividual intraethnic differences in CYP2D6 activity have been found. It is found that 5 10% Caucasians, 6 8% Afro Americans, and only 1% Asians have reduced CYP2D6 activity, and exhibit the PM phe notype. Expression of CYP2D6 has been shown to be polymorphic with up to now more than 80 genetic vari ants detected for the encoding gene, with more than 15 encoding for inactive enzyme. Probe drugs for CYP2D6 phenotyping are dextromethorphan, debrisoquin, sparte ine, and metoprolol. For safety concerns and drug avail ability, the preferred probe is dextromethorphan (DM) (Schmid et al. 1985).

PROCEDURE

Phenotyping: The method measures 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 30 mg DM (generally hydrobromide salt syrup) dose. Urine is collected from drug intake to 8 h 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 incidence of alleles coding for inactive enzymes varies 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 phe notype in Caucasians, with CYP2D6*4 most commonly associated with the PM phenotype. By far, the most fre quent null allele not encoding a functional protein prod uct is CYP2D6*4 with a frequency of 20 25% in Caucasians (Zanger et al. 2004). The frequency of the *17 allele associated with decreased enzyme activity is high in Black Africans and in Black Americans, but practically absent in Caucasian populations (Bapiro et al. 2002; Gaedigk et al. 2002; Zanger et al. 2003). Four potential subgroups ultrarapid metabolizers (UM), extensive metabolizers (EM), intermediate metabolizers (IM), and poor metabolizers (PM) 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 that allow to detect 86 100% of PM (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 MR >0.3 are PM. Subjects with DM/DX <0.03 are EM. Those with 0.03 < MR < 0.3 are IM.

No difference or slightly higher CYP2D6 activity in females has been found when comparing to male subjects (Hägg et al. 2001; McCune et al. 2001).

Relationship between phenotyping and genotyping is investigated by plotting log MR versus CYP2D6 allele combinations (Chou et al. 2003).

CRITICAL ASSESSMENT OF THE METHOD

The method is widely used due to easy and safe adminis tration. High intrasubject variability limits the test for discriminating between EM and UMs (Zanger et al. 2004).

The method is not appropriate in patients with renal impairment, due to reduced renal excretion of DM glucu ronide metabolites. Sparteine has been recommended as a probe for this population and to discriminate between the four phenotypes UM, EM, IM, and PM. The DM/DX MR does not allow for consistent differentiation between CYP2D6 EM 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 MRs calculated from plasma, saliva samples and those obtained from urine has been observed.

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B.24.1.5 CYP3A

PURPOSE AND RATIONALE

CYP3A is the predominant P450 subfamily (CYP3A4, CYP3A5, CYP3A7, and CYP3A43) in the human liver, and contributes significantly to the metabolism of many (at least 50%) drugs in numerous therapeutic classes. CYP3A4 is the major CYP present notably and predominantly in the liver and the small intestine, and interindividual variability in the level of its expression is very high 20 fold or more (Shimada et al. 1994). CYP3A5 shares rather similar tissue distribution with CYP3A4, but is preferentially expressed in the lung. It represents generally a few percentage of total CYP3A as compared to CYP3A4 (exceptions are esophagus 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 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 first 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 et al. 2004).

Currently, 40 and 24 alleles have been identified for CYP3A4 and CYP3A5, respectively. Expression of CYP3A5 varies greatly among individuals (Lamba et al. 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, DM, lidocaine, nifedipine, omeprazole, quinine, and 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 intraindividual differences in the liver and the intestinal CYP3A activity, phenotyping test results are related to the probe drug route of administration.

PROCEDURE

B.24.2 Phenotyping

Midazolam test: Midazolam is primarily metabolized to 1′ hydroxymidazolam by CYP3A. It is rapidly and completely absorbed after oral administration (Gorski et al. 1998). It is the probe of choice to assess intestinal and hepatic or hepatic CYP3A activities only, after oral (Thummel et al. 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 min infusion.

Blood samples are collected over a 6 h period. Numerous GC, GC/MS, HPLC/UV, or LC/MS methods have been developed for plasma midazolam assay (Lepper et al. 2004; Frison et al. 2001).

^{14}C erythromycin breath test or ERMBT: CYP3A4 catalyzes the *N* desmethylation of [^{14}C *N* methyl] erythromycin. The test consists of the measurement of a single breath expired $^{14}CO_2$ collection obtained at 20 min 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 Americans) 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 the review by Lamba et al. (2002).

EVALUATION

A complete pharmacokinetic profile is required to assess midazolam clearance, and is therefore more invasive than the ERMBT; however, the latter requires 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 anti cancer agents. The ratio 1′ hydroxymidazolam/midazolam has generally been found not useful for phenotyping.

Within a population of similar demographic and health characteristics, a four to sixfold range in the metabolic clearance of a CYP3A drug substrate is usual, with common individual outliers exhibiting high or low activity (Lamba et al. 2002).

CRITICAL ASSESSMENT OF THE METHOD

Midazolam clearance has been found to correlate with hepatic CYP3A levels (Thummel et al. 1994) as well as ERMBT results (Lown et al. 1992). However weak, incon stant, or lack of correlations between midazolam and ERMBT 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 capture CYP3A5 activity.

An ethnic difference that could be drug specific in CYP3A4 activity has been observed for few CYP3A4 sub strates (alprazolam and nifedipine), with a lower clearance in Asians than in Caucasians (Xie et al. 2001).

CYP3A4 and CYP3A5 genotyping tests could not explain sufficiently the interindividual variability observed in midazolam pharmacokinetics (Eap et al. 2004a).

MODIFICATIONS OF THE METHOD

The combined use of IV midazolam and oral ^{15}N midazolam or of the ERMBT and oral midazolam tests have been proposed to assess simultaneously the contri butions of liver and intestine in CYP3A activity (Gorski et al. 1998; McCrea et al. 1999). The administration of orally given midazolam followed by an intravenous administration has also been validated (Lee et al. 2002). A low oral 75 μg oral dose has recently been proposed, but needs large scale validation (Eap et al. 2004b).

Modifications of the ERMBT have been described to improve its predictability in drug clearance estimations in cancer patients (Rivory et al. 2000).

A single blood sample for midazolam assay at 4 h post dose has been reported as good estimator for IV or oral midazolam clearance determination (Lin et al. 2001).

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B.24.2.1 OTHER CYPS

This section summarizes succinctly the current knowledge on some other CYPs, its role in drug metabolism and its genetic impact have been more recently investigated as compared to other CYPs.

B.24.2.2 CYP2A6

CYP2A6, primarily expressed in the liver, is the major CYP (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). A couple of drugs is metabolized by CYP2A6: chlormethiazole, coumarin, disulfiram, halothane, valproic acid, and others (Oscarson 2001). CYP2A6 PM is 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 for PM status are CYP2A6*2 and CYP2A6*4, rather common in Orientals (15% in Chinese, 20% in Japanese). The important role of CYP2A6 in nicotine metabolism was shown in an epidemiological study, revealing that the CYP2A6 genotype was a major determinant for smoking behavior and susceptibility to tobacco related lung cancer (Fujieda et al. 2004).

Phenotyping has been performed in some countries with coumarin (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. Recent investigations using pilocarpine as probe demonstrated that PM status was associated with two inactive CYP2A6 alleles, CYP2A6*4A, CYP2A6*7, CYP2A6*9, or CYP2A6*10 (Endo et al. 2008).

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B.24.2.3 CYP2B6

CYP2B6 has been estimated to represent 1 10% of the total hepatic CYP content. It catalyzes bupropion hydroxylation, *S* mephenytoin *N* demethylation, and is involved in the metabolism of cyclophosphamide, ifosfamide, mianserin, efavirenz, artemisinin, and propofol (Turpeinen et al. 2006). CYP2B6*6 has been associated with reduced bupropion clearance in vitro (Hesse et al. 2004), but not in vivo whereas a moderate clearance increase was observed with CYP2B6*4 (Kirchheiner et al. 2003). Multiple gene polymorphisms have resulted in phenotypic null alleles (Lang et al. 2004). Pharmacokinetics of the anti HIV drug efavirenz has been associated with CYP2B6 G516T polymorphism (Saitoh et al. 2007).

Bupropion (150 mg dose) has been proposed for phenotyping, but it is recommended to administer body weight adjusted doses (Faucette et al. 2000). Efavirenz may also be a valuable probe for CYP2B6 (Ward et al. 2003).

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B.24.2.4 CYP2C8

CYP2C8 is involved in the metabolism of arachidonic acid, all trans retinoic acid, paclitaxel, amiodarone, amodiaquine, repaglinide, rosiglitazone, 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 et al. 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 subjects 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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B.24.2.5 CYP2E1

CYP2E1, an ethanol inducible CYP, activates some procarcinogens such as nitrosamines, is involved in the metabolism of endogenous substrates (steroids and bile acids), alcohols, xanthines, volatile chemicals (toluene, benzene, and halocarbons), but of few drugs (chlorzoxazone, etoposide, dapsone, and high dose acetaminophen) (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, toward assessment of its 6 hydroxylation (Ono et al. 1995; Lucas et al. 1999; 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 intraindividual 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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B.24.3 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 is large interindividual 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 thiopurine methyltransferase (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.

B.24.3.1 *N*-ACETYLTRANSFERASES

PURPOSE AND RATIONALE

N acetyltransferases type I (NAT1) and type II (NAT2) catalyze *N* and *O* acetylation reactions involved in the metabolism of drugs containing arylamino, hydroxyl, sulfhydryl groups and hydrazine structure, and also in environmental carcinogens (such as those present in tobacco smoke, or in diet such as charcoal broiled food) (Weber and Hein 1985). Pending on the drug, and on the interplay between CYPs 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 share common substrates (Meisel 2002) but coding genes loci are regulated independently. Main NAT2 drug substrates are isoniazid, sulfonamides, procainamide, hydralazine, acebutolol, aminoglutethimide, and dapsone.

Para aminosalicylic and para aminobenzoic acids are considered specific substrates for human NAT1, and sulfamethazine, isoniazid, procainamide, and dapsone are considered specific substrates for human NAT2 (Butcher et al. 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 the 1950s and is the first example of interindividual pharmacogenetic variability. Until 2007, about 30 and more than 50 variant alleles have been described for NAT1 and NAT2, respectively. At http://*N* acetyltransferasenomenclature.louisville.edu overviews on the NAT alleles can be found. 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 and 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 and Zanger 1997).

Probe drugs for NAT1 phenotyping is PAS, and for NAT2 phenotyping are caffeine, sulfamethazine, procainamide, isoniazid, and dapsone. In vivo testing for NAT2 has been proved 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 methyl xanthine (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 MR used is AFMU/1MX, but the AFMU/(AFMU + 1MX + 1MU) is more discriminating (Relling et al. 1992; Rostami 1995) and should be used when xanthine oxidase inhibitors may be present (Fuchs et al. 1999). Other ratios such as AFMU/(1MX + 1MU), or AAMU/1MX, AAMU/(AAMU + 1MX + 1MU) have been validated (Tang et al. 1991; Nyeki et al. 2002).

The most common methods to assay caffeine and its metabolite in urine used HPLC with UV detection (Grant et al. 1984; Krul and Hageman 1998) or mass spectrometry (Baud Camus et al. 2001).

Genotyping: Mutations of NAT2*5, NAT2*6, NAT2*7, NAT2*14, and NAT2*17 alleles are associated with a slow acetylation phenotype for homozygous subjects (Butcher et al. 2002).

There are large differences among ethnic groups regarding alleles' frequency. High frequency (>28%) of NAT2*5 alleles has been observed in Caucasians and Afri cans, and of NAT2*7 in Asians (>10%) and of NAT2*14 in Africans (>8%), this last one being <1% in Caucasians and Asians (Meyer and Zanger 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 et al. 1999). Higher activity has been observed in black as compared to white subjects (Relling et al. 1992), and a gender effect has generally not been observed (Kashuba et al. 1998).

CRITICAL ASSESSMENT OF THE METHOD

Depending on the probe drug used and on the experimen tal 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 dis tribution has been considered as a continuous variable (Meisel 2002). Due to slow postnatal 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 pheno typing tests have been reported (Meisel et al. 1997; Kita et al. 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 et al. 1999), or other drugs (Klebovitch et al. 1995). NAT activities are affected by anti inflammatory drugs. Of note, acetaminophen is an inhibitor of NAT2 in vivo (Rothen et al. 1998).

Discordances between caffeine and dapsone pheno typing data, and between NAT2 phenotyping status and genotyping have been observed in acutely ill patients infected with HIV (O'Neil et al. 2000), which may be due partly to non detection of rare NAT2 alleles (Alfirevic et al. 2003).

MODIFICATIONS OF THE METHOD

Some recent references for other used NAT2 phenotyping tests can be found for dapsone in Alfirevic et al. (2003), O'Neil et al. (2000), Queiroz et al. (1997), for sulfamethazine in Hadasova et al. (1996) and Meisel et al. (1997), and for procainamide in Okumura et al. (1997) and Mongey et al. (1999).

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B.24.3.2 Uridine Diphosphate Glucuronosyltransferases

Glucuronidation is a potent detoxification pathway. The uridine diphosphate glucuronosyltransferases (UGTs) are involved in the biotransformation of endogenous substances (bilirubin, biliary acids, and steroid hormones) and numerous drugs and carcinogens. Currently, 20 functional UGTs have been characterized with activity mainly expressed in the liver and the GI tract. There are three subfamilies: UGT1A, UGT2A, and UGT2B, with distinct 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 that 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 and Strassburg (2000), Fischer et al. (2001), Guillemette (2003), and Wells et al. (2004).

A decreased clearance has been observed for some drugs metabolized by glucuronidation in patients with Gilbert's syndrome. A clinically significant impact of UGT polymorphism has to date is only demonstrated for some anticancer agents: clearly for irinotecan, and with contradictory results for flavopiridol (Zhai et al. 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 et al. 1998; Innocenti et al. 2004; Iyer et al. 2002; Paoluzzi et al. 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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B.24.3.3 Methyltransferases

There are at least four enzymes catalyzing *S*, *N* and *O* methylation using *S*-adenosylmethionine, but only TPMT polymorphism has been found to have important clinical consequences. To date, no endogenous substrate of TPMT is known. TPMT is involved in the metabolism of mercaptopurine, azathioprine and thioguanine, narrow therapeutic index drugs in use for the treatment of patients with neoplasia or autoimmune disease, or of transplant recipients. About 0.3% of Caucasian subjects have no detectable enzyme activity and 10% intermediate activity (McLeod and Evans 2001). Four alleles TPMT*2, TPMT*3A, TPMT*3B, and TPMT*3C account for 80–95% of Caucasians with intermediate or low enzyme activities. Patients with low inherent TPMT activity are at

great risk for severe potentially life threatening myelosup pressive toxicity with treatment by the above mentioned drugs, whereas subjects with very high activity might be underdosed (Zhou 2006). Patients with two 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 et al. 2000; Evans 2004). A cut off concentration of 45.5 nmol thioguanine/gHb h^{-1} for this TPMT phenotyping test has been pro posed for assessing the need of the genotyping test (Wusk et al. 2004).

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B.24.3.4 Glutathione *S*-transferases and Sulfotransferases

Glutathione and sulfatation conjugations are important pathways for generally detoxifying endogenous substrates and xenobiotics (Commandeur et al. 1995). However, some produced metabolites (i.e., mercapturic acids, *O* sulfo conjugates) are toxic by different mechanisms, often by reaction with DNA and other cellular nucleophils.

Eight classes of glutathione *S* transferases (GSTs) have been described. The role of the glutathione pathway and the impact of enzyme polymorphism have been highlighted for detoxification and some disease suscepti bility, and routine phenotyping of some GSTs exists for clinical safety measurement, but currently there is no evidence of genotyping or phenotyping usefulness for drug dosage adjustment (Hayes and Strange 2000; Tetlow et al. 2004). GSTs are involved in the detoxification of chemotherapeutics, including platinum derivatives. Poly morphisms in the GSTP1 genotype might become a powerful tool to predict oxaliplatin induced cumulative neuropathy (Lecomte et al. 2006).

Soluble sulfotransferases are involved in the sulfona tion of endogenous substrates (notably steroids, neuro transmitters, and eicosanoids) and numerous xenobiotics (i.e., acetaminophen, and 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 and SULT1A3) by measuring platelet sulfotransferase activity (Glatt and Meinl 2004).

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B.25 PK/PD Approaches

Diether Rüppel · Willi Weber

Abbreviations: A, Amount (mass); a, Annum (year); B, Constant of HOMA expressing insulin production; α, Hill coefficient, proportionality constant; β, Factor expressing the effect of first phase insulin; C, Concentration; C_{50}, Concentration for 50% of the effect; c_o, Covariate; E, Effect; EF, Effect; E_{max}, Maximum effect; EMEA, European Medicines Agency; F, Function; FDA, Food and Drug Administration; FPG, Fasting plasma glucose; FSI, Fasting plasma insulin; G, Gluscose concentration; γ, Rate con stant in minimal model; G_B, Glucose base concentration; GIP, Glucose dependent insulinotropic polypeptide; GLP 1, Glucagon like peptide; h, Glucose constant in minimal model; I, Insulin concentration; IVGTT, Intravenous glu cose tolerance test; HbA_{1c}, Glycosylated haemoglobin, Alc; HOMA, Homeostatic model assessment; I_B, Insulin base concentration; IC_{50}, Concentration for 50% inhibition; K_0, Absorption rate; k_e, Elimination rate; k_{in}, Production rate; k_{out}, Elimination rate; l, Liter; n, Rate constant min imal model; NONMEM, Nonlinear mixed effect model ing; NLME, Non linear mixed effect; OGTT, Oral glucose tolerance test; p, Probability; $p_{1,2,3}$, Rate constants in min imal model; PD, Pharmacodynamics; PK, Pharmacokinet ics; PROC MIXED, Mixed effect package of SAS; QTc, Corrected QT interval of the electrocardiogram; R, Response, statistical software; S, Constant of HOMA expressing insulin sensitivity; SAS, Statistical software; t, Time; V_d, Dosing volume; X, Remote insulin concentration

PURPOSE AND RATIONALE

Pharmacokinetics (PK) describes the time course of the drug concentration in the body, normally in blood or plasma. Pharmacodynamics (PD) describes the time course of the drug effects, desired or not desired. PK/PD is the link between PK and PD describing how the time course of an effect depends on the time course of a drug.

Reviews on PK/PD are regularly published (Derendorf and Meibohm 1999; Derendorf et al. 2000; Csajka and Verotta 2006; Bonate 2006), and the more specialized literature is becoming more and more abundant. The book of Gabrielson and Weiner (2007) is an excellent introduction to PK/PD and pharmacokinetic modeling. This chapter is a comprehensive summary of the state of the art with references to basic and recent literature. PK/PD modeling is assessed by reviewing and discussing its application in diabetes modeling. In the last section, "Pharmacometrics" (Ette and Williams 2007), the general frame work of PK/PD, is briefly discussed.

B.25.1 Mathematical Models in Biology

Biology is complex, much too complex to be completely described in equations. And it is extremely difficult to obtain all the data needed for a detailed description. Models are reductions of reality to a mathematical system that can be handled, for example, by a computer. Models are simplified descriptions of a true biological process. They differ in the degree of simplification. There is no right model. A model cannot be better than the data without using previous knowledge. A dataset will never be complete. Models will differ depending on the available data. And in each model is part of the truth. The situation is not new. It is the moral of the famous parable of "the blind men and the elephant" probably originating from Asia a long time ago:

- *Six blind men were asked to determine what an elephant looked like by feeling different parts of the elephant's body. The blind man who feels a leg says the elephant is like a pillar; the one who feels the tail says the elephant is like a rope; the one who feels the trunk says the elephant is like a tree branch; the one who feels the ear says the elephant is like a hand fan; the one who feels the belly says the elephant is like a wall; and the one who feels the tusk says the elephant is like a solid pipe. And they started arguing and fighting and could not find out what was the truth. Finally a wise man explains to them: "All of you are right. The reason every one of you is telling it differently is because each one of you touched a different part of the elephant. So, actually the elephant has all the features you mentioned."*

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Depending on the data and the task, different parts of a system are described and different conclusions can be drawn. The question about the use of a model under development decides on the data needed. What is the resolution in time, in space, in concentration required? Will the model be used for interpolation only, or is it to simulate new situations by extrapolation? Pure empiric models can be used to interpolate in the limits of data used to develop the model. More mechanistic models are more difficult but more useful when cautious extrapolation beyond the limits of observed data is required. Finally, models may change if additional data are available.

B.25.2 Modeling in Pharmacometrics

The modeler can follow two principal approaches to cope with the complexity. One is to start with the complete knowledge expressed in all physiological equations and then to simplify as much as necessary so that the model can be processed in a computer. The other possibility is to fit a simpler empirical model. The inconvenience of the latter is that empirical models do not give any explanation of the underlying processes.

Linear functions, polynoms, or splines are pure empirical descriptions of the time course of observed data. The E_{max} model and its simplifications presented in the following are more useful. Fortunately, mechanistic biological models can be composed of relatively few, recurring algebraic constructs (Haefner 1996). However, the interdependencies may become very complex, and reduction of the complexity is necessary to make them processable.

Mixed-effects modeling does not only calculate the PK/PD parameters but also their statistical distribution in the population and is therefore called population PK/PD in this context.

PROCEDURE

B.25.3 Basic Concepts of Pharmacokinetics

Absorption, distribution, metabolism, and excretion are governing the concentration time course of a drug in blood or plasma. In principle, nonparametric, compartmental, and physiologically based models can be used for PK/PD modeling. The majority of PK/PD models use compartmental PK models to describe the concentration time course, in most cases in the central compartment. The central compartment is not necessarily the site of action, and concentrations in deeper compartments may enter into the PK/PD model. The drug concentration at the site of action may be accessible to measurements, but very often it is not and has to be calculated in the PK model. The compartment for drug action may be part of the pharmacokinetic model describing the concentration time function but can also be a small effect compartment with a nonmeasurable amount in mass balance. This will be discussed further as the link of a PK/PD model. A good introduction to PK is the standard textbook of Rowland and Tozer (1995). Population PK means modeling PK in a population attributing individual parameters to the individuals, which are drawn from a distribution calculated by a multivariate, mixed effects regression. Parts of the parameter variability are subsequently explained by demographic covariates making the model more deterministic.

B.25.4 Effects, Surrogates, and Biomarkers

PK deals with one dependent variable (drug concentration) and many covariates such as time, dose, and demographic variables. Drug concentration is a covariate in PD like all other covariates mentioned for PK. PD has usually several dependent variables as clinical end points, surrogates, and biomarkers. In most cases, only one is modeled at a time. It is an important decision of the modeler and his customer to choose an appropriate dependent variable. The dependent variable describing an effect should be meaningful, measurable, and appropriate for modeling. Dependent variables may be binary (alive, dead), categorical (several score levels), or continuous such as concentrations of an endogenous substance (glucose) or as a physical parameter (blood pressure, QTc, etc.).

B.25.5 Baselines

Drug responses are generally changes from baseline. Correct baseline measurements are essential. Often, baselines are measured at the beginning of a trial and cannot be measured during the trial. Sometimes, baselines are changing over time due to placebo effects, changing of habits and lifestyle during the trial, or due to disease progression. This has to be corrected by a model developed from placebo patients. In population PK/PD models, baseline and effect under drug application including their variability should be part of the model (and not only their ratio or difference).

B.25.6 General Considerations in Pharmacodynamics

In general, a drug effect E is depending on time t, drug concentrations C in the past until present, and additional covariates co through a function F

$$E(t) = F(t, C(-\infty, t), co(-\infty, t)) \qquad (B.25.1)$$

This is the description of a general dynamic system. In a static system, the effect depends only on the current time without any memory

$$E(t) = F(t, C(t), co(t)) \qquad (B.25.2)$$

The system may be time variant or invariant. For an invariant system, the response to a drug concentration is always the same and does not dependent on the time when it is applied. In a time variant system, the response is depending on the time, for example, through time changing covariates $co(t)$.

A relaxed system is at rest ($F = 0$) for drug concentrations $C = 0$

$$F(t, 0, co(t)) = 0 \qquad (B.25.3)$$

for all times t $\in [-\infty, \infty]$
and is excited ($F > 0$) by drug concentrations $C > 0$.

For a linear system, the effect of the sum of two concentrations is the sum of the effect of each concentration. Many effects are nonlinear, so that this simple additive procedure cannot be applied.

B.25.7 Basic Concentration Effect Relationships

Very often the effect is a continuous variable E like heart frequency or the concentration of endogenous substances like blood glucose. A well known relationship from receptor theory (or physical chemistry) is the Hill equation (Danhof et al. 2007; Csajka and Verotta 2006) sometimes called the sigmoid E_{max} model (Meibohm and Derendorf 1997)

$$E = E_{max} \frac{C^{\alpha}}{C_{50}^{\alpha} + C^{\alpha}} \qquad (B.25.4)$$

with effect E, maximum effect E_{max}, concentration C, the concentration of half effect C_{50}, and the Hill coefficient α. The relationship can be derived from the law of mass action by introducing some cooperativity of neighboring ligands. The equation has a mechanistic background. Often, data are not sufficiently informative or part of the information is blurred by some noise so that not all parameters can be determined. Sometimes the range of concentrations is not sufficiently large. In all these cases, the relationship has to be simplified. Very often it is difficult to determine the Hill coefficient α, which is then put to $\alpha = 1$.

$$E = E_{max} \frac{C}{C_{50} + C} \qquad (B.25.5)$$

Equation (B.25.5) is called the E_{max} model. Another simplification occurs when α becomes very large. In this case, the effect E turns out to be 0 below the threshold C_{50} and E_{max} above C_{50} (**threshold model**).

Equation (B.25.4) can be expressed with logarithmic concentrations:

$$E = E_{max} \frac{\exp\left(\alpha * \log\left(\frac{C}{C_{50}}\right)\right)}{1 + \exp\left(\alpha * \log\left(\frac{C}{C_{50}}\right)\right)} \qquad (B.25.6)$$

The linear logarithmic form for concentrations around $\log(C_{50})$ is derived by taking the derivative of (B.25.6) at $\log(C) = \log(C_{50})$:

$$E = E_{max} * \left(\frac{1}{2} + \frac{\alpha}{4} * \log\left(\frac{C}{C_{50}}\right)\right) \qquad (B.25.7)$$

This holds for concentrations around C_{50}: $C \in [0.2 * C_{50}, 0.8 * C_{50}]$ (log linear relationship).

Further simplifications are the limits for small and high concentrations in comparison to C_{50}

$$E = E_{max} \frac{C}{C_{50}} \quad \text{for small C} << \text{C}_{50} \quad \text{(linear relationship)} \qquad (B.25.8)$$

$$E = E_{max} * \left(1 - \frac{C_{50}}{C}\right) \quad \text{for large C} >> \text{C}_{50} \quad \text{(inverse proportional relationship).} \qquad (B.25.9)$$

E_{max} and C_{50} are not completely independent in (B.25.4) or (B.25.5). Fitting E_{max} and C_{50} can become difficult when E_{max} is not observed and has to be extrapolated from the curvature of the effect concentration function. As the ratio of E_{max} and C_{50} is more stable Schoemaker et al. (1998) proposed a different parameterization of (B.25.5):

$$E = \frac{S_0 E_{max} * C}{E_{max} + S_0 C} \qquad (B.25.10)$$

by introducing the ratio $S_0 = \frac{E_{max}}{C_{50}}$. Equation (B.25.10) is useful for small concentrations.

The effect may be a categorical variable or even a binary variable like (dead, alive) or (improvement, no improvement). In this case, the effect in the Hill equation

should express something like the probability of an event or improvement. Probability functions are often expressed with the help of the logit function (Colett 2003)

$$\log it = \log\left(\frac{p}{1-p}\right) \qquad (B.25.11)$$

which is the logarithm of the odds (probability for an effect divided by the probability of the effect not to occur). The probability of the effect is then the inverse function

$$p = \frac{\exp(\log it)}{1+\exp(\log it)} \qquad (B.25.12)$$

A natural link of probability and effect of the drug concentration can be derived by comparing (B.25.12) and the logarithmic form of the Hill Eq. (B.25.6). The equations are becoming identical when putting $E_{max} = 1$, assuming that the effect is simply the probability of the event and putting

$$\log it = \alpha * \log\left(\frac{C}{C_{50}}\right) \qquad (B.25.13)$$

Equation (B.25.13) is sometimes called the link function. For calculation purposes, (B.25.12) is often written as a logistic model

$$p = \frac{\exp(\beta_0 + \beta_1 * \log(C))}{1+\exp(\beta_0 + \beta_1 * \log(C))} \qquad (B.25.14)$$

with $\beta_0 = -\alpha * \log(C_{50})$ and $\beta_1 = \alpha$ (attention to units). In terms of a logistic distribution, the location parameter $\log(C_{50}) = \mu$ is the mean and $\pi^2/3\alpha$ is the variance.

The clinical endpoint may be lifetime. Lifetime analysis is a special topic and will not be discussed in this short overview. For more information, the book of Lawless is recommended (Lawless 1982). Examples can also be found in Weber and Rueppel (2006).

B.25.8 Classifying Types of Pharmacodynamic Models

Classifications may be appropriate for one purpose and wrong for another. Putting something into a box is one of the favorite games of children (and adults). Once in a box, it may become obvious that it is in the wrong box. It depends on the flexibility of the player to change the box or to create new boxes with appropriate labels. It is the same for classifying PK/PD models. Attributes (boxes) may be appropriate or not, they are always overlapping and never exhaustive. PK/PD models can be classified by the way concentration and effect are linked, by the type of response, by the way information is used, whether they are time variant or invariant (Derendorf et al. 2000).

B.25.8.1 Direct and Indirect Link

If effect and drug concentration are always in phase, this means if the effect course is always following the drug concentration despite the non steady state conditions, PK and PD can be directly linked like under steady state conditions. If the effect is lagging behind the concentration (counterclockwise hysteresis) an indirect link model has to be used. The hypothetical effect compartment is such a link (Sheiner et al. 1979). The effect is directly linked to the concentration in the effect compartment. The concentration in the effect compartment depends on the measured concentration in a central compartment by pharmacokinetic parameters describing the time course but is sufficiently small so that the mass balance (and the kinetics of the central department) is not modified.

B.25.8.2 Direct and Indirect Response

If effect and concentration in a central or an effect compartment are directly correlated, the model is called a direct response model. If the effect consists of stimulation or inhibition of a physiologic process, drug concentrations can often be correlated with production or elimination rates of endogenous substances. In this indirect response model, rates can again be linked via one of the basic pharmacodynamic models mentioned above. As an example the inhibition of the production rate of compound R could be modeled as

$$\frac{dR}{dt} = k_{in} * \left(1 - \frac{C}{C_{50}+C}\right) - k_{out} * R \qquad (B.25.15)$$

Four base indirect response models are discussed (Dayneka et al. 1993). Indirect response models are an alternative way to deal with time lags and hysteresis.

B.25.8.3 Hard and Soft Link

Hard and soft link models differ in the way they use information. In soft link models, both, concentration and effect observations enter into the model. In the case of indirect link models, an effect compartment with hypothetical concentrations serves as a buffer. The additional pharmacokinetic parameters for the effect compartment are governed

by the time course of the drug concentration and by the time course of the effect. Information on the link model comes from both sides, PK and PD.

Hard link models use physiologic knowledge to predict an effect. Observed PD data do not enter into the model but serve as comparison for the predictions. Often, the PK/PD link is based on in vitro studies and micro models like receptor ligand interactions.

B.25.8.4 Time Variant and Time Invariant

Models are called time invariant, when only concentrations and effects are varying over time, not the parameters of the model. The circadian rhythm plays an important role in physiological functions and makes some parameters of a model time dependent. Other examples for time variant models are tolerance and sensitization models.

B.25.9 Reversible, Nonreversible

Most of the drugs have reversible effects. Irreversible effects may occur in oncology (Karlsson et al. 1998) or antibiotic therapy (Dalla Costa et al. 1997). A short discussion of this topic can also be found in (Csajka and Verotta 2006).

B.25.10 Empirical Models

Empirical models are pure description of the data and have no physiological background. They are like a black box, transforming an input into an output in the limits of the inputs they have been tested. There is no understanding of what is happening in the black box. Extrapolation to input values never tested is quite dangerous. Linear models, polynoms, or splines are basic examples. Other models, like the sigmoid E_{max} models (Hill model), have already a mechanistic background: in receptor theory or in the physical Langmuir isotherm of gas absorption (law of mass action). Indirect response models with the production of endogenous substances have also mechanistic parts. In fact, the transition is smooth. Models may have empirical and mechanistic parts.

B.25.11 Mechanistic Models

Indirect response models reflect already a mechanism behind the production and elimination of an endogenous substance. The modeler puts the effect on the production rate k_{in} or the elimination rate k_{out} of the substance according to his knowledge. Several endogenous substances may be coupled by negative or positive feedback. The modeler takes this into account by using coupled differential equations as it will be discussed below when applying PK/PD methods in diabetes. There may be a whole cascade of mediators. Transit compartment models may be a way to cope with this phenomenon (Mager and Jusko 2007). In general, mechanism based PK/PD models differ from empirical models by incorporating mechanisms known from physiology or drug action. This prior knowledge is introduced into the model in the philosophy of Bayesian thinking. Receptor based PK/PD relationships were investigated in detail over years. Models and strategies are presented in the textbook of Kenakin (Kenakin 1997) or in the review article of Danhof et al. (2007).

EVALUATION

Plotting effects versus various covariates like time, concentrations, demographic variables will help to generate hypothesis about a future model. Is the effect time invariant? Is a hysteresis observed? Clockwise or counter clockwise? A strategy to decide about using an effect compartment or a direct response model has been discussed by Wakelkamp et al. (1998). Knowledge about the underlying process can also help to decide on this question and about the related physiology will also help to explore more mechanistic models.

The following items should be clearly addressed in a PK/PD model development:

1. Problem and purpose of the model
2. Assumptions in the modeling process (explicit and implicit assumptions)
3. Rationale of model development (Why is a model considered to be better than another?)
4. Validation strategy using internal or external data

B.25.12 One- or Two-Step Approach

PK and PD can be modeled in one step or in two steps. If it is modeled in one step, pharmacokinetic and pharmacodynamic parameters are calculated simultaneously and information goes in both directions: PK is influencing PD and vice versa. Very often the pharmacokinetic part is better known so that a sequential procedure is preferred: First, the PK is modeled and concentrations are simulated using individual PK parameters from the PK fit.

In a second step, the PK/PD model is developed by using observed PD and simulated PK data.

B.25.13 Population Approach

All pharmacokinetic and pharmacodynamic parameters have in general large distributions in the population. Simultaneously calculating parameters and their distributions, known as mixed effects modeling, is well established in PK (FDA 1999). It can be extended to PK/PD modeling. Population PK introduced in the 1970s by Sheiner and coworkers (1977) is discussed in a separate chapter of this book.

B.25.14 The Learning and Confirming Cycle

Classical clinical studies are self consistent, that is, a hypothesis is accepted or refused with the information coming from the study and nothing else. The learn and confirm approach (Sheiner 1997) consists of alternating learning and confirming cycles: a study to generate a hypothesis and a subsequent study to confirm the hypothesis (or to improve the model), followed by further hypothesis generating and confirming (or not confirming) studies.

B.25.15 Software

A comprehensive list and discussion of software used in PK/PD modeling can be found in the book of Ette and Williams (Ette and Williams 2007; Isukapalli and Roy 2007; Isukapalli et al. 2007; Wolk 2007). WinNonLin® (Pharsight®) is a wide spread software for PK and PK/PD modeling (Gabrielson and Weiner 2007). NONMEM® (ICON) is recommended for population PK/PD. NONMEM® is able to perform mixed effects modeling and to solve differential equations with huge data files. The subroutine PREDPP is the link to population PK and PK/PD applications. R and S plus® (Insightful®) are both object oriented programming languages, R (R project) being the free version of S plus®. Mixed effects modeling is possible with the NLME package (Pinheiro and Bates 2000). The advantages of R and S plus® are the various statistical packages, the possibilities of plotting and data management, and that many scientist working in population PK/PD are programming with R and are publishing solutions for various problems. SAS® is a commercial statistical software widely used in the medical area with similar functions as R. Its PROC MIXED routine allows mixed effects modeling. MATLAB® is a powerful software used in the mathematical or technical area and proposes also packages for pharmacokinetic and dynamic modeling. Applications of MATLAB® in Population PK/PD are also under development (MONOLIX project). Other often used software packages are Trial Simulator™ (Pharsight®), BUGS (PKBUGS from the BUGS project), and ADAPT II (Biomedical Simulation Resources). Versions and owners change rapidly. Actual information about these commercial or free software packages can be found on the corresponding Web sites in the Internet.

CRITICAL ASSESSMENT OF THE METHOD

B.25.16 Physiology of the Glucose Regulatory System

Glucose is the most important energy transport medium in vertebrates. But many endogenous substances are destroyed by glycosylation. HbA_{1c}, a glucosylated form of hemoglobin, is a biomarker for the glycosylation potential in the body. Fine control of the blood glucose concentration for example, enough during efforts, as low as possible in breaks is therefore important. In healthy subjects, fasting plasma glucose concentrations are normally maintained in the range of about (3.0 6.4) mmol/L.

Roughly speaking, glucose concentration is regulated in the following way: A glucose base concentration G_B assures functioning of various basic processes. The brain is one of the big glucose consumers. In the fasting equilibrium state, glucose is eliminated by consuming functions and is replaced at the same rate by cracking glycogen in the liver. Insulin has two effects on the glucose concentration: one on the elimination, the other on the production. Insulin increases the uptake of glucose in the liver, muscles, or in the fat tissue as glycogen or fatty acids. And it blocks the production of glucose from glycogen in the liver.

If the equilibrium is disturbed by higher glucose consumption, glucose concentration is decreasing followed by a decreasing insulin production. Lower insulin concentration decreases the inhibition of glucose production in the liver, that is, increases the glucose production and concentration. The process prevents hypoglycemia.

If the equilibrium is disturbed by food intake, glucose enters the body from the intestines and the glucose concentration increases. This stimulates the secretion of insulin. Insulin induces the storage of glucose in the form of fatty acids in cells reducing the glucose concentration. Insulin also inhibits the glucose production from

glycogen. In a negative feedback loop, decreasing glucose concentration stops insulin production. This process pre vents hyperglycemia.

The insulin amylin glucagon system is an important subsystem. Glucagon promotes cracking of glycogen to glucose in the liver. Glucagon is produced in the pancreas. Amylin is coproduced with insulin in the pancreas and inhibits the production of glucagon. Increasing insulin/ amylin production reduces the glucagon production, reduc ing the glucose production. Amylin works in the same sense as insulin and should be taken into account in more detailed pictures.

Glucose regulation is indeed more complicated. Insu lin has additional effects. It stimulates the conversion of glucose to fatty acids and stimulates the uptake of fatty acids or amino acids in the cells. In other processes, non carbohydrates (like fatty acids) can be converted to glucose. Or glucose can be converted in an anaerobic process to lactate and can be resynthesized from lactate in the liver. The processes of glucose uptake by the cells (stimulated by insulin) involves membrane processes, opening or clo sure of potassium channels, and membrane depolarization inducing increasing calcium concentrations in the cells.

In case of sudden glucose increase, insulin is produ ced in two phases. A first phase is lasting 5 10 min and a second phase is in the range of 1 2 h. Insulin is secre ted like many other hormones in a pulsatile fashion with rapid pulses (8 15 min) and ultradian oscillations (80 100 min). In case of food intake, glucose concentra tion is slowly increasing and the two phases are not clearly distinguished (Caumo and Luzi 2004). Food intake is also accompanied with secretion of incretin hormones GLP 1 and GIP from the cells in the gut wall. Both hormones stimulate glucose dependent insulin secretion (to prepare the system to the glucose input). Lifetime of GLP 1 or GIP is very short (<2 min [Hinnen et al. 2006]), like lifetime of insulin (∼10 min [Caumo and Luzi 2004]).

Hemoglobin is continuously glycosylated. The degree of glycosylation is the integrated history of the glucose concentration during its lifetime (up to 3 months). HbA_{1c} is a marker of glucose concentration over time but not a player in the glucose regulatory process. There is no feedback from HbA_{1c} to glucose concentration.

Depending on the mechanism of drug action these details should enter onto the PK/PD model. But modeling will need many simplifications. The question will be: What can be simplified? What can I do or what do I expect to do with the model? If I need information about the maxi mum level of glucose concentrations after food intake or glucose infusion, the incretin, insulin, glucagon, glucose, glycogen system has to be carefully put into equations. If I need information about the fasting glucose, not consid ering glucose regulation after food intake, the number of coupled processes to take into account will be reduced. Modeling will then happen on another timescale.

B.25.17 Diabetes

Two forms of diabetes are reported today. Diabetes type 1 corresponds to a complete inability of producing insulin in the pancreas. Type 2 corresponds to an insufficient insulin secretion in the pancreas and reduced sensitivity of the liver and peripheral cells to insulin. This should be reflected by modified parameters in the above described glucose regulatory system. As the disease progresses slowly over time, this should be incorporated into the parameter changing at the long term. Antidiabetic drugs have various mechanisms of action. Putting this into equations for the glucose regulation system is an exiting and critical step in PK/PD modeling.

This short overview will follow the concept of time scales (seconds, minutes, hours, days, weeks, month, years) used in modeling diabetes.

B.25.18 Modeling the Glucose Regulatory System

Since the 1960s various models with different degrees of complexity have been proposed. The model proposed by Cobelli et al. (1982) describing the insulin glucose glucagon system involves seven coupled differential equa tions and several additional equations. The popular minimal model (Bergman et al. 1979) was developed for the interpretation of the intravenous glucose tolerance test (IVGTT). It still includes three coupled differential equa tions. It is already less mechanistic but has mathematical limitations (de Gaetano and Arino 2000). The widespread homeostatic model assessment (HOMA) model of fast ing glucose plays on another timescale and under steady state conditions (Wallace et al. 2004). The article of Landersdorfer and Jusko (2008) is an excellent overview of the state of the art in diabetes modeling with special attention to modeling the effects of drugs.

B.25.19 Modeling the Effects of Drugs

The frequently used indirect response models describe drug effects on the endogenous glucose and insulin pro duction and elimination. Biophase distribution models

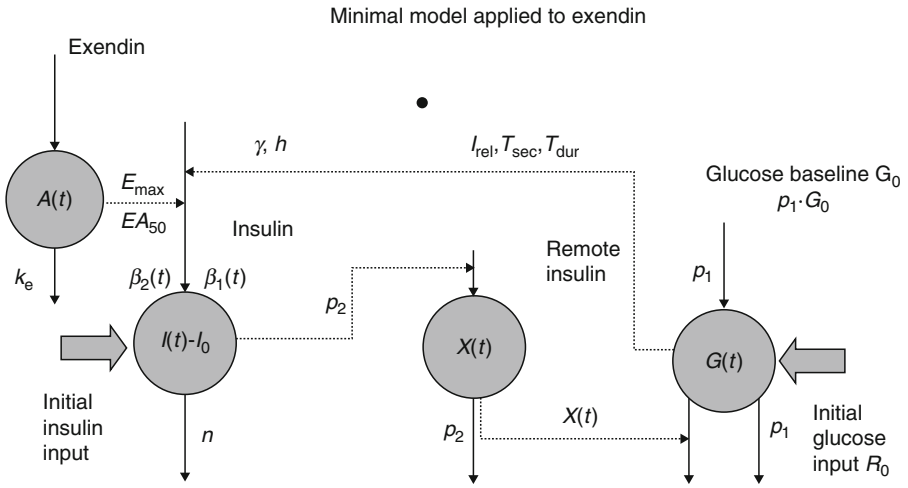


Figure B.25-1

Application of the minimal model (Bergman et al. 1979) to exendin (Agersø and Vicini 2003; Mager et al. 2004). The mathematics of the model is documented in (B.25.16 B.25.25). G is the plasma glucose concentration, I the insulin concentration. X stands for the virtual remote insulin concentration modeling the time delay between glucose and insulin time course. β_1 and β_2 correspond to the first insulin secretion with or without exendin

using effect compartments (Sheiner et al. 1979) are less used in diabetes modeling. Diabetes type 1 patients cannot produce insulin. Treatment consists therefore mainly of exogenous insulin administration. Diabetes type 2 has several causes like insufficient insulin production or redu ced sensitivity to insulin. There are various modes of action for the different classes of antidiabetic drugs. Choosing the right subsystems of the glucose regulatory system is a decisive part of PK/PD modeling.

B.25.20 Modeling at the Short-Term Timescale

The short term is interesting for food intake and diagnos tic challenges like the IVGTT or the oral glucose tolerance test (OGTT). A defined amount of glucose is applied, and the increasing and decreasing glucose concentration over time is observed. The timescale is minutes and hours. In the glucose clamp technique, the glucose concentration is monitored, and glucose is infused intravenously to keep the blood glucose at a constant level. The amount of glucose infused per time is measured. Bergman et al. (1979) devel oped the minimal model widely used for the interpre tation of these experiments. The basic idea is to couple insulin I and glucose G regulation by introducing an addi tional, virtual, remote insulin X imitating the time delay between the release of insulin and reduction of glucose concentration.

$$\frac{dG}{dt} = -(p_1 + X(t)) * G(t) + p_1 * G_B \qquad (B.25.16)$$

$$\frac{dX}{dt} = p_2 * (I(t) - I_B) - p_3 * X(t) \qquad (B.25.17)$$

$$\frac{dI}{dt} = \gamma * (G(t) - h) * t - n * I(t) \qquad (B.25.18)$$

With initial conditions

$$G(0) = \frac{Dose}{V_d} + G_B \qquad (B.25.19)$$

$$X(0) = 0 \qquad (B.25.20)$$

$$I(0) = I_B \qquad (B.25.21)$$

and parameters p_1, p_2, p_3, γ, n, and base values G_B, I_B and h. *Figure B.25 1* illustrates the minimal model and its application to exenatide, a GLP 1 analog. The minimal model (Bergman et al. 1979) was modified and adapted to special experimental conditions. It is widely used since it was published in 1979. But the minimal model has several problems (Pacini and Bergman 1986). One is that it does not allow a real stable equilibrium. It works only when applying the "correct procedure." In one version, the insu lin data serve to fit the glucose data, in the other, the glucose data to fit the insulin data. Insulin and glucose cannot be fitted together (Pacini and Bergman 1986). De Gaetano and Arino investigated the stability of the

differential equation system and concluded that the virtual, remote insulin would increase without bounds. They proposed a unified model that can be fitted simultaneously to insulin and glucose concentrations (de Gaetano and Arino 2000). This model served as starting point for Silber et al. to develop an integrated model of glucose and insulin to explain the IVGTT (Silber et al. 2007). The other problem with the minimal model is that it does not deal with the first and second insulin phase. An additional term representing the first phase was added to the original minimal model by Agersø et al. when trying to model a GLP 1 analog (Agersø and Vicini 2003). This will be discussed in the next paragraph.

B.25.20.1 Effect of GLP-1 Analogs

Drugs can play a role in different processes in the regulation of glucose concentrations: glucose elimination, sensitivity of insulin or remote insulin, and insulin production. The incretin hormone GLP 1 is secreted by the gut cells and stimulates insulin secretion after food intake. Agersø et al. investigated the action of NN221, a long acting GLP 1 derivative (Agersø and Vicini 2003). They modified the original minimal model by adding a glucose independent term $\beta(t)$ describing the first phase insulin secretion after exposure to increasing glucose concentration (food intake):

$$\beta(t) = \frac{I_{\text{rel}}}{T_{\text{dur}}\sqrt{2\pi}} * e^{\frac{(t - T_{\text{sec}})^2}{2T_{\text{dur}}^2}} \qquad (B.25.22)$$

I_{rel} is the amplitude of the first phase insulin, T_{sec} describes the time at which maximum insulin excretion occurs, and T_{dur} is the duration of the first phase insulin. The insulin differential equation reads now

$$\frac{dI}{dt} = \beta(t) + \gamma * (G(t) - h) * t - n * (I(t) - I_0) \qquad (B.25.23)$$

They fitted all parameters and found T_{sec} = 3.5 min and T_{dur} = 1.9 min. They found an influence of NN221 on γ and I_{rel}. This means that NN221 influences the first and second phase insulin.

Mager et al. (2004) developed this model further by application to exendin 4, another GLP 1 analog (synthetic exendin is called exenatide). They put the effect of exendin on γ

$$\gamma = \gamma_0 + \frac{E_{\max} * A}{EA_{50} + A} \qquad (B.25.24)$$

where γ_0 is the original γ without drug effect and $A(t)$ is the amount of exendin governed by the differential equation

$$\frac{dA(t)}{dt} = k_0(t) - k_e * A \qquad (B.25.25)$$

They were able to describe the experimental data and to calculate the model parameters (Mager et al. 2004). They did not model the effect of exendin on I_{rel} found by Agersø et al. (It is also important to distinguish between the messenger effect of GLP 1 and its analogs from the gut to the insulin production system on the one hand and the pulsatile insulin production on the other.) $1/p_1 \approx$ 0.7 h (Agersø and Vicini 2003) indicates the timescale of the process. With these models, the time course of glucose after glucose injection and drug application can be simulated under various conditions. *Figures B.25 2* and *B.25 3* show such simulations for healthy volunteers and patients with diabetes type 2. The simulation parameters are from Mager et al. (2004). The serum glucose lowering effect of 3.6 nmol exendin applied over 60 min is higher for healthy volunteers than for patients.

B.25.21 Modeling at the Intermediate Timescale

The time course of fasting glucose concentration is interesting on the medium timescale of days to weeks. It is therefore not necessary that a model follows the timescale of food intake with a timescale of minutes and hours.

A frequently used model for fasting glucose is the HOMA model (Wallace et al. 2004). It is based on two coupled differential equations for fasting plasma glucose *FPG* and fasting serum insulin *FSI* (see *Fig. B.25 4*).

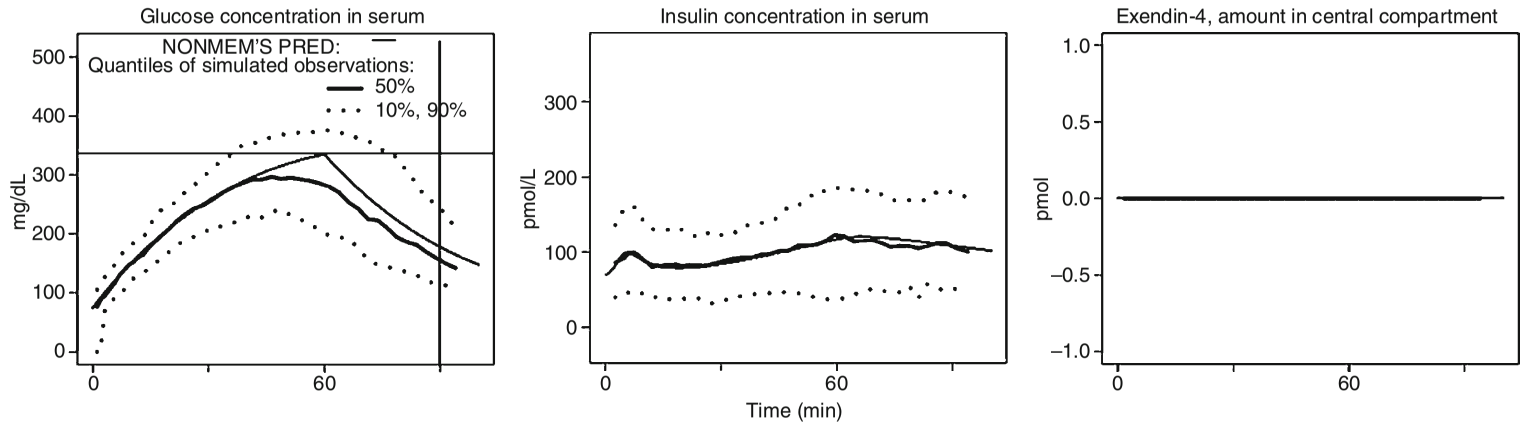
$$\frac{dFSI}{dt} = B * \left(FPG - 3.5\frac{\text{mmol}}{\text{l}}\right) * k_{\text{inFSI}} - FSI * k_{\text{outFSI}} \qquad (B.25.26)$$

$$\frac{dFPG}{dt} = \frac{k_{\text{inFPG}}}{S * FSI} - FPG * k_{\text{outFPG}} \qquad (B.25.27)$$

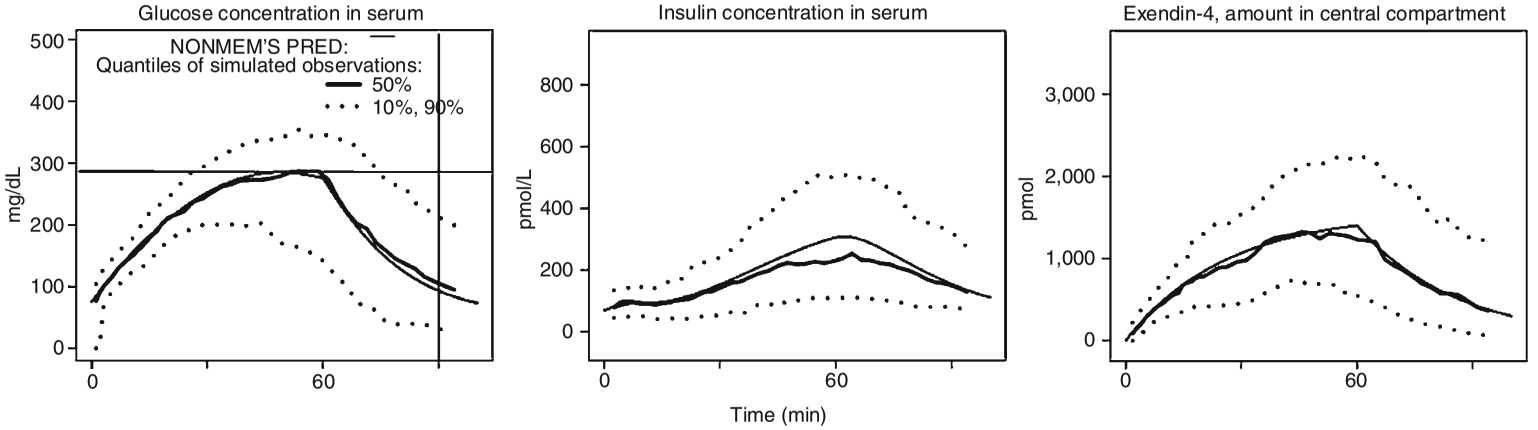
B is the amount of insulin produced per glucose concentration, essentially reflected by the beta cell mass and the productivity of beta cells. S is the hepatic insulin sensitivity.

B.25.21.1 Under Steady State Conditions (HOMA)

$$\frac{dFPG}{dt} = \frac{dFSI}{dt} = 0 \qquad (B.25.28)$$



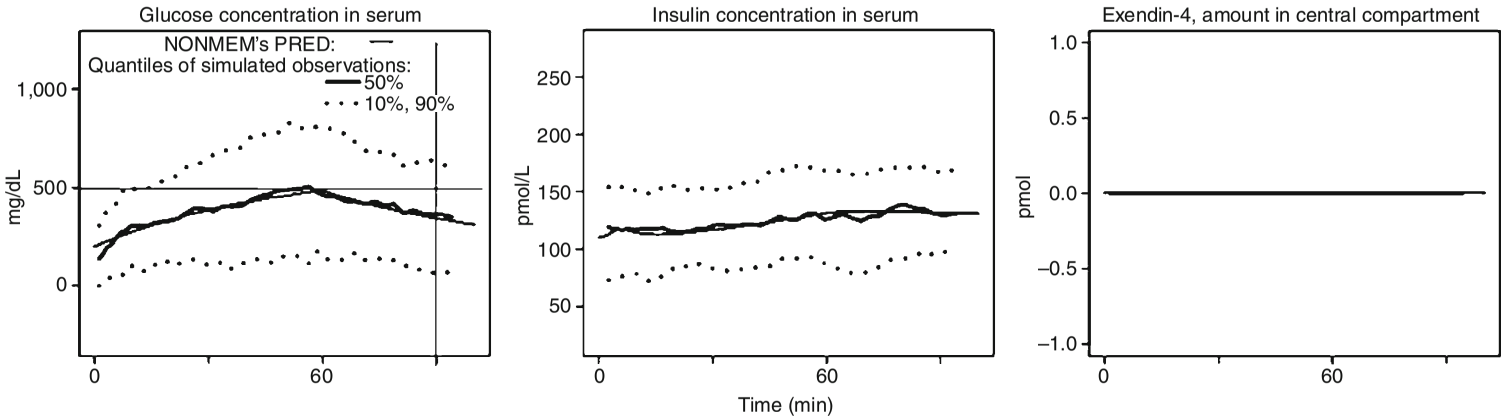
State = healthy; $G_0 = 75$; $I_0 = 70$; $T_{dur} = 1.71$; $T_{sec} = 3.41$; $I_{rel} = 49.9$; $V = 97.3$; $p_1 = 0.0228$; $p_2 = 0.0339$; $p_3 = 5.6e{-}06$; SI ($=p_3/p_2$) = 0.000165; *gamma* = 0.000552; $h = 63.2$; $n = 0.165$; $E_{max} = 0.00281$; $E_{A50} = 18.76$; $k_e = 0.0386$; *dose;glucose* = 50,000; *duration.glucose* = 60; *dose.exendin* = 0; *rate.exendin* = 0; *indiv* = 20



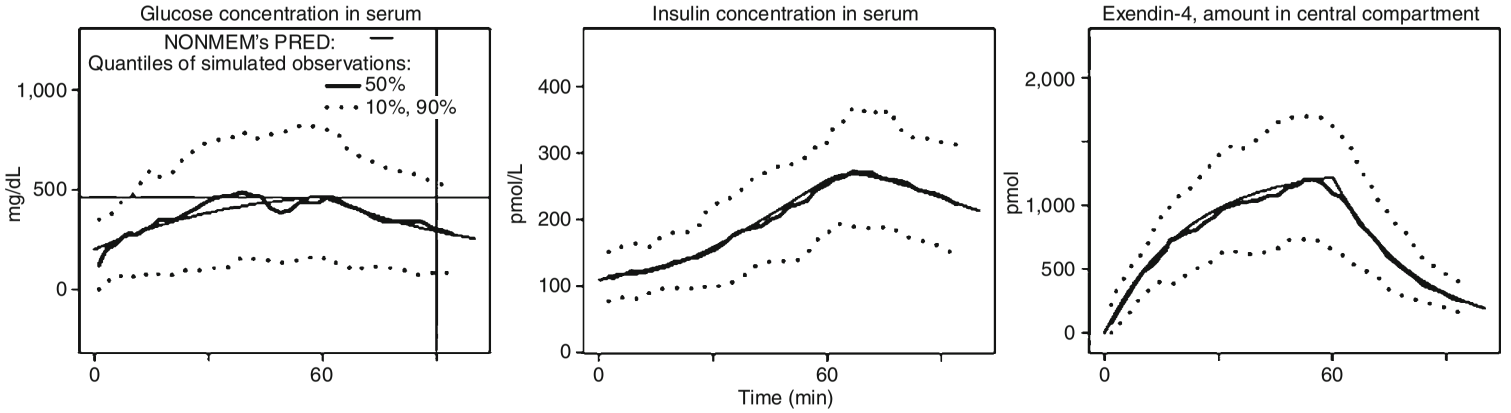
State = healthy; $G_0 = 75$; $I_0 = 70$; $T_{dur} = 1.71$; $T_{sec} = 3.41$; $I_{rel} = 49.9$; $V = 97.3$; $p_1 = 0.0228$; $p_2 = 0.0339$; $p_3 = 5.6e{-}06$; SI ($=p_3/p_2$) = 0.000165; *gamma* = 0.000552; $h = 63.2$; $n = 0.165$; $E_{max} = 0.00281$; $E_{A50} = 18.76$; $k_e = 0.0386$; *dose.glucose* = 50,000; *duration.glucose* = 60; *dose.exendin* = 3,600; *rate.exendin* = 60; *indiv* = 20

Figure B.25-2

Simulations with the model from *Fig. B.25-1* in healthy volunteers. All constants are taken from Mager et al. (2004). Patients get a glucose infusion of 50 g over 60 min starting at 75 mg/dL plasma glucose concentration. In the upper part of the figure, no exendin is applied. Maximum glucose concentration of the population mean (thin continuous curve) is 334 mg/dL. After 90 min, the plasma glucose concentration decreases to 178 mg/dL. In the lower part of the figure, 3.6 nmol exendin (15 μg) is applied over 60 min. Maximum plasma glucose concentration is now 285 mg/dL and after 90 min the glucose level decreases to 88 mg/dL



state = diabetes; G_0 = 200; I_0 = 110; T_{dur} = 1.76; T_{sec} = 3.54; I_{rel} = 13; V = 99.4; p_1 = 0.0209; p_2 = 0.0161; p_3 = 1.49e–06; SI (=p_3/p_2) = 9.25e-05; *gamma* = 0.000243; h = 144; n = 0.204; E_{max} = 0.00154; E_{A50} = 7.77; k_e = 0.046; *dose.glucose* = 50,000; *duration.glucose* = 60; *dose.exendin* = 0; *rate.exendin* = 0; *indiv* = 20



state = diabetes; G_0 = 200; I_0 = 110; T_{dur} = 1.76; T_{sec} = 3.54; I_{rel} = 13; V = 99.4; p_1 = 0.0209; p_2 = 0.0161; p_3 = 1.49e–06; SI (=p_3/p_2) = 9.25e-05; *gamma* = 0.000243; h = 144; n = 0.204; E_{max} = 0.00154; E_{A50} = 7.77; k_e = 0.046; *dose.glucose* = 50,000; *duration.glucose* = 60; *dose.exendin* = 3,600; *rate.exendin* = 60; *indiv* = 20

Figure B.25-3

Simulations with the model from *Fig. B.25-1* in diabetes type 2 patients. All constants are taken from Mager et al. (2004). Patients get a glucose infusion of 50 g over 60 min starting at 200 mg/dL plasma glucose concentration. In the upper part of the figure, no exendin is applied. Maximum plasma glucose concentration of the population mean (thin continuous curve) is 472 mg/dL. After 90 min, the glucose concentration decreases to 333 mg/dL. In the lower part of the figure, 3.6 nmol exendin (15 μg) is applied over 60 min. Maximum plasma glucose concentration is now 465 mg/dL and after 90 min the glucose level decreases to 290 mg/dL. The beneficial effect of exendin for the diabetes patients is the accelerated decrease of the glucose concentration whereas the peak levels are not significantly decreased

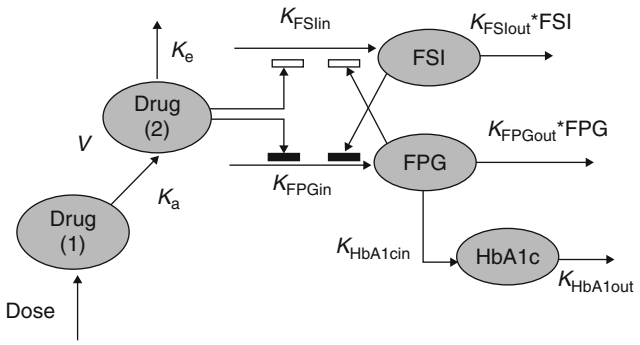


Figure B.25-4

Model of fasting plasma glucose (FPG), fasting serum insulin (FSI), HbA_{1c}, and the effect of an antidiabetic drug. The model uses the differential Eqs. (B.25.34), (B.25.35), and (B.25.41). Enhancing effects are visualized by white rectangles, inhibiting effects by black rectangles. Pharmacokinetics of the drug is described by a one-compartment model with absorption compartment. B and S are the well-known HOMA parameters (Wallace et al. 2004) for insulin production and sensitivity. Effects EF_S or EF_B can be modeled in different ways, simple E_{max} models are proposed here. The model is discussed in de Winter et al. (2006). The paper also contains predictions for the effect of metformin, pioglitazone, and gliclazide

So that

$$B = \frac{FSI}{(FPG - 3.5\frac{\text{mmol}}{\text{l}})} * \frac{k_{\text{outFSI}}}{k_{\text{inFSI}}} \qquad (B.25.29)$$

and

$$S = \frac{1}{FPG * FSI} * \frac{k_{\text{inFPG}}}{k_{\text{outFPG}}} \qquad (B.25.30)$$

B is normalized to 1 for a standard person, S is the insulin sensitivity for a standard person normalized to 1. A stan dard person has $FPG = 4.5$ mmol/l and $FSI = 5$ mU/l. It follows

$$\frac{k_{\text{inFSI}}}{k_{\text{outFSI}}} = 5\frac{\text{mU}}{\text{mmol}} \text{ and } \frac{k_{\text{inFPG}}}{k_{\text{outFPG}}} = 22.5 * \frac{\text{mmol} * \text{mU}}{\text{l}^2} \qquad (B.25.31)$$

$$B = \frac{FSI}{(FPG - 3.5\frac{\text{mmol}}{l}) * 5 * \frac{\text{mU}}{\text{mmol}}} \qquad (B.25.32)$$

$$S = \frac{1}{FPG * FSI} * 22.5\frac{\text{mmol} * \text{mU}}{\text{l}^2} \qquad (B.25.33)$$

For non standard persons or under drug applications, B and S are different from 1. B and S are measures of disease worsening or improvement after drug adminis tration. The HOMA model is widely used (Wallace et al. 2004).

B.25.21.2 Effect of Gliclazide, Pioglitzone, and Metformin

The effect of a drug may be modeled as an effect on the beta cell mass B or the insulin sensitivity S. De Winter et al. (2006) used the coupled differential equations with the relationships for k_{in} and k_{out} derived from the steady state conditions and introduced the effect parameters EF_B and EF_S. They investigated gliclazide, metformin, and pioglitazone. The differential equations for fasting glucose and insulin read now

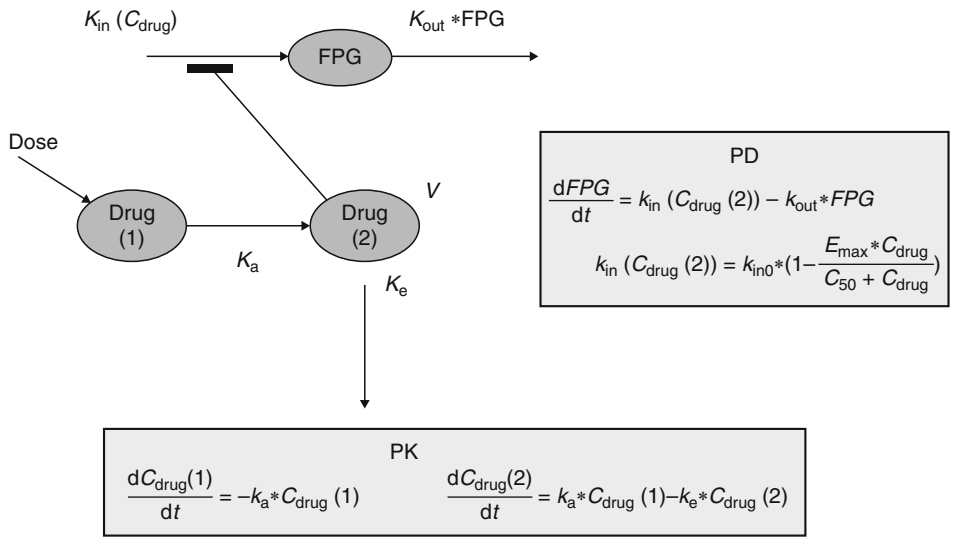


Figure B.25-5

Simplified model of fasting plasma glucose (FPG) and effect of an antidiabetic drug. The effect on insulin is incorporated in the inhibiting effect of the drug. The model uses the differential Eq. (B.25.28). A similar model is used by Hong et al. (2008)

$$\frac{dFSI}{dt} = EF_B * B * (FPG - 3.5\frac{\text{mmol}}{\text{l}}) * k_{\text{inFSI}} - FSI * k_{\text{outFSI}} \quad (B.25.34)$$

$$\frac{dFPG}{dt} = \frac{k_{\text{inFPG}}}{EF_S * S * FSI} - FPG * k_{\text{outFPG}} \quad (B.25.35)$$

Figure B.25 4 illustrates the complex situation. De Winter et al. took data from several trials with defined dose ranges for each of the compounds. EF_B and EF_S were modeled as step functions between 1 and maximum effect. They succeeded to estimate the maximum effect together with some disease progression parameters discussed in the long term timescale section below. Their publication also contains concentration time curves for FPG, FSI, and HbA_{1c} after application of the three compounds. The model is only a rough description as it covers several doses and does not take into account individual drug concentrations. However, it includes long term effects discussed in the next paragraph.

Hong et al. (2008) investigated a simplified model for metformin using only the FPG differential equation and putting S and FSI into the parameter k_{inFPG}. They modeled the fasting glucose equation as

$$\frac{dFPG}{dt} = k_{\text{inFPG}}\left(1 - \frac{C_p}{IC_{50} + C_p}\right) - FPG * k_{\text{outFPG}} \quad (B.25.36)$$

using the metformin concentration C_P (see *Fig. B.25 5*). The effect of metformin was modeled as inhibition of glu cose production with the half effect concentration IC_{50}. Metformin is orally applied. The PK of metformin was modeled with a 1 compartment model and first order absorption. The posterior Bayes estimates for the PK parameters were used to simulate the metformin concen trations at all times needed for the PK/PD model. k_{in} and k_{out} are proportional with the fasting plasma glucose FPG_0 without medication at steady state as proportional factor:

$$FPG_0 = \frac{k_{\text{inFPG}}}{k_{\text{outFPG}}} \quad (B.25.37)$$

The remaining fit parameters are k_{outFPG} and IC_{50}. Hong et al. succeeded to fit IC_{50} by mixed effects modeling with NONMEM when putting k_{outFPG} to a fixed value of 0.8 h^{-1} giving physiological arguments. Freely fitting gave k_{outFPG} = 0.03 h^{-1}. Other values for k_{outFPG} found in the literature are 0.02 h^{-1}, 0.04 h^{-1}, or 0.06 h^{-1} (Landersdorfer and Jusko 2008). In fact, the fitted k_{outFPG} is essentially indicating the timescale at which the effect of the drug works (15 h 50 h), the other parts of the system are remaining in equilibrium. Modifications of these models were investigated by Lima et al. (see Landersdorfer and Jusko 2008) or Benincosa et al. (see Landersdorfer and Jusko 2008).

Frey et al. (2003) used an effect compartment to model the effect of glicazide. Their model is based on the AUC of glicazide. They also introduced a term for the disease progression discussed in the next paragraph.

B.25.22 Modeling at the Long-Term Timescale

Disease progression is a long term phenomenon in diabetes. Frey et al. (2003) used a linear disease progression model for FPG based on findings of the UK Prospective Diabetes Study (UKPDS)

$$FPG = FBG_B + \alpha * t \qquad (B.25.38)$$

with $\alpha = 0.84 \frac{\text{mmol}}{\text{l} \cdot a}$.

De Winter et al. (2006) used a different disease progression term. Beta cell activity B and insulin sensitivity S are worsening over time:

$$B = \frac{1}{1 + e^{b_0 + r_B * t}} \qquad (B.25.39)$$

$$S = \frac{1}{1 + e^{s_0 + r_s * t}} \qquad (B.25.40)$$

with bases b_0 and s_0 and slopes r_B and r_S in the exponential model. They succeeded to fit these parameters together with EF_B and EF_S (efficacy parameters discussed above).

The degree of glycosylation of hemoglobin, HbA_{1c}, integrates the glucose concentration over weeks up to 3 months. De Winter et al. also introduced a third differential equation in their model dealing with $HbA_{1c.}$

$$\frac{dHbAlc}{dt} = FPG * k_{\text{inHbAlc}} \quad HbAlc * k_{\text{outHbAlc}} \text{ with } \frac{k_{\text{inHbAlc}}}{k_{\text{outHbAlc}}} = HbAlc(0) \,(0 = \text{dosing time}) \qquad (B.25.41)$$

Again, HbA_{1c} was fitted simultaneously with fasting glucose and insulin. Hamren et al. (2008) used a transit compartment approach (Savic et al. 2007) to model the fasting plasma glucose and HbA_{1c} relationship. The time scale discussed in these publications is days, weeks, and months.

B.25.23 Time Variance

Insulin secretion seems to be higher during the night (sleep). Attempts to separate this phenomenon from other factors and to quantify it are reported in the literature (Van Cauter et al. 1991). Circadian rhythm is observed for many endogenous processes. Models based on minutes and hours should take care of this time variance. For models with a timescale of days, the phenomenon will be less or not important.

B.25.24 Discussion

The application of the general features of PK/PD modeling to diabetes shows: Accurate and meaningful PK/PD modeling is improved by incorporating the underlying physiologic and biochemical processes. It is important to have a good understanding of the disease. Diabetes type 1 is the inability to produce insulin. In other words, there is no beta cell function so that HOMA B approaches 0. Therapy is the application of insulin. In diabetes type 2 patients, the beta cell function is reduced (decreasing HOMA B), and the insulin sensitivity is reduced (decreasing HOMA S). Drugs could have several effects: on the insulin production or elimination, on the glucose production (from glycogen), or elimination (uptake by cells, elimination via kidneys). The modeler describes this in the different parts of the differential equations. Diabetes is evolving at the long term. Drugs have short and sometimes medium term effects after single doses and medium or long term effects after repeated doses. It is very important to use the appropriate timescale when developing PK/PD models. Computers have limited resources. Applying an inadequate timescale will increase computing times or can even produce unsolvable problems (Holford et al. 2008). Several glucose insulin models are competing but also complementing each other, showing again that there is no true model. Models are describing different aspects and are applicable under defined conditions. Much depends on the question that should be answered.

MODIFICATIONS OF THE METHOD

PK and PK/PD are parts of Pharmacometrics, a science developing since the last 15 to 20 years. The recent book of Ette and Williams (2007) is an excellent overview on pharmacometrics. They explain: "Pharmacometrics is therefore the science of developing and applying mathematical and statistical methods to (a) characterize, understand, and predict a drugs pharmacokinetic and pharmacodynamic behavior; (b) quantify uncertainty of information about that behavior; (c) rationalize data driven decision making in the drug development process and pharmacotherapy. In effect, pharmacometrics is the science of quantitative pharmacology" (Williams and Ette 2007).

FDA and EMEA issued guidelines on population PK (FDA 1999; EMEA 2007) and other related topics as

pediatrics (EMEA 2006) or QTc interval prolongation (FDA 2005). Scientists from the FDA published a series of articles about the impact of pharmacometrics on their decisions (Gobburu and Marroum 2001; Bhattaram et al. 2005; Bhattaram et al. 2007; Lesko 2007; Powell and Gobburu 2007; Wang et al. 2008). The articles of Bhattaram et al. and Wang et al. explain many situations in selected case studies from various therapeutic areas.

In 2005, the AAPS Journal published a whole series on population PK (AAPS 2005). The textbook of Bonate is also a very practical introduction to pharmacokinetic and pharmacodynamic modeling and simulation (Bonate 2006). Issues to implement pharmacometrics in actual drug development are discussed in the article of Grasela et al. (2005). The article of Barrett et al. is focused on the background and the profession of pharmacometricians and the future of their science (Barrett et al. 2008).

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Human Studies in Clinical Pharmacology

C.1 Methodologies of PD Assessment: Scales

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PURPOSE AND RATIONALE

Measurement and scaling are fundamental processes in the empirical sciences and especially in drug research and development. Both topics are related as well to the description, characterization, or quantification of objects and processes within an experiment or clinical study, as to the outcome of some intervention. Measurement and scaling are two aspects of the quantification of an object or process, the first may be merely regarded as the use of an existing scale to quantify the object, the latter, scaling, can be regarded as "... the assignment of objects to numbers according to a rule" (Stevens 1951). So we can conclude that measurement is only possible, if some scale is defined. The use of the word "scale" in literature is not unique. In the present paper, we will use *scale* synonymously for a questionnaire or psychological test, regardless of who is responding to it, whether the questionnaire or test is used by experts, physicians, patients, relatives, or caregivers. In this more strict sense, a scale is not just a response scale but a response value resulting from a formal rule. The way many psychometricians are defining the term *scale* includes a concept covered by tasks or questions, as well as a concept of verbalization of the response options that are presented to the respondent, a model for proper quantification of the response, and some evidence on reliability and validity. The scale defined in this sense is the standardized quantification of a response of someone in a well defined test situation (e.g., Lienert and Raatz 1998). We will show that it is of importance to define quantification before data generation, that is, prior to the experiment in order to prevent oneself from serendipity. In social sciences and medicine, a *scale* is an instrument for classifying, ordering, or measuring entities with respect to quantitative attributes or traits. The definition of exact standards of measurement is related to units that refer to specific conditions and quantitative attributes. The natural sciences are using the metric international system of measurements (système international d'unités, SI), in which scientifically, some quantities are designated as the fundamental units. The first fundamental units, referring to specific empirical conditions and quantitative attributes, were:

- Meter (m) SI unit of length
- Second (s) SI unit of time
- Kilogram (kg) SI unit of mass
- Kelvin (K) SI unit of temperature
- In 1971, the last of today's accepted seven basic units was Mol (mol), the SI unit of amount of substance

from which all other needed units can be derived. In social science and clinical medicine, units of measurement are not yet standardized. The importance of a unified system may be demonstrated by the failure of the NASA Mars Climate Orbiter, which was destroyed on a mission to the Mars in September 1999 instead of entering the planet's orbit, due to miscommunications about the value of forces: the computer programs used different units of measurement (ftp://ftp.hq.nasa.gov/pub/pao/reports/1999/MCO report.pdf.). This is not the only example of erroneous transfer of results from one system to another, and we cannot exclude similar shortcomings in the research and development of new drugs especially when relating data between experimental studies and within and between research programs.

Therefore, the measurement concept in drug research and development is of crucial importance right from the beginning of the search for a new substance or the modification of a known drug through the assessment of patient's benefit after approval by authorized bodies.

The measurement of hypothesized effect as well as the confirmation of known effects of a substance or compound needs a theory based quantification strategy to make the hypothesis testable and enable new hypotheses. There are two major strategic mistakes that should be avoided from the perspective of methodology: usage of measurement tools of unknown validity or reliability in explorative or pivotal clinical trials and usage of well known measurement instruments in the wrong experimental setting or populations.

In clinical trials, scales may either be used as diagnostic tools for the classification or severity grading of patients,

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and as criteria for selection of patients, or as outcome variables. In some justified case, scales may be used for both diagnostic purposes and outcome variables. In case of diagnostic tools it is important that the scale has sufficient content and construct validity to cover essential aspects of the target disease, symptom, or syndrome. These criteria are prerequisites for the correct selection of valid samples from the target population for the clinical drug development plan. The observer rated scales, in contrast to the patient rated scales, sometimes are available without explicit recommendations on who should use them for rating (expert, physician, medicinal personal, etc.). It is mandatory to define the accountable respondent in the study protocol and to make sure that this rater will be trained for the correct use of the scale throughout the study.

Scales that will be used as outcome variables should be reliable and valid in a way that they cover important aspects of the symptoms and signs associated with the target disease or syndrome. They should further be sensitive to drug induced effects.

In a draft guideline on patient reported outcome (PRO) measures, the FDA stated that these measurement tools are of increasing importance in drug development.

> Self-completed questionnaires that are given directly to patients without the intervention of clinicians are often preferable to the clinician-administered interview and rating. Self-completed questionnaires capture directly the patient's perceived response to treatment, without a third party's interpretation, and may be more reliable than observer-reported measures because they are not affected by interobserver variability (FDA 2006).

There are various possible concepts of capturing the response to treatment in a PRO. It will very much depend on the target of the clinical program. A concept of PRO measures may be, for example, one of the following:

- Discrete symptoms or signs, for example, sensation of pain and frequency of seizures
- Overall condition, for example, depressiveness, complaint of asthma, and urinary incontinence
- Feelings about the health condition, for example, worry about getting worse and avoiding crowded places
- Feelings about the treatment, for example, feeling the treatment is effective and feeling relief of depressive mood since the start of treatment
- General assessments, for example, improvement in physical functioning, treatment satisfaction, and overall quality of life ratings
- Specific assessments, for example, decreased pain intensity, how bothersome the symptoms are, and health related quality of life (HRQL) ratings

Clinicians have recognized that understanding the patient's perspective on the impact of disease and treatment on functioning and well being is important for pharmaceutical, biologic, and medical device product development and evaluation. Pharmaceutical companies are increasingly incorporating health related quality of life (HRQL) and other concepts of patient reported outcome instruments into clinical trial programs for new drugs with the expectation that these outcomes will help inform physicians and patients on the beneficial effects of these treatments (e.g., Wilke et al. 2004). PROs may be further useful in differentiating the patient benefits among competing products with similar clinical efficacy and translating clinical effects into outcomes more meaningful to patients, their relatives, and their treating physicians. For a first classification of the various scales, see *Table C.1 1*.

Technically speaking, a diagnostic scale may be constructed as a questionnaire or checklist asking for a step by step response from one item to the next or it may be used as a guidance for the experienced physician to conduct a patient interview (e.g., Hamilton Depression Scale). Diagnostic scales should reflect relevant diagnostic criteria in DSM IV and correlate with criteria from structured or semi structured interviews for DSM IV or ICD 10 (http://www.who.int/classifications/icd/en/) diagnoses. The ongoing discussions about the scientific usefulness of diagnostic systems such as DSM IV or ICD 10 (e.g., Kendell and Jablensky, 2003) may be taken into account. Both systems may not provide distinct classification in some disciplines, for instance, in psychiatry, that cannot always be transcribed into neurobiological pathways and genetic entities.

We want to emphasize the necessity to make sure that the physicians or experts have been individually trained in the use of the diagnostic scales. Rater training should be an integrated part of any clinical explorative and pivotal trial to help to guarantee valid diagnosis and reliable selection of study populations in accordance to the study protocol. For outcome variables, there should be data available that show relevant correlations to diagnostic criteria in DSM IV or ICD 10. The method of rating in questionnaires intended to deliver values as outcome variables may ask for direct judgment (e.g., the treatment helped me: Yes/No), for comparative judgment within the measurement situation (e.g., I am rather tired neither nor I am rather attentive), or comparative judgment between measurement situations. (My health status improved since

Table C.1-1

Taxonomy of observer- and patient-rated scales in clinical development of drugs

Scope	Source of information	Content	Purpose/target
Diagnostic tool; entry criteria	Physician/Expert	o Overall health status o Symptoms, signs, or syndrome o Functional status	o Classification o Identification o Ranking
	Patient	o Overall health status o Symptoms/signs, individually or as a syndrome associated with a medical condition o Functional status o Activities of daily living o Perception/worries about health o Health-related quality of life	
	Relative/Caregiver	o Functional status in daily situations o Efforts in caring for the patient/carer burden	
Outcome criterion	Physician/Expert	o Clinical Global Impression of health status, severity of illness, change in condition, therapeutic effect, side effects, etc. (e.g., AMDP&CIPS 1990) o Overall health status o Health-related quality of life status and/or change o Symptoms, signs, or syndrome o Functional status	o Primary efficacy endpoint o Two or more coprimary endpoints o Secondary endpoint(s) o Composite endpoint o Safety and tolerability information
	Patient	o Overall health status or change in status o Symptoms/signs, individually or as a syndrome o Functional status or change o Activities of daily living (status or change) o Perception/worries about health o Patient satisfaction with the treatment and its results (e.g., Asadi-Lari et al. 2004) o Health-related quality of life	
	Relative/caregiver	o Functional status/change in status in daily situations o Carer burden (status or change)	

the last time when I was in the hospital: Yes/No.) The level of measurement, depending on the definition of response options, has several implications to scale trans formation, to statistical analysis, and in conclusions from the studies. Some of these will be discussed in the follow ing sections.

PROCEDURE

Scaling is the branch of measurement that involves the construction of an instrument that associates quali tative constructs with quantitative metric units. Scaling evolved out of efforts mostly in psychology and edu cation to measure constructs like intelligence, motiva tion, authoritarianism, self esteem, and so on. In many ways, scaling remains a mostly misunderstood aspects of social research measurement. Remarkably it attempts to do one of the most difficult of research tasks, measure abstract concepts, which have no obvious empirical conditions.

In this text, we define *item* of a test or questionnaire to be a question, a task or any expression which is combined with a response option of a specific format. The response option may be binary, multicategorical, ordered categori cal, or a response line with one or more anchors (e.g., visual analog scale, VAS). The rating of the expression may in relation to the question be

- Direct (e.g., are you satisfied with the results of this treatment: Yes/No)
- Comparative (e.g., my headache is much better than before treatment)
- Magnitude (any frequency or intensity rating, e.g., for pain)

According to S.S. Stevens (1951), measurements in science are generally on four levels (see *Table C.1 2*). The taxonomy may be extended by a fifth measurement level, the absolute scale (e.g., number of objects, probabilities).

Often people do not understand what scaling is. It is important to distinguish between a scale and a response format. A response format is the way someone collects responses from people on an instrument by using a dichotomous response options like Agree/Disagree, or Yes/No. Or, the researcher might use an ordered categor ical or interval response categories like a 1 5 rating. Attaching a response format to an object or statement is not scaling. Scaling involves procedures independently done of the respondent so that one can come up with a numerical value for the object. In true scaling research, we use a scaling procedure to develop our instrument (scale) and we also use a response scale to collect the responses from participants. But just assigning a 1 5 response scale for an item is not scaling!

Scales are generally divided into two broad categories: unidimensional and multidimensional. The unidimen sional scaling methods were developed in the first half of the twentieth century and some of them have been named after their inventor. Among the various scaling methods, the *psychophysical scaling* has a separate theoretical back ground. Psychophysics is a psychological discipline that has its roots back in the work of G. T. Fechner, E. H. Weber, and Wilhelm Wundt, founder of the first laboratory for experimental psychological at the University of Leipzig, 1879. Psychophysics deals with the relationship between physical stimuli and subjective correlates, in general the percept. Psychophysicists employ experimental stimuli that can be objectively measured, such as pure tones varying in intensity, or lights varying in luminance or frequency. All the traditional senses have been studied including the enteric perception, and the sense of time. Regardless of the sensory domain, three main procedures of investigation have been used: the definition of absolute threshold, discrimination threshold, and various scaling procedures using constant or systematically varied stimuli characteristics. The absolute threshold is the level of inten sity or frequency at which the subject can just detect the

Table C.1-2

Levels of measurement

Level	Statistical description	Example	Test statistics
Nominal (or categorical)	Modus, frequency	Gender	Nonparametric tests
Ordinal	Median, percentile	Degree of agreement	
Interval	Arithmetic mean, standard deviation	Body temperature in Celsius or Fahrenheit	
Ratio	Geometric mean	Age, body weight	parametric tests

presence of the signal. The difference threshold is defined as the magnitude of difference between two stimuli of differing intensity or frequency that the subject is able to detect. The just noticeable difference, also named difference limen (DL), is the difference in stimuli properties the subject notices with a defined proportion of the cases (mostly $p = 0.50$).

The determination of critical flicker fusion frequency and that of critical fusion frequency are examples of psychophysical measurement that have often been used and are still in use in psychopharmacology.

The *visual analog scale* (VAS) is in most cases a 100 mm horizontal line with two named poles or verbal anchors, like "not at all" and "very much" or similar wordings. The typical use of VAS is, for example, the following:

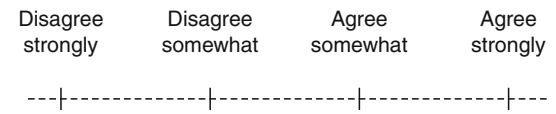
How severe was your pain today? Please place a mark on the line below to indicate how severe your pain was!

When using VAS as a measurement instrument one tries to quantify a sensation, a trait or any other entity on a ratio scale level assuming further, that the entity's characteristics is ranging across a continuum from "none" to "very severe" or "very intensive" or within a similar concept. The assessment is highly subjective, in a practical way "imprecise" in regard to the positioning of the tic mark. VAS may be of value when looking at the intraindividual change of the entity, but they are most likely of less value for comparing groups. It has been argued that a VAS is trying to deliver interval or even ratio measures. But there are no convincing arguments for the values being more than ordinal data. Despite the simplicity and "face validity" of this measurement method, data handling and interpretation have to be done with caution.

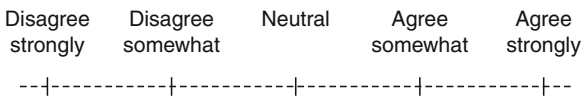
Thurstone Scaling. Thurstone was one of the first scaling theorists. He invented three different methods for developing a unidimensional scale: the method of equal appearing intervals, the method of successive intervals, and the method of paired comparisons. The three methods differed in how the scale values for items are constructed, but in all three cases, the resulting scale is rated the same way by respondents. The method of equal appearing intervals is explained as it is the easiest method. Because this is a unidimensional scaling method, the concept one is trying to scale is reasonably thought of as one dimensional. When starting the procedure, the description of this concept should be as clear as possible so that the person(s) who are going to create the statements (items) have a clear idea of what the investigator is trying to measure. Next, the developer will ask people to generate similarly worded statements about the concept. Then the participants are asked to rate each statement on an 11 point response scale with a predefined criterion, like how favorable the statement appears to them with regard to the construct. Next the ratings will be analyzed. For each statement, one needs to compute the median and the Interquartile Range. The median is the value above and below which 50% of the ratings fall. The first quartile (Q1) is the value below which 25% of the cases. The median is the 50th percentile. The third quartile, Q3, is the 75th percentile. The Interquartile Range is the difference between third and first quartile, or Q3 Q1. To facilitate the final selection of items for the scale, one might write the parameters into a table, may be we want to sort the statements in the table of medians and Interquartile Range in ascending order by median and, within that, in descending order by Interquartile Range.

For the final scale, one should select statements that are equally distributed across the range of medians. Within each median value, try to select the statement that has the smallest Interquartile Range. Once the items for the final scale are selected, one should test the new scale in an independent sample of eligible patients or members of the target population.

Likert scaling is a unidimensional scaling like Thurstone scaling. The term "Likert scale" is used in various ways in literature. Sometimes the term actually seems to describe Likert or Likert like items. Likert scales are the four to nine point scales much used in clinical trials and in many other fields of research. The scale is often used as a semantic differential, that is, with a given statement the response options correspond to, for example, agree strongly and disagree strongly. Example:



The five point scale is probably the most commonly used version, which includes in comparison to the four point scale a midpoint "neutral."



The assumption with the VAS as well as behind numeric rating scales, including Likert scale, is that the

geometrical distance between markers on a line or of tic boxes in combination with verbal expressions and/or numbers are homologous. This is one of the reasons why the graphical layout of those items should guarantee equally spaced elements of the response format.

After definition of the concept, verbalization of the statements, tests of the draft version and confirming those items that form a reasonable scale, the final score for the respondent on the scale is the sum of their ratings for all of the items (sometimes called a "summated" scale). On some scales, one will have items that are reversed in their meaning from the overall direction of the scale. To cumulate item scores, one will have to inverse the response score of this item. Likert scales, like other item scales may be problematic in comparisons across groups. The expectation of a researcher would normally be that the mean response will vary across treatment groups. The problem is that in many cases the variances will also differ. The variance has to be less at the ends of the scale, as there is no alternative response to one side of the endpoint. For example, with a five point scale, the variance would be expected to be largest at the midpoint, 3, and smallest at the extremes, 1 and 5. A possible solution to this problem might be to use the arc sine square root transformation of the scores. The responses are divided by 5, to yield a number between 0 and 1. The square root is taken (still between 0 and 1). The angle whose trigonometric *sine* is that number is the transformed response and can be used for further statistical analysis.

Osgood et al. (1957)'s *semantic differential* was designed to measure the connotative meaning of concepts. The respondent is asked to choose where his or her position lies on a scale between two bipolar adjectives (e.g., "Adequate Inadequate," or "Valuable Worthless").

Sometimes it may be difficult to find properly defined poles of the differential! Therefore, many researchers prefer unipolar item scales (e.g., mood scales and multi item pain scales).

Guttmann Scaling. This method is also known as cumulative scaling. Like with the other examples of item response scaling, this method starts with the definition of the construct of interest, the generation of a large set of statements that are judged by some experts or members of the target group how favorable (Yes/No rating) the expressions are in regard to the construct. Following this, we construct a matrix or table that shows the responses of all the respondents on all of the items. We then sort this matrix so that respondents who agree with more statements are listed at the top and those agreeing with fewer are at the bottom of the matrix. For respondents with the same number of agreements, we sort the statements from left to right from those that most agreed to those that fewest agreed to. In case of only a few items we can easily examine this matrix. In larger item sets, the method of choice may be the *scalogram analysis* to determine the subsets of items from our pool that best approximate the cumulative property. After review of these items we select our final scale elements. In many cases, there is no perfect cumulative scale and the researcher will have to test for goodness of fit. These statistics will estimate a scale score for each item that are used in the calculation of a respondent's score.

In the late 1950s and early 1960s, measurement theorists developed more advanced techniques for creating multidimensional scales. *Multidimensional Scaling* (*MDS*) is a data reduction technology normally using the direct similarity or dissimilarity matrix. MDS fits a set of points in a space such that the distances between the points are as closely as possible to a given set of dissimilarities between a set of objects, for example, ratings. MDS does not make distribution assumptions necessary. As MDS is a spatial method there are metric assumptions, for example, the distance from some point A to B shall be the same as from B to A. This might sound strange to the reader, but in some situations two points A and B may not be bidirectional equidistant. Consider for instance the distance between home and work, which may be due to specific situations in the morning and the evening not of identical length. If the equidistance assumption cannot be fulfilled, one should not use MDS. Anyway, the question whether to use or construct one or multidimensional scales depends very much on the concept that has to be defined in the clinical development plan. If the construct is unidimensional we also will use unidimensional scales, if the construct is of known multidimensionality, one should consider multidimensional scales or several one dimensional scales. Both ways will offer their special advantages with regard to the upcoming point of decision on the clinical trial outcome results.

The application of mathematical models to response data from questionnaires and educational and psychological tests is discussed and described in test theory. Test theory is a body of theory that offers mathematical modeling of response data in order to predict, describe, or estimate a person's trait, ability, attitude, or any other construct. There are in general two different test theories, which we have to deal with in order to understand the steps from item pool generation to item construction, definition of adequate response options to testing the first draft questionnaire, and confirmation of the final measurement instrument. The methods are also very

helpful for the reevaluation of known scales and items. The two main test theories are:

1. *Classical Test Theory*, which assumes that for each person we have a true score of some ability or character istic, T, which would be obtained if there were no errors in our measurement. Because instruments used for measure ment (and sometimes the users of those too) are imper fect, the score that is observed for each entity, for example, a person's ability, most times is different from the person's true abilities. It is concluded that the difference between the true score and the observed score is the result of measurement errors. Classical test theory is dealing with the relation of the true score T, the error E, and the observed score X. Formally:

$X = T + E.$

Further assumption: True score T and error E are not correlated, $\rho(T,E) = 0$.

The most important concept is that of reliability. The reliability of the observed test scores X, denoted as $\rho^2(X, T)$, is defined as the ratio of true score variance $\sigma^2 T$ to the observed score variance $\sigma^2 X$. Because it can be shown, that the variance of the observed scores to equal the sum of the variance of true scores and the variance of error scores, it follows that

$$\rho^2(X, T) = \frac{\sigma^2 T}{\sigma^2 X} = \frac{\sigma^2 T}{\sigma^2 T + \sigma^2 E}.$$

The reliability of test scores becomes higher as the proportion of error variance in the test scores becomes lower and vice versa. The reliability is equal to the pro portion of the variance in the test scores that could be explained if we knew the true scores. The square root of the reliability is the correlation between true and observed scores.

2. *Item Response Theory* (*IRT*), also known as latent trait theory is a set of probabilistic models and the appli cation of mathematical models to response data from tests and questionnaires measuring abilities, characteristics, or other variables. IRT models apply functions to quantify the probability of a discrete outcome, such as a correct response to an item, in terms of *person and item parame ters* (for further details see e.g., Rost 2004).

Person parameters may, for example, represent the cognitive ability of a patient or the severity of a patient's symptom. Item parameters may include item difficulty (location), discrimination (slope), and random guessing (lower asymptote). IRT do not only apply to discrete binary data, but may also deal with ordered categorical data to indicate level of agreement, etc. One of the pur poses of IRT is to provide a framework for evaluating how well assessments work, and how well individual questions on assessments work. In drug development programs, IRT may be very helpful to collect and construct items and maintain item pools for clinical trials in a defined indica tion and develop or adopt new scales within the concep tual framework of the clinical program.

The performance of an item in a test is described by the *item characteristic curve* (ICC). The curve gives the prob ability that a person with a given ability level will answer the item correctly, or give an answer in line with the expectations according to the construct definition. Per sons with lower ability ($\theta < 0.0$) have less of a chance to answer correctly or agree on a Yes/No item, while persons with high ability are very likely to answer correctly.

IRT models can be divided into two families: one dimensional and multidimensional models. One dimensional models require a single trait (e.g., ability) dimension θ. Multidimensional IRT models analyze response data arising from multiple traits. However, because of the greatly increased complexity with increas ing number of included traits, the majority of IRT research and applications utilize a one dimensional model. The models are further on named according to the number of parameters estimated. The one parameter logistic model (1PL) assumes that there is only minimal guessing by the respondent and that items have equivalent discrim inations, so that items can be described by a single param eter (b_i). The 1PL uses only b_i, the 2PL uses b_i and the parameter a_i, and the 3PL uses b_i, a_i, and item parameter c_i.

A given model describes the probability of a correct response (or a Yes/No response option where one is defined as correct and the other as incorrect in the frame of some syndrome or disease theory) to the item as a function of a *person parameter*, which is in the case of multidimensional item response theory, a vector of person parameters. For simplicity we will stay with the model of only one person parameter. The probability of a correct response depends on one or more item parameters for the item response function (IRF). For example, in the three parameter logistic (3PL) model, the probability of a correct response to an item i is given by:

$$p_i(\theta) = c_i + \frac{(1 - c_i)}{1 + e^{\ ai(\theta\ \ bi)}}$$

where θ signifies the person parameter; e is the constant 2.718; and a_i, b_i, and c_i are the item parameters.

As you can see from Fig. C.1 1, the item parameters simply determine the shape of the IRF. The figure depicts an example of the 3PL model of the ICC with an explana tion of the parameters. The parameter b_i represents the item location (item difficulty). It is over the point on θ where the IRF has its maximum slope. The simulated

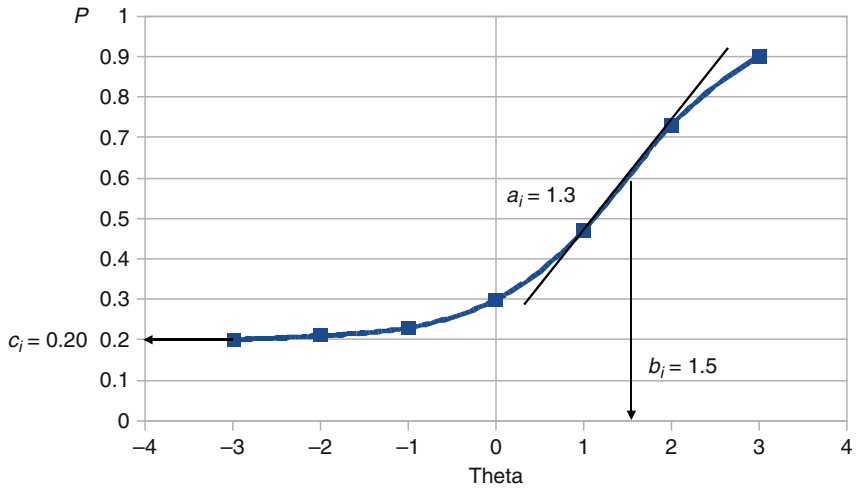


Figure C.1-1

IRF for hypothetic item data with item parameters $b_i = 1.5$, $a_i = 1.3$, and $c_i = 0.2$

example item is of medium to higher difficulty, since $b_i = 1.3$, which is to the right of the center of the distribu tion. The item parameter a_i represents the (rather good) discrimination, the degree to which the item discriminates between persons in different regions on the latent contin uum. This item parameter characterizes the slope of the IRF where the slope is at its maximum. The item param eter $c_i = 0.20$ indicating that persons with low ability may endorse correct response.

One of the major contributions of item response the ory is the extension of the concept of reliability. Tradition ally, reliability refers to the precision of measurement (i.e., the degree to which measurement is free of error). And traditionally, it is measured using a single index, such as the ratio of true and observed score variance (see above). This index is helpful in characterizing an average reliabil ity. But IRT makes it clear that precision is not uniform across the entire range of test scores. Scores at the edges of the test score range generally have more error associated with them than scores closer to the middle of the range.

Item response theory elaborated the concept of item and test information to replace reliability. Information is also a function of the model parameters. According to Fisher information theory (named after the inventor and famous statistician R.A. Fisher), the item information supplied in the case of the Rasch model (Rasch 1960) for dichotomous response data is simply the probability of a correct response multiplied by the probability of an incorrect response:

$$I(\theta) = p_i(\theta)q_i(\theta).$$

The standard error (SE) is the reciprocal of the test information at a given trait level:

$$SE(\theta) = \frac{1}{\sqrt{I(\theta)}}.$$

Intuitively, we can agree to the conclusion that more information implies less error of measurement.

After this short excursion into some basics of test theory, we may agree that measuring is not just assigning numbers to empirical objects or events (see the straight forward definition of S.S. Stevens 1951). In the classical definition, measurement is the estimation of ratios of quantities. Quantity and measurement are mutually defined: quantitative attributes are those, which make measuring possible. In terms of representational theory, numbers are assigned based on similarities between the structure of number systems and the structure of qualita tive systems. A property is quantitative if such structural similarities can be established. As we can see, this defini tion is much stronger than the definition of S.S. Stevens (1951).

EVALUATION

When searching and selecting suitable scales for the clinical development plan, essential information about the scale characteristics and properties are of major importance.

When starting drug development in a known or new indication, an extensive literature search for measurement

models and scales in the target area should be performed and evaluated with regard to:

- Completeness and representativeness with regard to the concept of interest
- Relation to medical and mathematical measurement models available
- Published indices or data for at least scale reliability and validity
- Sufficient evidence on satisfying scale properties
- Evidence for validated linguistic and/or cultural versions, as in many cases pharmacological drug development will increasingly often be performed in multicultural, multilingual studies

Further we should find instructions for the standardized application of the test or questionnaire, procedures of training raters for the proper use of those instruments are as important as the mostly cited scale properties reliability and validity. Among others we need information about the way the items shall be presented, the scoring rule, for which experimental conditions and in which population the indices and coefficients are valid. It is almost always necessary to consider some kind of reevaluation of the selected scale(s) for the own new project.

The necessity of a conceptual framework before starting the clinical development program of a drug is known for decades in the field of pharmacodynamic research and development. It has been explicitly named in the now finalized guideline for patient reported out come measures (FDA 2009). The conceptual framework should combine three major concepts:

1. Treatment or interventional concept
2. Target population for the intended treatment
3. Measurement concept (including the endpoint model)

Figure C.1 2 gives an overview of some important milestones in the process from the development of a concept to the final definition of the endpoint model and the entrance of the findings in a clinical study protocol.

CRITICAL ASSESSMENT OF THE METHOD

The selection of scales for a drug development program should always be guided by the program goals and claims that are intended to reach for. Sometimes the decision to select and use known scales may be influenced by their

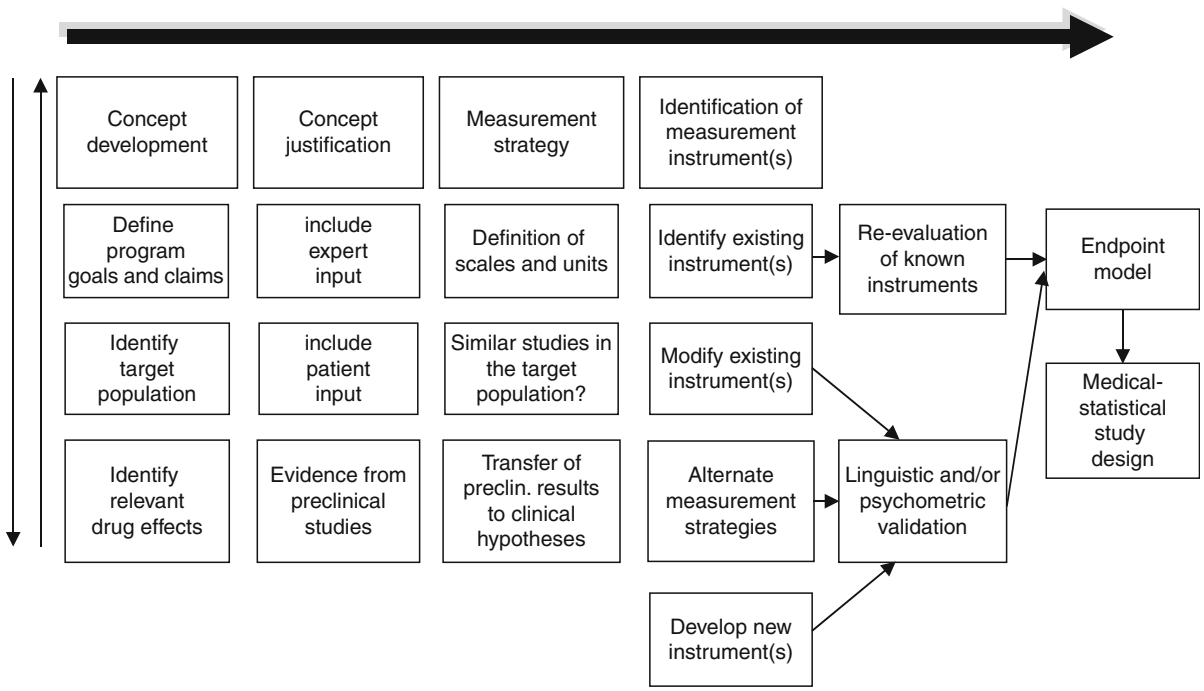


Figure C.1-2

Critical path description from concept development to endpoint model. The *arrows* shall indicate the flow of information necessary during the process

availability, their application in similar clinical programs or clinical studies, and published data. In case of diagnos tic scales, this strategy may be appropriate as long as the drug development program is within a well known tradi tional indication and patient population. Drug develop ment in special populations, for example, for children or very old persons, will, in many cases, make modification and/or development of diagnostic scales, scales for inclu sion criteria assessments and above all, of outcome mea sures necessary. The following criteria in case of selection of known scales may be helpful for the decision. For what purpose and how was the scale developed to care for sufficient content validity? Does the item concept (content and response scaling) match the intended construct to be investigated? Is the scale a single item or multi item scale and does it appropriately fit the one dimensional or multidimensional construct? In the following paragraphs, the reader will find some information about item and scale development methods which might be helpful to answer these questions during search, selection, and decision on eligible scales for the planned clinical program.

Item analysis and construction. The item may be seen as the unit of a scale. Wording, layout, and arrangement of response options are of crucial importance to direct the respondent's attention to the task or question and to encourage to an open unbiased response to it. Therefore, the wording of the statements or expressions should always be comprehensible and readable to the intended respondent. In case of diagnostic scales, the items must be coherent to relevant criteria of the disease or the syn drome. Patient reposted diagnostic scales should avoid as much as possible technical or medical terms and replace them by more daily speech terms. The input of patients from the target population is mandatory, where structured interviews may be more informative than unstructured interviews. With regard to the response options and the response scaling, the response scale should reflect both temporal properties of the disease or symptom(s) as well as the temporal aspects of the hypothesized time and mode of action of the drug, all of these should be ade quately reflected in the study design and protocol. Typical response options used in clinical trials are intensity or frequency ratings related to "yesterday," "this morning," "the last week," "the last month," "since the last visit," and others. The selection of this kind of temporal anchoring of assessments must be in line with the nature of the symp tom or the disease and must consider reasonable memo rization of the construct, symptom, or process. Answers about the past are less reliable when they are very specific, because the questions may exceed the subjects' capacity to remember accurately. This might invite the respondent to random guessing or cause responses in direction of a presumed desired way. Items with comparative judg ments, for example, How is your pain today: "no pain" "pain as usual" "pain more severe than usual" are obsolete because of the undefined anchor "usual."

Implementation of a scale into the clinical development plan and single studies. There is a common misconception that if someone adopts a validated instrument to a clinical study or evaluation program, he/she does not need to check the reliability and validity of the instrument with the own population, study design and data, and make modifications if necessary. The modified instrument has to be tested again for its scale properties in a separate sample from the target population before using it in explorative or pivotal clinical trials. If the investigated symptom has temporal properties in the way that its appearance or intensity is changing during daytime, or its appearance or intensity may vary from day to day, the investigator might consider the implementation of a patient's diary in the study design. FDA supports the use of diaries where appropriate, but "If a patient diary or some other form of unsupervised data entry is used, the FDA plans to review the protocol to determine what measures are taken to ensure that patients make entries according to the study design and not, for example, just before a clinic visit when their reports will be collected" (Federal Register Vol. 71, Nr. 23, pp. 10; 334 337). In recent years, systems for use of electronic questionnaires, PRO and diaries increasingly frequently offered by spe cialized companies and used by sponsors to facilitate data selection. The eventual advantages of the administration of well controlled electronic questionnaires may be the following (R. Piazza and J. Dustin, presentation at the invivodata ePRO conference, Florida, April 2008):

- Increased accuracy of data
  - Prevents errors
  - Ensures required data is collected
  - No illegible data
- Increased compliance
  - Clear insight into subject's behavior regarding input of data
- Increased visibility into data
  - Rapid and continuous access to data
  - Improved ability to manage data
- Increased study power
  - More data is "valid/usable," potentially fewer sub jects needed

Validation, reliability, and compliance with Part 11 electronic data requirements (http://www.fda.gov/ora/compliance ref/Part11/) are important issues that will be

raised by the FDA, and the sponsor will have to successfully address them. Are paper questionnaires and electronic questionnaires equivalent? The answer is most likely no, because any modification in a scale (including method of administration) will certainly need some additional validity evidence (see FDA 2006, draft PRO guideline).

Scale properties. A questionnaire or test as an instrument to measure entities or a construct should have specific properties in order to be accepted as a scale. The instrument should be standardized in the way and the circumstances it shall be presented and used, the evaluation of the ratings shall be defined and objective in a way that the results of this evaluation is independent of the evaluating person, and there should be important data available on its reliability and validity. In psychology, validity has two distinct fields of application. The first involves test validity, a concept that has evolved in psychometrics, dealing with theory and technique of psychological and educational measurement "... refers to the degree to which evidence and theory support the interpretations of test scores entailed by proposed uses of tests" (AERA et al. 1999) The second involves study design, pointing to the fact that different types of studies are subject to different types of bias. For example, recall bias is likely to occur in cross sectional or case control studies where subjects are asked to recall exposure to life events or other special events. Subjects with the relevant condition (e.g., the degree of disease or syndrome to be investigated) may be more likely to recall relevant events that they had experienced than subjects who do not have the condition or have a lower degree of it.

In contrast to test validity, assessment of the validity of a research design does not involve data collection or statistical analysis but rather evaluation of the design in relation to the desired conclusion on the basis of prevailing standards and theory of research design.

Test validity, on which we are focusing here, can be assessed in a number of ways. Test validation typically involves more than one type of evidence in support of the validity of a measurement method (e.g., structured interview, questionnaire, test, etc.). The various types of validity include content related, construct related, and criterion related evidence with the subtypes concurrent and predictive validity according to the timing of the data collection. In the following we will present and discuss some of the various aspects.

Construct validity evidence involves the empirical and theoretical support for the interpretation of the construct. A good construct validity has a theoretical basis which is translated through clear operational definitions involving measurable indicators. Construct validity evidence includes statistical analyses of the internal structure of the test including the relationships between responses to different test items. They also include relationships between the test and measures of other constructs. Researchers should establish both of the two main types of construct validity, *convergent* and *discriminant*, for their constructs.

Convergent validity is assessed by the correlation among items that make up the scale or instrument measuring a construct (internal consistency validity), by the correlation of the given scale with measures of the same construct using scales and instruments proposed by other researchers, if appropriate, with already accepted in the field (criterion validity), and by correlation of relationships involving the given scale across samples. *Internal consistency* is one type of convergent validity that seeks to assure there is at least moderate correlation among the indicators for a concept. Cronbach Coefficient Alpha is commonly used to establish internal consistency (as well as an aspect of reliability and for evidence of construct validity) with at least Alpha of 0.60 considered acceptable for exploratory purposes, Alpha of 0.70 considered adequate for confirmatory purposes, and Alpha of 0.80 considered good for confirmatory purposes.

Simple factor structure is another test of internal consistency, seeking to demonstrate for a valid scale that indicator items for a given construct load unambiguously on their own factor. This tests both convergent and discriminant validity.

Rasch models, one parameter logistic models (1PL), are also internal consistency tests used in item response theory for binary items. Rasch models for polytomous items are also available. They are generalizations of 1PL Rasch model. Like Guttman scales, Rasch models test that the included items which are measuring a construct will form an ordered relationship (see Rasch 1960). A set of items may have ordered internal consistency even though they do not highly correlate (additive internal consistency as tested by Cronbach Alpha or factor structure). Ordered internal consistency reflects the difficulty factor, which means that correct response to a more difficult item will predict the response on less difficult items but not vice versa.

When factor analysis is used to validate the inclusion of a set of indicator variables in the scale for a construct, the researcher is assuming a linear, additive model. Linearity is assumed as part of correlation, which is the basis for clustering indicator variables into factors. With additivity is meant that items will be judged to be internally consistent if they are mutually highly correlated. However,

items may lack high intercorrelation but have a strong ordered relationship. For this reason, many researchers prefer to use a Rasch model for scale construction, in preference to additive models like Cronbach Alpha or factor analysis.

Discriminant validity, the second major type of con struct validity refers to the principle that the indicators for different constructs should not be highly correlated. Discriminant validity analysis refers to testing statistically whether two constructs differ as opposed to testing con vergent validity by measuring the internal consistency within one construct. In constructing scales, some researchers reject an indicator if it correlates more highly with a construct different from the one which was intended to be measured. Some researchers use $r = 0.85$ as a rule of thumb cutoff value for this assessment. Con struct validity is not distinct from the support for the substantive theory of the construct that the test is designed to measure, which is an issue for measurement models in drug development. Experiments designed to reveal aspects of the causal role of the construct may contribute to construct validity.

Content validity evidence involves the degree to which the content of the test matches a content domain associ ated with the construct. Content related evidence typi cally involves subject matter experts evaluating test items against the test specifications. Content validity is also called *face validity*, and has to do with items seeming to measure what they claim to do. In content validity, one is also concerned with whether the items measure the full domain implied by their label. Failure of the researcher to establish credible content validity may easily lead to rejec tion of his or her findings. One should consider the use of surveys of panels of content experts and/or additional focus groups of representative subjects or not only in case of PROs, asking patients, are ways in which content validity may be established.

It is a challenging task to make sure that the measures operationalizing the entity by experts or common sense sufficiently address the concept of the later scale. There could also be a *naming fallacy*. Indicator items may display construct validity, yet the label attached to the concept may be inappropriate.

Criterion validity evidence involves the correlation between the test and a criterion variable (or several vari ables) taken as representative of the construct. The corre lation with known and accepted standard measures or criteria is of interest. Ideally these criteria are direct objec tive measures of what is being measured. Where direct objective measures are unavailable, the criteria may be merely closely associated. For example, employee selection tests are often validated against measures of job perfor mance. If the test data and criterion data are collected at the same time, this is referred to as *concurrent validity* evidence. If the test data is collected first in order to predict criterion data which is collected at a later point in time, then this is referred to as *predictive validity*.

Reliability. According to classical test theory reliability is not a fixed property of a test, but a property of test scores that is relative to a particular population. A reliability coefficient is computed for a sample. This is because test scores will not be equally reliable in every population or even every sample. For instance, as is the case for any correlation, the reliability of test scores will be lowered by restriction of range. Also note that test scores are perfectly unreliable for any given individual *i*, because, as has been noted above, the true score is a constant at the level of the individual, which implies it has zero variance, so that the ratio of true score variance to observed score variance, and hence reliability, is zero. The reason for this is that, in the classical test theory model, all observed variability in *i*'s scores is random error by definition (see above). Classical test theory is relevant only at the level of populations and samples, not at the level of individuals. Reliability cannot be estimated directly since that would require one to know the true scores, which according to classical test theory is impossible. Estimates of reliability can be obtained by various means. However, there is no one standard method. The method of assessing reliability must reflect the medical use of the instruments. Some of the statistical and psychometrical methods are as follows.

Frequently the *p* value is cited as evidence of reliabil ity: a significant Pearson correlation means a correlation significantly different from 0. But one should scatterplot the data and check for biased values. The concordance correlation coefficient addresses the concept of agreement. However, it can be misleading in that it summarizes the fit around the line of identity and therefore, like the Pearson correlation, a value close to one may not denote lack of variability around the line.

If a cut point is to be used to classify patients, agree ment of the classifications could be examined, using Kappa indices. Kappa is commonly used to measure reliability or agreement for nominal or ordinal variables; however, it also has limitations. If one method is a gold standard then predictivity (sensitivity, specificity, or allied statistics) should be determined. Receiver operating characteristic (ROC) type analyses have much to offer, it can be argued that in paying attention to the misclassification, rather than the consequences of misclassification, there may not result an appropriate comparison (Obuchowski, 2005).

The intraclass correlation coefficient or its analogs (Bland and Altman, 1996). However, the value of this method depends heavily on the sample used, and without repeated measurements, estimates of precision are impossible.

The following are the conventional views of the various concepts of reliability of test scores (for details, see Aera et al. 1999):

Stability of the response. The same form of a test on two or more separate occasions to the same group of examinees (test–retest). On many occasions, this approach is not practical because repeated measurements are likely to change the rater (patient or observer). For example, the rater will adapt the test format and thus tend to score higher in later tests. A careful implementation of the test–retest approach is recommended. If appropriate and possible, parallel test forms of the scale will of great help in case of repeated measurement, which is the rule in most clinical trials, to control for instance memory and/or training bias. Extensive training of observers before entering the clinical trial is another method of reducing this kind of potential bias.

An aspect of reliability of special interest in drug development is the instrument's *sensitivity to change (responsiveness)*. In more general terms it is that the measured scores are changing in direct correspondence to actual changes in the entity under treatment. There is a growing recognition that assessing the effect of an intervention should not only focus at the statistical significance of the differences in outcome measures between the experimental and the control group, but should also focus at the relevance or importance of these outcomes. Estimating the magnitude of the difference between change scores in both groups, the difference between mean change scores may be expressed in standard deviation units with the effect size index (ES). One of the possible definitions has been developed by Cohen Unfortunately, there is no agreed standard method for the estimation and the interpretation of the magnitude of intervention related change over time or responsiveness assessed with outcome measures. For further details, see Middel and van Sonderen (2002) who are discussing advantage and limitations of several ES proposals.

Form equivalence is related to two or more different forms of test or questionnaire (sometimes called parallel version) based on the same content and administered in an identical way to the respondent. The presentation of a test (or questionnaire) one time as a paper–pencil test version, the next time as a computer based test version is not regarded as being parallel versions and can not be exchanged in a setting assuming equally valid and reliable. After alternate/parallel forms have been developed, they can be used for different persons or for several measurement occasions with the same person in a trial. This method is for instance very common in educational examinations to prevent communication between participating people. A person who took form A earlier could not share the test items with another person who might take form B later, because the two forms have different items. We should always consider the use of parallel test versions in trials with intraindividual repeated measurements when we cannot exclude considerable training effect or change in the strategy of responding to the items caused by experience with the test.

Internal consistency is defined as the association of responses to a set of questions designed to measure the same concept. It is normally expressed by the coefficient of test scores obtained from a single test or survey. Usually, internal consistency is measured with Cronbach Coefficient Alpha, or its algebraically equivalent, the Kuder–Richardson Formula 20, when the data are dichotomous, or the Spilt–half method based on the assumption that two halves of a test is parallel except for having different variances. Cronbach Alpha, which is the most frequently used and easily available procedure in nearly every commercial, statistical software package, is defined by:

$$\alpha = \frac{n\bar{r}}{(1 + \bar{r}(n-1))}.$$

Here the Coefficient Alpha is based on the average size of item to total score correlations, sometimes named standardized Alpha. One could also use the item to total score covariances that may be more informative when the items have different variances.

To describe the logic of internal consistency more vivid, let us assume patients participating in a postmarketing survey about drug D for the treatment of a symptom S are asked to rate statements about their satisfaction with the treatment. One of the statements is "Drug D helped me very much in getting rid of the symptom." Another statement is "After intake of the drug I frequently experienced unusual headache." A third statement is "If the symptom will come back, I will use drug D again." People who strongly agree with the first statement would most probably agree with the third statement, and vice versa. Patients, who agree with the second statement, will most probably disagree with statement one, and depending on the anticipated need for future treatment, or the availability of alternatives, will more or less disagree with the third statement. If the rating of the statements is patternless high and low among the participants of the survey, the responses are said to be inconsistent. When no pattern can be found in the

patients' responses, probably the test is too "difficult" and patients just guess the answers randomly. Of course, dif ferent conclusions could be drawn from inconsistent results, like, the items may be reworded or items addressing similar aspects of patient's satisfaction may be added to the survey to capture the intended construct in a more reliable way. As we have already seen, internal consistency is a measure based on the correlations (or covariances) between different items or statements on the same questionnaire or test. It measures whether several items that presumably measuring the same construct are producing similar scores. The procedure of Cronbach Alpha is a statistic calculated from the pairwise corre lations between items. The coefficient ranges between 0 and 1. In case where some or many items are negatively correlated with the total score, the coefficient can take on negative values even less than −1.0. One can check the effect of those items by reversing the item scoring and run the procedure again. As a rule of thumb, Alpha of 0.6 0.7 indicates acceptable reliability and Alpha of 0.8 or higher indicates good reliability. High reliabilities (0.95 or higher) are not necessarily desirable, as this indicates that the items may be entirely redundant. The goal in designing a reliable instrument is for scores on similar items to be related (internally consistent), but for each to contribute to some part a unique information.

In 2004, Lee Cronbach, the inventor of Coefficient Alpha as a way of measuring reliability, reviewed the historical development of Alpha: "I no longer regard the formula as the most appropriate way to examine most data. Over the years, my associates and I developed the complex generaliability (G) theory" (Cronbach 2004, p. 403). Discussion of the G theory is beyond the scope of this contribution. Cronbach did not object the use of Coefficient Alpha, but he recommended that researchers should take the following into consideration while employing this approach:

- Standard error of measurement is the most important piece of information to report regarding the instru ment, not a coefficient.
- Independence of sampling.
- Heterogeneity of content.
- How the measurement will be used: Decide whether future uses of the instrument are likely to be exclu sively for absolute decisions, for differential decisions, or both.
- Number of conditions for the test.

When ratings are by an observer rather than the patients themselves, reliability is called intra observer or *intra rater reliability*. The comparison between the rating of several raters on the identical entities (objects, persons, etc.) is called the interobserver or *inter rater reliability*. Statistical methods for measuring agreement between categorical outcomes are well established. Cohen (1960) developed the kappa statistic as an agreement index for two binary variables. It has an appealing interpretation as a measure of chance corrected agreement. Later, Cohen (1968) generalized the original kappa to the weighted kappa coefficient for ordinal discrete outcomes. Since its development, kappa with its extensions (Cohen, 1960, 1968; Fleiss, 1971, 1981, Fleiss and Cohen 1973 and others) have been well studied in the literature and broadly applied in many areas.

As a first summary from the previous presentation and discussion, we may draw the conclusion that although there has been some considerable discussion about the scientific value of validity evidence and the relation between reliability and validity, we should keep to the following message that reliability is a *necessary* but not *sufficient* condition for validity.

MODIFICATION OF THE METHOD

The psychometrical methods of development and evalua tion of measurement tools may be even more useful for drug development if they are integrated in the concepts of treatment, target population, and measurement in an early phase of drug development. They should not be restricted to endpoint definitions for single clinical stud ies. The added value of the methods is among others:

- To develop complex measurement models combining subjective and objective measurements
- To select and evaluate known items and scales
- To construct and develop new items and scales
- To scale values and scores across phase I to phase III studies

The methodology of measuring and scaling human traits, states, expectations, and opinions should be an integrated part of drug development plans, one class of scales, the PRO measures will increasingly contribute to the development of new, effective, and safe medicinal drugs and translate pharmacological and clinical study outcomes into meaningful information for physicians, patients, and their relatives in everyday practice.

We would propose to extent measurement concepts beyond the clinical disease models and related target populations to concepts of preclinical experiments on one side and to cost benefit quantifications on the other. This integrated measurement model should be in line with other concepts of modern drug development (e.g., EMEA/ 127318/2007).

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C.2 Methodologies of Pharmacodynamic Assessment

Claudia Pfeiffer · Peter Ruus

C.2.1 General Introduction

In 2009, spending on new drug development was esti mated to be in the range of 1 billion per drug reaching the market (Health Economy 2009). Since the costs are exponentially increasing during clinical development and late stage failures are costly, early clinical milestones are needed to decide about continuation or discontinuation of further clinical development. In this regard, the imple mentation of pharmacodynamics in early clinical studies, conducted in healthy subjects or in the target population, should be considered crucial information in the develop ment process to separate the wheat from the chaff.

Pharmacodynamics can be defined as the study of the biochemical and physiological effects of drugs and their mechanisms of action or as defined by Leslie Z. Benet (Benet 1994) what does the drug to the body.

Pharmacodynamic studies are conducted to observe and/or quantify one or more effects of the drug or the actions thought to be the basis of these effects. In an antihypertensive study, this may mean monitoring dia stolic and systolic blood pressure, in a gastric antisecretory study measuring gastric acid output and stomach pH, or in an antidiabetic study measuring *fasting or postprandial* blood glucose, insulin, C peptide or HbA1c. The ultimate objective of a pharmacodynamic study is to *get an estima tion* on whether the drug is capable of eliciting a clinically meaningful response. Other objectives may be to charac terize the time course of response and/or to identify the variables, e.g., dosage or dosing intervals, which influence the response (Rodda et al. 1988). This information sup ports the optimization of dose selection applied in the dose range finding study (Phase IIb).

C.2.2 Design of Pharmacodynamic Studies

Pharmacodynamic studies may be conducted either in healthy volunteers or in patients from a clearly defined target population. Since a high degree of interpatient variability is associated with a pharmacodynamic study, a cross over design with adequate washout intervals sep arating treatment periods may be preferable. A parallel design rather than a cross over design should be employed if the treatment creates a "permanent" or sustained change in a patient's condition, as in an antipyretic, antiinfective, or analgesic study.

A parallel design is a complete randomized design in which each patient receives one treatment in a random fashion. The simplest parallel design is the two group parallel design, which compares two treatments. For Phase II and Phase III clinical trials, the parallel design probably is the most frequently used design. However, it may not be an appropriate design for Phase I studies, e.g., bioequivalence, food effect studies. The variability in observations consists of interpatient and intrapatient var iabilities, and the assessment of bioequivalence between treatments is usually made based on the intrapatient var iability. A parallel design, however, is not able to identify and separate these two sources of variations because each patient in the parallel design usually receives the same drug during the entire course of the study. Patients in cross over trials serve as their own control, and thus, the variability is generally lower as in parallel group studies. As a result, in general, for parallel design studies a higher sample size is necessary than for cross over studies to demonstrate a similar effect. Although the parallel design is not widely used for Phase I studies due to the incapa bility of identifying and removing the interpatient vari ability from the comparison between treatment groups, a parallel design may be more appropriate than a cross over design in some situations, especially for first in man studies, first multiple dose studies for new compounds and some pharmacodymic studies. It cannot be recommended to use cross over designs for drugs known to have a very long half life. In a cross over design, a sufficient length of washout is necessary to eliminate the possible carry over effects and consequently, the study may take considerable time. In addition, if the study is to

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be conducted with very ill patients or diseases where a deterioration, an improvement or a irreversible outcome during the course a study can be expected, parallel design is usually recommended. For example, for infectious dis eases, a cross over design is not suitable. Another problem may be dropouts. These are patients who discontinue the treatment before the study is completed. Dropouts cause difficulties for analysis and interpretation for both types of design. However, in cross over trials, it is extremely diffi cult if the patient can provide no direct information on one treatment because measurements are not available for this treatment.

If a long duration of treatment is needed to be able to see an effect of the study compound on the clinical end point, the study duration of a cross over study might be too long. The study duration of a cross over study is at least two times the treatment duration plus the duration of the washout phase. Long treatment duration in cross over studies may result in significant treatment by period inter action effects or sequence effects, which affects the inter pretation of the study results. One such cause for this type of interaction is the carry over effect.

A parallel design may be considered as an alternative to a cross over design in the following situations: If the interpatient variability is relatively small compared to the intrapatient variability; the drug is potentially toxic, and/ or has a very long elimination half life; the population of interest consists of very ill patients; the cost for increasing the number of patients is much less than that of adding an additional treatment period; the duration of the study will be too long due to the response time of the drug.

A cross over design is a modified randomized block design in which each block receives more than one treat ment in different periods. A block may be a patient/ patients or a group of patients/patients. Patients in each block receive a different sequence of treatments. A cross over design is called a complete cross over design if each sequence contains each treatment. For a cross over design, it is not necessary that the number of treatments in each sequence is greater than or equal to the number of treat ments to be compared. We shall refer to a cross over design as a $g \times p$ cross over design if there are g sequences of treatments, which are administered in p different periods. As an example, for bioavailability/bioequivalence studies, the cross over design is viewed favorably due to the following advantages: Each patient serves as his/her own control. It allows a within patient comparison between treatments. It removes the interpatient variability from the comparison between treatments. With a proper randomization of patients to the sequence of treatment administrations, it provides the best unbiased estimates for the differences (or ratios) between treatments. The use of cross over designs for clinical trials has been greatly discussed in the literature. See, for example, Senn (2002).

The variability associated with an observed pharma codynamic effect has numerous sources. Factors influenc ing the response, which should be taken into account or controlled, include weight, sex, age, diet, concomitant medication, circadian rhythm effects, and the presence, severity, and time course of existing pathological condi tions. However, even when all known sources of variation are considered, pharmacodynamic effects are usually not identical in all patients or even for the same patient on different days. Whether parallel or cross over in design, pharmacodynamic studies should be placebo controlled, may be either single or repeated dose, and may include a range of doses.

To demonstrate a pharmacodynamic effect, the sample size should be large enough that a specified difference from placebo, either in the proportion of responders or in the magnitude of response, can be detected with ade quate power at an appropriate significance level. Depending on the magnitude of the specified difference and the anticipated variation in response, 10 18 patients are often sufficient to meet this requirement in a cross over study with quantifiable response. If prior information is available from literature or previous studies, the mean square error (from models similar to those intended for use in the study being designed) is used in the power calculation. However, as is usually the case, when data from a placebo group or the verum group alone are avail able, the power calculation for a one sample test of the difference from placebo may be performed with an arbi trary within patient correlation, often 0.5. This is accom plished by using $\rho = 0.5$ in the following formula: $V(x\ \ y) = V(x) + V(y) - 2\ \rho\ \sigma_x\sigma_y$, where $V(x) = \sigma_x^2 = V(y) = \sigma_y^2 =$ between patient variance, x = posttreatment value, and y = pretreatment value. To detect a specified difference in the pharmacodynamic effect from placebo in a parallel study, the standard deviation from the placebo group can be used in the power calculation for a two sample test. A parallel study with quantifiable or dichotomous response typically requires 20 40 patients per treatment group. If, however, the primary objective is to detect differences in response between doses, prior relevant phar macodynamic data are required, and increases in sample size may be necessary for both parallel and cross over design studies due to smaller differences to be expected.

Measurements of the pharmacodynamic effect should be taken not only following treatment administration

but also prior to treatment administration. For each individual, a series of baseline measurements are needed to demonstrate clearly that a patient has the condition and degree of severity required for admittance to the study and for continued participation in each treatment period. The number of response measurements and times at which they are to be taken will vary for each drug tested, however, as many response measurements should be taken as needed to completely characterize the effect of the drug. Moreover, statistical comparison of baseline values can test if the treatment groups were balanced at baseline or if an imbalance in one or more of the baseline values might bias the results of the study. (Rodda et al. 1988).

Since the future of the drug may be dependent on the accuracy and reliability of the pharmacodynamic measurements, the best techniques available should be employed. In addition to monitoring the pharmacodynamic effect or response, determinations of drug concentrations in appropriate biological samples (plasma, urine, saliva, stool, etc.) may be performed. Sampling should be scheduled in at least a subset of the same times at which response measurements are scheduled to facilitate the analysis and interpretation of the blood level response relationship.

C.2.3 Randomization

In the statistical theory of design of experiments, randomization involves the random allocation of patients/patients to treatment groups. For example, if an experiment compares a new drug against a standard drug, then the patients should be allocated either to the new drug or to the standard drug using randomization. Randomized experimentation is not haphazard. Randomization reduces bias by equalizing other factors that have not been explicitly accounted for in the experimental design (according to the law of large numbers). To avoid any bias in the assignment of study treatment to patients or patients, the randomization of patients to treatment groups is the only way to ensure that the assigned treatment cannot be predicted, that the treatment is independent of the patient's characteristics, and that uncontrolled variability factors are distributed at random between the groups. As long as the numbers of patients are sufficient, randomization is an effective method for balancing confounding factors between treatment groups.

Stratification enables separate randomization within patients or patients that share a common prognostic factor. Randomization should take place as late as possible before effecting the treatment of the patient and the results of the randomization should be unpredictable to those individuals (patient and investigator) involved in the study (Chow and Liu 1991).

C.2.4 Analysis of Pharmacodynamic Studies

Pharmacodynamic effects refer to occurrence of a disease, symptom, sign, or laboratory abnormality that constitutes one of the target outcomes of the trial. Pharmacodynamic effects may be assessed by examining the proportion of patients at each response measurement time for which a clinically meaningful response is observed. For an antiliperdemic trial, a clinically meaningful response may be the reduction in lipids, and for an antidiabetic trial, the response may be defined as the decrease in blood glucose by 30 mg/dl. For an antisecretory study, a clinically meaningful response may be defined as an increase in stomach pH above clinically meaningful value, and for an antihypertensive drug, a clinically meaningful response may be defined as a decrease in diastolic blood pressure of at least 10 mmHg. Clinical efficacy can further be characterized by the time required to reach a clinically meaningful response, i.e., onset of action, the magnitude, and time of peak response, and the period of time for which a clinically meaningful response is continuously sustained, i.e., duration of action. Blood drug concentrations at the times during which a clinically meaningful response is demonstrated may also be examined (Rodda et al. 1988).

In pharmacodynamic studies with blood glucose lowering drug for the treatment of diabetes, typical pharmacodynamic measurements are profiles of blood glucose, insulin, or C peptide. These profiles can be measured for 12 or 24 h at close time points from the fasting value in the morning until the first meal and in broader time points afterward. From these profiles, parameters will be derived to characterize the effect of the compound. Typical parameters are the area under the curve (AUC) for certain time intervals, the mean value over all measurements, or the postprandial blood glucose. To assess the drug effect in comparison to the initial value before start of treatment, the change from baseline is often being used as response criteria. The baseline measurement or the average of the baseline measurements may be used as a covariate, or to determine the change in response from baseline.

Differences in pharmacodynamic effects or response between treatments may be analyzed at each time point of collectively (repeated measures) via standard parametric/nonparametric statistical methods appropriate for

cross over or parallel designs. A confidence interval may be calculated within the frame of the statistical model used. Confidence intervals are computed in addition or alternatively to significance tests.

The analyses of a cross over design study and a parallel design study will be illustrated in the examples in Parts A and B for a parametric approach.

C.2.5 Descriptive Analyses

Descriptive analysis for the study endpoints may be presented in addition. Descriptive analyses normally include the presentations of descriptive statistics for study endpoints. Typical statistics for quantitative parameter are: number of non missing observations, mean, standard deviation or standard error of the mean, minimum, median, maximum, geometric mean, coefficient of variation. Geometric mean and coefficient of variation will be provided mainly for log transformed parameters. For qualitative parameter data, e.g., proportion typical statistics are the number of non missing observations and percent.

The relationships between response, time, and blood drug concentration may be graphically displayed by plots of response versus time, response versus concentration, concentration versus time, and/or mean or median profiles. Correlation and regression analysis and between dosage group comparisons may be performed where appropriate.

To assess the comparability of treatments groups or sequences at baseline, appropriate statistical tests can be performed. For continuous response variables, typically simple Analysis of Variance Models (ANOVA) may be performed with treatment as fixed effect: (response = treatment + error).

PURPOSE AND RATIONALE

The primary objective of a pharmacodymic study is to assess the pharmacodymic effect of the new compound. Pharmacokinetics, safety, and tolerability are of secondary interest. For some compounds, the pharmacodynamic effect can be observed only in patients having the disease to be treated and cannot be observed in healthy volunteers. In the following, two pharmacodynamic studies will be presented, a single dose 2 treatment, 2 period, 2 sequence, placebo controlled cross over study (Part A), and a multiple dose placebo controlled, parallel group study (Part B). The intention of both studies is to demonstrate a pharmacodynamic effect of new antidiabetic compounds for the treatment of Type II diabetes. Both studies have been performed in Type II diabetic patients.

C.2.6 Part A

PURPOSE AND RATIONAL

The design and the analyses of a pharmacodynamic cross over study to assess the effect of a subcutaneous (sc) injection of an antidiabetic drug on insulin secretory responses in patients with type 2 diabetes mellitus, with a focus on first phase insulin release, are presented below (Part A).

C.2.6.1 Protocol Title

Restoration of first phase insulin release in patients with type 2 diabetes mellitus with subcutaneous injection of 20 µg X001 in a double blind, randomized, placebo controlled, 2 period, 2 treatment, 2 sequence cross over intravenous glucose challenge study.

C.2.6.1.1 Primary Objective

The primary objective of this study was to test whether X001 restores first phase insulin response in patients with diabetes mellitus type 2.

C.2.6.1.2 Secondary Objective

The secondary objective of this study was to test whether X001 improves second phase insulin response.

C.2.6.1.3 Study Design

Single center, double blind, randomized, placebo controlled, single dose, 2 period, 2 treatment, 2 sequence cross over study comparing X001 injection with saline injection (placebo) in patients with type 2 diabetes mellitus. Patients were admitted to the unit the evening prior to experimentation (Day 1). On Day 1, after an overnight fast, patients were prepared for the experiment and rendered euglycemic (blood glucose concentration of 5.5 mmol/l/100 mg/dl) by an intravenous insulin infusion

for 3 h, stopped 30 min prior to an intravenous glucose challenge of 0.3 g/kg bodyweight over 30 s. Patients received a subcutaneous dose of 20 µg of X001 or placebo 2 h prior to the glucose challenge according to randomization.

C.2.6.1.4 Inclusion Criteria

Male and female patients with type 2 diabetes mellitus on diet and exercise with or without metformin treatment, age 18 65 years, BMI 25 35 kg/m^2, systolic blood pressure from 90 to 155 mmHg and diastolic blood pressure from 45 to 100 mmHg.

C.2.6.1.5 Exclusion Criteria

Patients with type 2 diabetes mellitus on other oral antihyperglycaemic medication than metformin.

C.2.6.1.6 Treatments

Subcutaneous (s.c.) injection of 20 µg X001 or placebo once injected in the morning at around 11:00 in fasted conditions.

EVALUATION

Standard safety/tolerability criteria (e.g., adverse events, biochemistry, hematology, urinalysis, ECG, vital signs) and X001 plasma concentrations, pharmacokinetic parameters C_{max}, T_{max}, AUC_{last}, AUC were assessed in this study but are out of the scope of this chapter.

C.2.6.2

C.2.6.2.1 Criteria for Pharmacodynamic Evaluation

Primary Endpoints

Primary endpoint is the first phase insulin response INS $AUC_{(0–10\ min)}$ assessed by calculating the area under the insulin concentration curve AUC during the first 10 min after the iv glucose bolus, subtracted for basal insulin concentration.

Secondary Endpoints

Second phase insulin response INS $AUC_{(10–120\ min)}$, first and second phase C peptide response C peptide $AUC_{(0–10\ min)}$, C peptide $AUC_{(10–120\ min)}$, glucagon concentrations, glucose disappearance constant kg were considered as secondary endpoints.

C.2.6.2.2 Pharmacodynamic Sampling times

Blood was collected for the determination of insulin, C peptide, glucose, and glucagon concentrations. Sampling times were at −300, −240, −180, −120, −60, −30, −15, −10, −5, 0, 2, 4, 6, 8, 10, 12, 15, 20, 30, 45, 60, 90, and 120 min relative to glucose bolus injection.

C.2.6.2.3 Determination of Sample Size

The study was planned to have 20 evaluable patients. With a total of 18 patients, a threefold increase in log INS $AUC_{(0–10\ min)}$ after s.c. injection of X001 in comparison to placebo could be detected with a power of 90% and a type one error of 5% assuming a within standard deviation of 0.9.

C.2.6.2.4 Pharmacodynamic Population

The full analysis population encompassed all patients who received at least one injection of X001 or placebo.

C.2.6.2.5 Statistical Analysis

The basal insulin concentration was calculated as the mean insulin concentration between −5 and 0 min before glucose bolus. The basal C peptide concentration was calculated as the mean C peptide concentration between −5 and 0 min before glucose bolus. First and second phase insulin responses were calculated as area under the insulin secretion curve within 10 min respectively 10 120 min after i.v. glucose challenge, subtracted for the mean basal insulin concentration. First and second phase C peptide responses were calculated as area under the C peptide secretion curve within 10 min respectively 10 120 min after i.v. glucose challenge, subtracted for the mean basal C peptide concentration. AUC was calculated using trapezoidal rule.

Glucose disappearance constant kg was calculated as 100× the slope of the regression line fitted to the natural

logarithm of glucose concentrations from time 10 to 30 min after the glucose bolus.

The following hypothesis was tested:

$$H0 : X001 = \text{Placebo versus } H1 : X001 \neq \text{Placebo}$$

Prior to the analyses described below, all pharmacodynamic parameters were log transformed (base e).

Log transformed INS $AUC_{(0–10\ min)}$, INS $AUC_{(10–120\ min)}$, C peptide $AUC_{(0–10\ min)}$, C peptide $AUC_{(10–120\ min)}$, and glucose disappearance constant kg was analyzed using linear mixed models:

$$\text{Log (Parameter)} = \text{Sequence} + \text{Period} + \text{Treatment} + \text{Sex} + \text{Patient (Sequence)} + \text{Error},$$

with fixed terms for sequence (X001 placebo vs. placebo X001), period (Day 1 vs. Day 2), and treatment (X001 vs. placebo), and with an unstructured R matrix of treatment (i,j) variances and covariances for patient within sequence blocks, using SAS PROC MIXED.

The following SAS code was used:

```
proc mixed order = internal;
CLASS sequence treatment period sex patient;
MODEL LOG paramter = sequence treatment period
  sex patient/outp = predicted outpm = randeff
 ddfm = kr;
REPEATED treatment/patient = patient(sequence)
 TYPE = FA0(2);
ESTIMATE "X001 Placebo" treatment 1 1/CL;
CONTRAST "X001 Placebo" treatment 1 1;
```

Estimates and 90% confidence interval for the ratios of geometric means of X001 versus placebo were calculated within the linear mixed effects model framework and then converting to ratios by the antilog transformation.

A significance level of $p < 0.05$ were used. No adjustments were made for multiple testing.

For insulin, C peptide, and glucagon concentrations mean profiles were provided.

Results

The results of the statistical analyses are presented in *Table C.2 1* (Results of the ANOVA) und *Table C.2 2* (estimates and confidence intervals for the ratios). Analysis of variance (ANOVA) revealed significant treatment effects for insulin, C peptide, and glucose disappearance constant. 20 µg X001 present maximum plasma concentration 2 h after subcutaneous injection. X001 enhances (restores) first phase insulin release in response to an intravenous glucose challenge 2 h after injection about sixfold and second phase insulin response about threefold, also confirmed by about six and twofold increases in C peptide release, respectively, and accelerates glucose disposition about twofold in patients with diabetes mellitus type 2. X001 does not affect glucagon release in this setting (*Fig. C.2 1*).

Table C.2-1
Treatment effect on Insulin AUC, C-Peptide AUC, and Glucose disappearance constant - ANOVA

Parameter	Effect	*p*-value
AUC of insulin 0 10 min	Sequence	0.381
	Period	0.610
	Sex	0.712
	Treatment	<0.001
AUC of insulin 10 120 min	Sequence	0.767
	Period	0.858
	Sex	0.602
	Treatment	<0.001
AUC of C-peptide 0 10 min	Sequence	0.229
	Period	0.278
	Sex	0.890
	Treatment	<0.001
AUC of C-peptide 10 120 min	Sequence	0.660
	Period	0.223
	Sex	0.386
	Treatment	<0.001
Glucose disappearance constant	Sequence	0.068
	Period	0.107
	Sex	0.449
	Treatment	<0.001

C.2.6.3 Summary

CRITICAL ASSESSMENT OF THE METHOD

The primary stimulus for insulin secretion is the beta cell response to changes in ambient glucose with a usually biphasic pattern of insulin release. First phase insulin release occurs within the first few minutes after exposure to an elevated glucose level (0 10 min); this is followed by a more enduring second phase of insulin release (10 120 min). Numerous studies have demonstrated that first phase insulin secretion is lost in patients with type 2 diabetes, but also in impaired glucose tolerance (Cavaghan et al. 1997), older individuals (Kahn et al. 1992),

Table C.2-2

Estimates of insulin AUC, C-Peptide AUC, and glucose disappearance constant ratios with 90% confidence interval

Parameter	Comparison	Estimate	90% CI
AUC of insulin 0 10 min	X001 vs. placebo	6.60	(5.00 8.72)
AUC of insulin 10 120 min	X001 vs. placebo	2.96	(2.65 3.29)
AUC of C-peptide 0 10 min	X001 vs. placebo	6.09	(4.20 8.83)
AUC of C-peptide 10 120 min	X001 vs. placebo	2.08	(1.88 2.31)
Glucose disappearance constant	X001 vs. placebo	1.75	(1.62 1.89)

first degree relatives of subjects with type 2 diabetes (Cnop et al. 2007) as well as women with either polycystic ovary syndrome (Dunaif et al. 1996) or a history of gestational diabetes (Ward et al. 1985). In this regard, first phase insulin release is considered an important and early marker of beta cell dysfunction.

Since loss of first phase insulin secretion is a prevailing feature in a type 2 diabetic population, restoration of first insulin release in this population is considered to best highlight drug related beneficial pharmacological properties on beta cell function. For this reason, the effect of X001 on first phase insulin release was selected as primary objective. Since X001 was shown to have pharmacodynamic effects after the very first dose, the study could be conducted according to a placebo controlled single dose design. Due to the well known dependency with an increasing first phase insulin response in patients with an impaired insulin sensitivity (Kahn et al. 2008), a cross over design was required to limit variability.

X001 has a delaying effect on gastric emptying, and an oral glucose load may furthermore induce significant incretin effects (Ritzel et al. 1995). Taking this into consideration, the intravenous glucose challenge overcoming the variability by variable gastrointestinal absorption and activation of the endogenous incretins should be the preferred option when determining the acute insulin response and was therefore also applied in the study exploring X001.

As shown by Brunzell (Brunzell et al. 1976), first phase insulin release is attenuated or absent in subjects with blood glucose values exceeding 6.4 mmol, which may be present in a type 2 diabetic population at start of treatment. For this reason, at the day first phase insulin release was determined, all patients were fasted and, if necessary, treated with exogenous insulin to have a blood glucose of about 5.5 mmol/l at the time of the IV glucose challenge (0.3 g/kg b.w.). Insulin was interrupted about 30 min before starting the IV glucose challenge. Since insulin has a half life of about 4 6 min, the impact of the exogenous insulin on the test results was considered negligible.

The study revealed a nearly sixfold increase of the first phase insulin release and a threefold increase for the second phase insulin secretion, underlining the large signal to noise ratio with this parameter and the tested population.

Overall, the procedures as applied in the study testing X001 on first phase insulin secretion were shown to be suitable as long as the study is conducted by an experienced study center.

MODIFICATIONS OF THE METHOD

The study described above is a classical Phase I like study and therefore not easily to implement in a Phase III program. In addition, a lot of compounds do not exert pharmacodynamic effects from the very first dose. Therefore, numerous studies have been published (Song et al. 2007) to explore beta cell function using indices. In this regard, the Homeostasis Model Assessment (HOMA) B appears to be frequently used, probably due to the simplicity of calculation:

$$\text{HOMA B} = 20 \times \text{fasting insulin } (\mu\text{IU/ml}) / \text{fasting glucose(mmol/ml)} - 3.5$$

There are a number of other methodologies and indices estimating the beta cell function going beyond this chapter. A comprehensive review is provided by Kahn (Kahn et al. 2008).

C.2.7 Part B

PURPOSE AND RATIONAL

The design of a study investigating pharmacodynamics following dose titration of subcutaneously administered

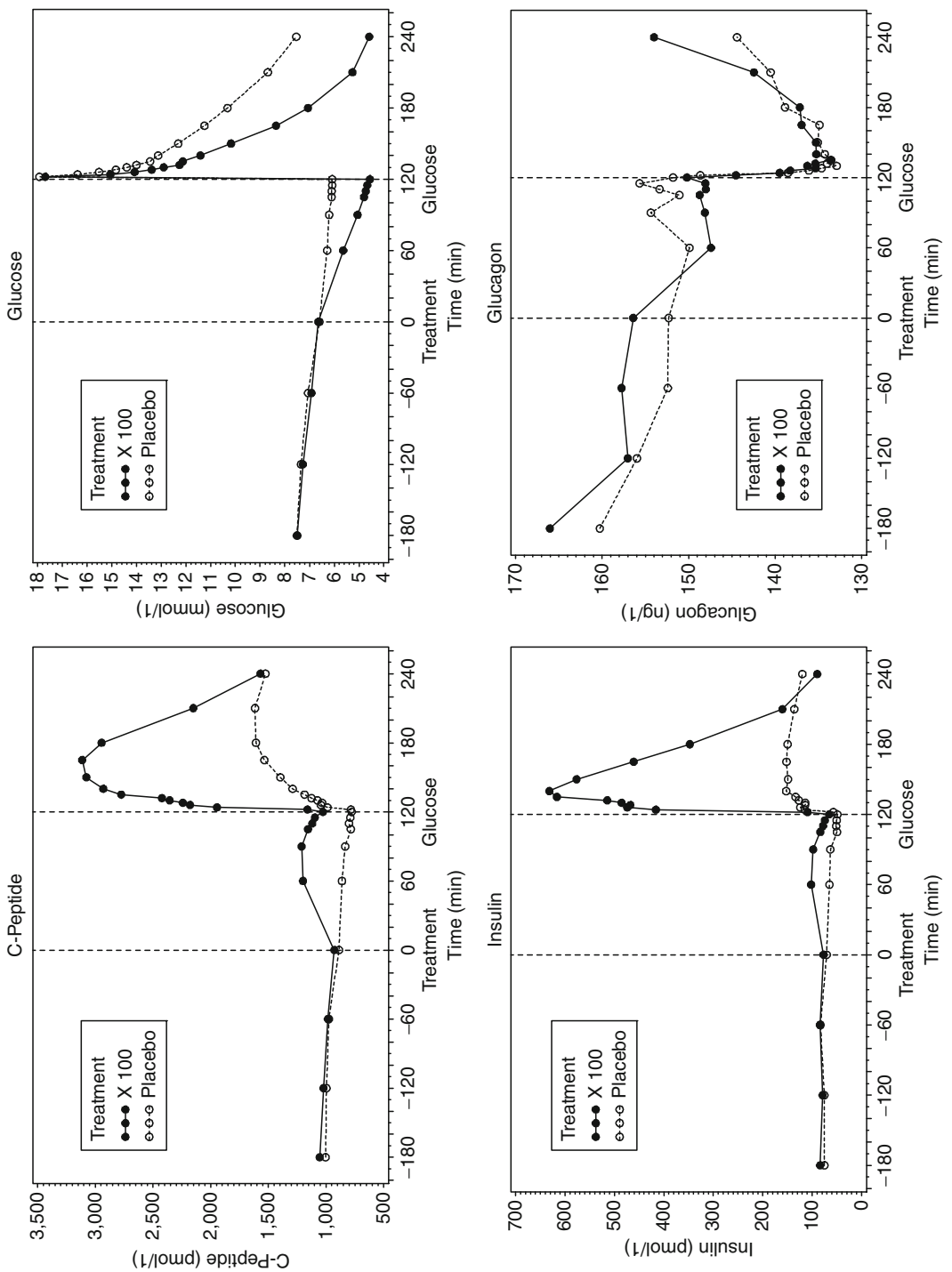

Figure C.2-1
Mean time profiles for C-Peptide, Glucose, Insulin, and Glucagon

X002 in patients with type 2 diabetes mellitus in a double blind, randomized, placebo controlled parallel group Phase IIa study is presented below. The description is limited to pharmacodynamic data although safety/tolerability and pharmacokinetics data were also obtained.

PROCEDURE

Pharmacodynamics, safety, tolerability, and pharmacokinetics following dose titration of subcutaneously administered X002 in patients with type 2 diabetes mellitus were investigated in a multicenter, double blind, randomized, placebo controlled parallel group Phase IIa study. Eligible patients were randomly assigned to one of the following treatments: QD regimen (X002 in the morning, placebo in the evening), BID regimen (X002 in the morning and in the evening), and placebo treatment (placebo in the morning and in the evening). Each patient received a subcutaneous injection 15 min prior to the morning and evening meals. The starting dose was 5 μg X002 (or matching volume of placebo solution) per subcutaneous (s.c.) injection administered either QD or BID. If safety and tolerability are permitted, the dose per injection was then to be increased every fifth day in increments of 2.5 μg. The maximum treatment period was not to exceed 28 days, with a theoretical maximum dose of 20 μg X002 QD or BID. In case of dose limiting adverse events, the patients could continue at the current dose level, or continue with the preceding dose, or they could be discontinued from treatment with study medication. Decisions on the dose progression in each patient were made by the investigator, and were based upon evaluation of blinded safety and tolerability data.

On Day 1, and Day 4 of each dose level, blood glucose was determined before and after the three standardized test meals (breakfast, lunch, dinner). Serum insulin, C peptide, and glucagon were measured on Day 1, Day 12 (fourth day of the 10 μg dose level), and Day 28 (last dosing). Fasting blood glucose was measured in the morning on the third day of each dose level. To explore gastric emptying rate, 13C octanoic acid breath test was determined after the standardized breakfast test meal on Day 1, Day 12 (fourth day of the 10 μg dose level), and Day 28 (last dosing).

The primary objective of the study was to assess the effects of individually increased once daily (QD) or twice daily (BID) doses of X002 in a stepwise manner on the increase in blood glucose induced by a standardized breakfast test meal and to identify an individually well tolerated dose associated with a statistically significant and clinically meaningful effect on postprandial blood glucose. Secondary pharmacodynamic objectives were to assess the effects of individually increased once daily (QD) or twice daily (BID) doses of X002 in a stepwise manner on the increase in blood glucose induced by a standardized lunch and dinner test meals, the increases in insulin, C peptide, and glucagon, induced by the standardized test meals, insulin sensitivity as evaluated by means of the homeostasis model assessment (HOMA), on morning fasting blood glucose (FBG), on body weight, and on gastric emptying.

C.2.7.1 Sample Size Justification

A sample size of 16 in each group (X002 QD, X002 BID, placebo) was expected to have 90% power to detect a difference in mean AUC of change in postprandial blood glucose of 300 h·mg/dl (the difference between a Group 1 mean of 280 h·mg/dl and a Group 2 mean of −20 h·mg/dl) assuming a common standard deviation is 250 h·mg/dl using a two group *t* test with a 0.05 two sided significance level. Assuming a 20% dropout rate, 20 patients per group were to be randomized. Thus, 60 male and female patients with stable type 2 diabetes mellitus (20 patients per treatment group) were to be included in this study.

C.2.7.1.1 Inclusion Criteria

Male and female patients, $\geq$18 to $\leq$70 years of age, with stable (in previous 3 months) type 2 diabetes mellitus treated with up to two oral hypoglycemic agents (sulfonylurea, metformin).

EVALUATION

The following pharmacodynamic variables were analyzed: change from baseline (Day 1) on the fourth day of each dose level, on the fourth day of the highest individually well tolerated dose and on Day 28 in the postprandial blood glucose, Insulin and C peptide AUC at breakfast, lunch, and dinner, the morning fasting blood glucose levels on the third and fourth day of each dose level, the average 7 point daily blood glucose profile calculated as the mean of the blood glucose measurements before meals (at 0:14 h, 5:14 h, 10:14 h after the morning injection), 90 min after meals (at 1:45 h, 6:45 h, 11:45 h after the morning injection), and at bedtime (at 14:55 h after the morning injection); the maximum postprandial blood glucose after breakfast, lunch and dinner. Change in HbA1c from baseline measured on Day 29 was calculated. For insulin sensitivity from the HOMA the beta cell

function was derived (= [20 × fasting insulin (μIU/mL)]/[fasting glucose (mmol/L) − 3.5]) and the insulin resistance (= [fasting insulin (μIU/mL) × fasting glucose (mmol/L)]/22.5). From the gastric emptying time (from a 13C octanoic acid breath test) for the test breakfast meal on the fourth day of the 10 μg dose level and on Day 28, the lag phase tlag, the emptying half life t1/2, and the gastric emptying coefficient (GEC) were analyzed. The analyses of pharmacodynamic parameters (including the primary analysis variable) were based on the actual sampling times.

AUCs were calculated as the area under the concentration time curve of the respective variable for the time intervals 0:14 4:55 h for breakfast, lunch and dinner: 0:14 h after the morning injection (just before standardized breakfast intake) until 4:55 h after the morning injection and relative to the pre meal value determined for lunch and dinner test meal.

The highest well tolerated dose was determined for each patient programmatically based on the question "Would you consider increasing the dose level for this patient?", which was to be answered in the CRF by the investigator on Day 29 (or after last dose of study medication in case of premature withdrawal). If this question was answered with "no", then the cases were discussed by the sponsor and the initial response on the CRF could be revised if there was an appropriate rationale for doing so after reviewing the relevant safety data. Otherwise, the dose below the maximum given dose level that the patient received was used as his or her individually well tolerated dose. In all other cases, the individually well tolerated dose was equal to the maximum given dose. The individually well tolerated dose for each patient was documented in addition.

The primary analysis variable was the area under the blood glucose concentration time curve from (area under the curve [AUC][0:14 4:55 h]) the fourth day of the highest individually well tolerated dose. The change compared to baseline (Day 1) was evaluated.

C.2.8 Study Populations

The analyses of pharmacodynamic variables were performed using the modified intention to treat (mITT) population (including the primary variable). Patients were included in the analysis of the variable in question if they had a baseline value and at least one value at the highest individually well tolerated dose. Patients randomized but not treated with study medication were excluded.

C.2.9 Statistical Methods

C.2.9.1 Demographics and Baseline Characteristics

Demographic and background characteristics were summarized for the analysis population of all treated patients as well as by treatment group to assess the comparability of the three treatment groups at baseline and to characterize the population.

Continuous variables were summarized using the following descriptive statistics: number of patients (N), mean, standard deviation, median, minimum, and maximum. Absolute and relative frequency distributions are provided for categorical data.

Baseline demographic variables were tested for overall between group homogeneity using all patients with available data. The continuous variables age, BMI, time since diagnosis of diabetes, age at diagnosis of diabetes, skin thickness, and baseline pharmacodynamic variables were compared using an analysis of variance (ANOVA) model with treatment as fixed effect. The general association Cochran Mantel Haenszel (CMH) test was used for categorical data such as sex, BMI categories, race, and smoking status. Baseline gastric emptying parameters of tlag and $t_{1/2}$ were compared between treatment groups using an ANOVA model applied on the ranked values and with treatment included as fixed effect. The statistical methods for the analysis of baseline data were used in a purely exploratory manner. Although patients were allocated at random to treatment groups, it is possible that one or more baseline variables could differ considerably between groups. In these cases, additional analyses were to be performed to determine what effect the baseline imbalance had on the primary analysis variable.

The primary analysis was based on the primary variable, i.e., the change from baseline in the blood glucose $AUC_{[0:14\ h–4:55\ h]}$ at test breakfast on the fourth day of the highest individually well tolerated dose. An analysis of covariance (ANCOVA) model was used to analyze differences in the primary variable between the treatment groups. The following model was used:

$$\text{Response} = \text{Treatment} + \text{Baseline} + \text{Random Error} \quad (C.2.1)$$

where:

- Response = Change from baseline in the blood glucose $AUC_{[0:14\ h–4:55\ h]}$ at test breakfast;
- Treatment = X002 QD, X002 BID, placebo;
- Baseline = Covariate ($AUC_{[0:14–4:55\ h]}$ of Day 1).

The estimates and the corresponding 95% confidence intervals for the comparisons of X002 QD placebo, X002 BID placebo, X002 BID X002 QD are provided along with adjusted means and standard errors for each treat ment group.

In addition, descriptive statistics (N, mean, standard deviation, median, minimum, and maximum) are presented for each treatment group.

The same ANCOVA model (1) with the respective Day 1 AUC as baseline was used to analyze the change from baseline in $AUC_{[0:14–4:55\ h]}$ for the postprandial changes in blood glucose, insulin, and C Peptide relative to the pre meal value determined for the breakfast, lunch, and dinner test meals using the respective AUC values from the fourth day of each dose level as the response variable.

The ANCOVA model (1) was also performed to ana lyze the morning fasting blood glucose values collected on the third and fourth day of each dose level, the 7 point blood glucose values, and the average 7 point blood glu cose profile using the respective change from baseline (Day 1) as the response variable and the corresponding baseline value.

Adjusted means with the corresponding standard error are provided for each treatment group and estimates together with the corresponding 95% confidence intervals are presented for the same pairwise treatment compari sons as described for the primary analysis variable.

In addition, for the values and the changes from base line, descriptive statistics (N, mean, standard deviation, median, minimum, and maximum) are presented for each treatment group.

Results are presented for the highest individually well tolerated dose as well as separately for each dose level including patients who received the respective dose.

Mean profiles of blood glucose, insulin, and C peptide per treatment group (using the per protocol sampling time) were plotted against time for each dose level.

The change in HbA1c from baseline to Day 29 was evaluated, and treatments were compared using the ANCOVA model (1)where:

Response = HbA1c change from baseline to Day 29;
Treatment = X002 QD, X002 BID, placebo;
Baseline value = Baseline HbA1c measured on Day 1.

Insulin sensitivity (HOMA) parameters were analyzed using the same ANCOVA model as described in (1). Data were ln transformed (base e) before the analysis. Adjusted means with the corresponding standard error are provided for each treatment group, and estimates together with the corresponding 95% confidence inter vals are provided for the same pairwise treatment comparisons as described for the primary analysis variable. Descriptive statistics (N, mean, standard devia tion, geometric mean, geometric standard deviation, median, minimum and maximum) for the absolute (untransformed) values and for the change from baseline are provided for each treatment group.

Results are presented for the highest individually well tolerated dose as well as separately for each dose level including patients who received the respective dose.

Gastric emptying

Treatment groups were compared on the fourth day of the 10 µg dose level and on Day 28 using the ANCOVA model (2) applied on the ranked gastric emptying parameters tlag and t1/2where:

- Response = Ranked value for tlag or t1/2, respectively;
- Treatment = X002 QD, X002 BID, placebo;
- Baseline value = Covariate (ranked baseline value for tlag or t1/2, respectively).

The estimates and the corresponding 95% confidence intervals for the comparisons of X002 QD placebo, X002 BID placebo, X002 BID X002 QD are provided along with adjusted means and standard errors for each treat ment group.

C.2.9.2 Multiplicity Issues

Due to the explorative nature of the study, the type 1 error alpha was not adjusted for multiplicity and all tests were performed at the alpha level of 0.05.

C.2.9.3 Results – Anthropometric Data

Baseline body weights were slightly higher in the X002 QD group (mean = 89.4 kg) than in the placebo (83.8 kg) and X002 BID (83.7 kg) groups. Overall, there was a slight decrease in body weight during the study in each treat ment group. The differences to placebo for changes in body weight relative to baseline were not statistically sig nificant between any of the treatment groups at any of the treatment levels. Skin thickness was generally comparable across the treatment groups at baseline.

C.2.9.4 Results – Pharmacodynamics

Treatment differences compared to placebo at each dose level and at the highest well tolerated dose for the primary,

and key secondary pharmacodynamic variables are summarized in the tables below for blood glucose parameters, HbA1c, insulin sensitivity parameters, and gastric emptying parameters.

Highly significant differences to placebo were seen in the X002 QD and BID groups for the change from baseline to Day 4 at the highest well tolerated dose in the postprandial blood glucose AUC at breakfast. *Table C.2 3*. Furthermore, the differences to placebo for the change from baseline in the postprandial blood glucose AUC were all highly significant at all dose levels for the meals at which X002 had been administered, i.e., at breakfast for the X002 QD group, and at breakfast and dinner for the X002 BID group. In addition, at lunch, the differences to placebo for the change from baseline in the postprandial blood glucose AUC were all highly significant at all dose levels for the X002 QD group, and in the X002 BID group, the differences to placebo were statistically significant for dose levels of 12.5 µg and higher. At dinner, the differences to placebo were statistically significant in the X002 QD group at all dose levels apart from 5 and 10 µg. Thus, X002 caused a clear attenuation of the rise in

Table C.2-3

Change from baseline in the postprandial blood glucose AUC at breakfast, lunch, and dinner (mITT population)

Postprandial blood glucose AUC (h.mg/dL)						
Meal Treatment group Dose level	N	Adjusted mean ± SE			95% CI of difference	*p*-value
		Baseline mean	Change from baseline	Difference vs. placebo		
Breakfast						
Placebo						
10.0 µg	22	1007.4 ± 38.18	51.4 ± 36.12			
20.0 µg	22	1007.4 ± 38.18	0.4 ± 45.35			
Highest individually well-tolerated dose	22	1007.4 ± 38.18	3.0 ± 44.62			
X002 QD						
10.0 µg	21	947.6 ± 39.08	346.7 ± 36.09	295.3 ± 50.93	(397.2; 193.5)	<0.0001
20.0 µg	19	947.6 ± 39.08	387.4 ± 47.83	387.0 ± 65.50	(518.1; 255.9)	<0.0001
Highest individually well-tolerated dose	21	947.6 ± 39.08	378.3 ± 44.58	381.3 ± 62.92	(507.1; 255.4)	<0.0001
X002 BID						
10.0 µg	21	863.6 ± 39.08	316.7 ± 37.21	265.3 ± 53.23	(371.8; 158.9)	<0.0001
20.0 µg	21	863.6 ± 39.08	361.3 ± 47.08	360.9 ± 67.12	(495.3; 226.6)	<0.0001
Highest individually well-tolerated dose	21	863.6 ± 39.08	360.0 ± 45.97	362.9 ± 65.75	(494.5; 231.4)	<0.0001
Lunch						
Placebo						
10.0 µg	22	905.0 ± 40.04	0.4 ± 39.53			
20.0 µg	22	905.0 ± 40.04	102.9 ± 52.28			
Highest individually well-tolerated dose	22	905.0 ± 40.04	103.2 ± 52.14			
X002 QD						
10.0 µg	21	890.0 ± 40.98	204.4 ± 40.36	204.0 ± 56.44	(316.9; 91.2)	0.0006

Table C.2-3 (Continued)

Postprandial blood glucose AUC (h.mg/dL)						
Meal Treatment group Dose level	N	Adjusted mean ± SE			95% CI of difference	*p*-value
		Baseline mean	Change from baseline	Difference vs. placebo		
20.0 µg	19	890.0 ± 40.98	186.1 ± 56.21	289.0 ± 76.64	(442.4; 135.6)	0.0004
Highest individually well-tolerated dose	21	890.0 ± 40.98	174.3 ± 53.24	277.5 ± 74.44	(426.4; 128.6)	0.0004
X002 BID						
10.0 µg	21	848.4 ± 40.98	95.7 ± 40.56	95.3 ± 56.86	(209.1; 18.4)	0.0988
20.0 µg	21	848.4 ± 40.98	144.5 ± 53.75	247.4 ± 75.26	(398.0; 96.7)	0.0017
Highest individually well-tolerated dose	21	848.4 ± 40.98	152.9 ± 53.50	256.1 ± 74.99	(406.1; 106.1)	0.0011
Dinner						
Placebo						
10.0 µg	22	889.9 ± 47.76	42.0 ± 36.41			
20.0 µg	22	889.9 ± 47.76	66.7 ± 49.75			
Highest individually well-tolerated dose	22	889.9 ± 47.76	64.0 ± 50.67			
X002 QD						
10.0 µg	21	875.2 ± 48.88	139.0 ± 37.19	97.0 ± 51.98	(201.0; 7.0)	0.0670
20.0 µg	19	875.2 ± 48.88	133.0 ± 53.45	199.7 ± 72.93	(345.7; 53.7)	0.0082
Highest individually well-tolerated dose	21	875.2 ± 48.88	115.1 ± 51.76	179.1 ± 72.35	(323.8; 34.4)	0.0162
X002 BID						
10.0 µg	21	823.3 ± 48.88	224.0 ± 37.37	182.0 ± 52.36	(286.7; 77.3)	0.0010
20.0 µg	21	823.3 ± 48.88	280.9 ± 51.10	347.6 ± 71.58	(490.9; 204.4)	<0.0001
Highest individually well-tolerated dose	21	823.3 ± 48.88	293.6 ± 52.01	357.5 ± 72.88	(503.3; 211.8)	<0.0001

Differences vs. placebo are calculated as X002 minus placebo.
CI confidence interval, *mITT* modified intention to treat, *N* number of evaluable patients, *SE* standard error

blood glucose induced by standardized test meals at all doses up to 20 µg, which was the highest dose tested and was the highest well tolerated dose for most patients.

Except for the homeostasis model assessment (HOMA) parameters, the differences to placebo were also statistically significant at the highest well tolerated dose for the key secondary variables in the X002 BID group, and for the average 7 point blood glucose profile and glycohemoglobin (HbA1c) in the X002 QD group. Only the difference to placebo for fasting blood glucose on the fourth day of treatment at the highest well tolerated dose in the X002 QD group failed to achieve statistical significance, although the difference to placebo for fasting blood glucose on the third day of treatment was statisti cally significant in this treatment group ($p = 0.0145$).

For body weight, the difference to placebo for change from baseline at the highest well tolerated dose was not statistically significant for the QD or BID regimen, indi cating that the blood glucose lowering effects observed with X002 were not attributable to weight loss.

In almost all cases, the magnitude of the differences to placebo for these pharmacodynamic variables tended to be dose dependent in both the X002 QD and BID groups.

For HbA1c, which is the most important prognostic parameter for blood glucose control, there was a clear linear relationship between values measured at baseline and on Day 29, and all patients treated with X002 achieved a decrease in HbA1c value during the study (Table C.2 4).

Even though the changes in the HOMA parameter beta cell function compared to placebo were not statistically significant, pronounced mean increases were recorded between baseline and the fourth day of treatment with X002 QD (an increase of 57.34 [μIU/mL]/[mmol/L] at the highest well tolerated dose) and BID (an increase of 63.74 [μIU/mL]/[mmol/L]), whereas values remained relatively unchanged after treatment with placebo (an increase of 14.87 [μIU/mL]/[mmol/L]), suggesting an improvement in the insulin secretory capacity of the beta cells (Tables C.2 5 and C.2 6). These results were supported by insulin and C peptide data, especially in the X002 BID group, which had the highest mean posttreatment changes in insulin and C peptide values despite having had the lowest mean baseline fasting blood glucose values. Other pharmacodynamic effects induced by X002 were suppression of glucagon after test meals, and decreases in the gastric emptying rate (Table C.2 7).

Conclusions

X002 administered QD and BID caused a clear attenuation of the rise in blood glucose induced by standardized test meals at all doses up to 20 μg, which was the highest dose tested and was the highest well tolerated dose for most patients. These findings were supported by secondary pharmacodynamic data, in particular data for HbA1c and beta cell function (Figs. C.2 2 C.2 4).

CRITICAL ASSESSMENT OF THE METHOD

The selection of the pharmacodynamic methodologies applied in the presented study was facilitated by a huge body of published data for the pharmacodynamic properties of the compound class of X002. In preclinical and a first single dose clinical study in patients, X002 was already characterized as a glucagon like peptide (GLP) 1 agonist. GLP 1 is an endogenous hormone secreted from intestinal L cells to orally ingested nutrients and has substantial pharmacodynamic effects on the endocrine pancreas, on the gastrointestinal tract, and in the brain (Ritzel et al. 1995; Deacon 2004).

X002, as GLP 1 agonist, was therefore expected to stimulate meal induced insulin secretion, to delay gastric emptying, and eventually to lower blood glucose. With the aim to take advantage of the potential dual mechanism of X002 on intestinal glucose absorption and glucose stimulated insulin secretion, both considered to contribute to blood glucose lowering effects in a type 2 diabetic population, the presented study included an oral glucose challenge.

Regarding an oral glucose challenge, there are several options available such as the classical oral glucose tolerance test (as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water) (Ref WHO/NCD/NCS/99.2), a liquid mixed meal (e.g., Sustacal, Ensure), or a standardized solid mixed meal. Since the latter was considered to mainly reflect daily life and since it was hypothesized that the delaying effect on gastric emptying of X002 is maximized by ingestion of solid food, a test breakfast was implemented in the presented study. The standardized breakfast was administered 15 min after administration of the test drug. The caloric value per meal ranged to 450 kcal, with 50% carbohydrates, 23% fat, and 27% protein (2 ¼ slices of white bread, 45 g low fat cottage cheese, one teaspoon

Table C.2-4

Changes in HbA1c on Day 29 relative to baseline (mITT population)

HbA1c (%)						
		Adjusted mean ± SE				
Dose level (μg)	*N*	Baseline mean	Change from baseline	Difference vs. placebo	95% CI of difference	*p*-value
Placebo	20	8.87 ± 0.239	0.61 ± 0.159			
X002 QD	19	8.54 ± 0.245	1.17 ± 0.161	0.550 ± 0.227	(1.004; 0.096)	0.0184
X002 BID	21	8.38 ± 0.233	1.16 ± 0.154	0.550 ± 0.223	(0.997; 0.103)	0.0169

Differences vs. placebo are calculated as X002 minus placebo

CI confidence interval, *mITT* modified intention to treat, *N* number of evaluable patients, *SE* standard error

Table C.2-5

Changes in insulin sensitivity parameters from homeostasis model assessment relative to baseline (mITT population) beta cell function

Insulin sensitivity parameters (μIU/ml)/(mmol/l)						
Parameter Treatment group Dose level (μg)	N	Adjusted mean ± SE			95% CI of ratio	*p*-value
		Baseline	During treatment	Ratio to placebo		
Beta cell function						
Placebo						
10.0 μg	21	70.04 ± 1.123	70.14 ± 1.171			
20.0 μg	21	70.04 ± 1.123	84.91 ± 1.139			
Highest individually well-tolerated dose	21	70.04 ± 1.123	84.91 ± 1.139			
X002 QD						
10.0 μg	15	58.39 ± 1.147	102.40 ± 1.201	1.46 ± 1.274	(0.90; 2.38)	0.1245
20.0 μg	15	58.39 ± 1.147	115.73 ± 1.162	1.36 ± 1.220	(0.91; 2.03)	0.1265
Highest individually well-tolerated dose	15	58.39 ± 1.147	115.73 ± 1.162	1.36 ± 1.220	(0.91; 2.03)	0.1265
X002 BID						
10.0 μg	14	46.24 ± 1.152	113.97 ± 1.215	1.62 ± 1.293	(0.97; 2.73)	0.0655
20.0 μg	14	46.24 ± 1.152	109.98 ± 1.174	1.30 ± 1.236	(0.85; 1.98)	0.2278
Highest individually well-tolerated dose	14	46.24 ± 1.152	109.98 ± 1.174	1.30 ± 1.236	(0.85; 1.98)	0.2278

margarine, ¼ teaspoon mayonnaise or olive oil, 100 g fruit yogurt, one apple or peach).

This test meal was specifically adapted to South African habits. In an US study with a similar study design and duration, the test meal was composed of one bagel, one cheese slice, orange juice, soft corn margarine, and 2% milk corresponding to 55% carbohydrates, 15% protein, and 30% fat (Fineman et al. 2003) underlining the flexibility in selecting the components of a test meal according to country specific habits and the target population included in the study.

At the time X002 was studied, very limited information was known in patients about the magnitude of the glucose lowering effect, the onset and the duration of the pharmacodynamic activity, safety and tolerability, and no clinical information was available about the pharmacokinetic profile. In addition, first clinical data with single doses suggested the maximum tolerated dose in the range of 10 μg X002 with nausea and vomiting as dose limiting adverse events. On the other hand, data came up suggesting that dose titration of GLP 1 agonists may improve gastrointestinal tolerability (Fineman et al. 2004).

The constellation of the data resulted in a dose escalation design using a starting dose 50% of the potential MTD and slowly increases to a maximum dose of 20 μg (which was eventually well tolerated in most of the included patients at the end of the 4 week study). Blood glucose after a test breakfast was monitored up to the lunchtime and glucose was analyzed as under the curve (AUC) considered to maximize the chance to detect pharmacodynamic

Table C.2-6

Changes in insulin sensitivity parameters from homeostasis model assessment relative to baseline (mITT population) insulin resistance

Insulin sensitivity parameters (μIU/ml)/(mmol/l)						
Parameter Treatment group Dose level (μg)	*N*	Adjusted mean ± SE			95% CI of ratio	*p*-value
		Baseline	During treatment	Ratio to placebo		
INSULIN RESISTANCE						
Placebo						
10.0 μg	21	6.79 ± 1.125	6.49 ± 1.101			
20.0 μg	21	6.79 ± 1.125	5.74 ± 1.092			
Highest individually well-tolerated dose	21	6.79 ± 1.125	5.74 ± 1.092			
X002 QD						
10.0 μg	16	5.18 ± 1.145	5.67 ± 1.114	0.87 ± 1.158	(0.65; 1.17)	0.3611
20.0 μg	16	5.18 ± 1.145	4.75 ± 1.104	0.83 ± 1.144	(0.63; 1.08)	0.1655
Highest individually well-tolerated dose	16	5.18 ± 1.145	4.75 ± 1.104	0.83 ± 1.144	(0.63; 1.08)	0.1655
X002 BID						
10.0 μg	15	4.72 ± 1.150	4.79 ± 1.120	0.74 ± 1.164	(0.54; 1.00)	0.0513
20.0 μg	15	4.72 ± 1.150	4.68 ± 1.109	0.82 ± 1.149	(0.62; 1.08)	0.1492
Highest individually well-tolerated dose	15	4.72 ± 1.150	4.68 ± 1.109	0.82 ± 1.149	(0.62; 1.08)	0.1492

Ratios to placebo are calculated as X002/placebo

CI confidence interval, *mITT* modified intention to treat, *N* = number of evaluable patients, *SE* standard error

properties of X002 if present. As demonstrated by the study results, AUC of glucose after a test breakfast was a robust marker to establish the potency of PDY effect of X002. However, although the slow dose titration turned out to improve tolerability and the pharmacodynamic effect was clearly demonstrated, the selection of the dose regimen for the confirmatory dose range trial based on the 4 week trial alone became a challenge (as described in the next chapter). A dose titration in steps of 2.5 μg every fifth day up to 20 μg was considered not applicable for an ambulatory study in patients. Once and twice daily were equally effective and the highest dose was administered for only 4 days. This led eventually to the need of doing a dose range finding study with a significant number of dose levels to avoid missing the dose with the best benefit/risk ratio. Whenever possible, the dose regimen in a PDY study serving proof of concept should be as close as possible to Phase IIb requirements.

X002 showed from the very first dose a pronounced pharmacodynamic effect with nearly complete attenuation of the rise of postprandial blood glucose after the test meal. Due to the early onset of blood glucose lowering effects, there was also a significant reduction of HbA1c for both dose regimens of X002, despite the only 4 week treatment within the study. It should be kept in mind that for com pounds with later onset of action close to the pharmaco dynamic profile of metformin or glitazones, the detection of effects on HbA1c would require at least a 3 month study.

As a GLP 1 agonist, X002 has insulin releasing prop erties as clearly shown for the compound class. However, due to the pronounced pharmacodynamic effect on postprandial blood glucose of X002, the postprandial serum insulin levels in patients on X002 were lower compared to the placebo group. This phenomenon has been also described in other studies within the

Table C.2-7

Changes in gastric emptying parameters relative to baseline (mITT population)

Gastric emptying (minutes)								
Parameter	Baseline value				Change from baseline			
Treatment group Dose level	N	Mean ± SD	Median	Range	N	Mean ± SD	Median	Range
t_{lag}								
Placebo								
10.0 µg	17	150.6 ± 53.12	134	94 284	17	10.6 ± 72.71	7	115 186
Highest individually well-tolerated dose	17	150.6 ± 53.12	134	94 284	17	13.7 ± 66.40	3	168 93
X002 QD								
10.0 µg	17	150.6 ± 56.31	125	90 270	17	106.6 ± 106.67	78	81 274
Highest individually well-tolerated dose	17	150.6 ± 56.31	125	90 270	17	130.8 ± 158.07	136	79 355
X002 BID								
10.0 µg	14	154.9 ± 65.12	146	69 307	13	102.7 ± 102.77	79	29 313
Highest individually well-tolerated dose	14	154.9 ± 65.12	146	69 307	14	121.8 ± 126.71	122	89 331
$t_{1/2}$								
Placebo								
10.0 µg	17	237.9 ± 95.12	199	143 520	17	4.6 ± 134.02	8	290 300
Highest individually well-tolerated dose	17	237.9 ± 95.12	199	143 520	17	24.1 ± 133.13	2	382 186
X002 QD								
10.0 µg	17	245.3 ± 103.04	198	133 499	17	169.0 ± 176.65	189	260 393
Highest individually well-tolerated dose	17	245.3 ± 103.04	198	133 499	17	211.5 ± 278.50	250	233 588
X002 BID								
10.0 µg	14	235.6 ± 113.86	197	104 511	13	189.5 ± 190.61	229	51 670
Highest individually well-tolerated dose	14	235.6 ± 113.86	197	104 511	14	202.3 ± 229.98	197	162 532

mITT modified intention to treat, *N* = number of evaluable patients, *SD* standard deviation

compound class (Fineman et al. 2003), demonstrating that the isolated evaluation of serum insulin levels without consideration of the prevailing blood glucose at the time insulin was determined could lead to puzzling constellations.

Gastric emptying time was determined by adding 100 mg 99% ^{13}C octanoic acid to the solid test breakfast and analyzing the $^{13}C/^{12}C$ ratio in collected breath, as described by Zahn (Zahn et al. 2003). Despite a high variability, the methodology turned out to have nearly no interference with other study specific measures and could be easily implemented in the investigation schedule. As seen by the data, the methodology was specific enough to reveal the delaying effect of X002 on gastric emptying even with a sample size as low as 20 patients per arm.

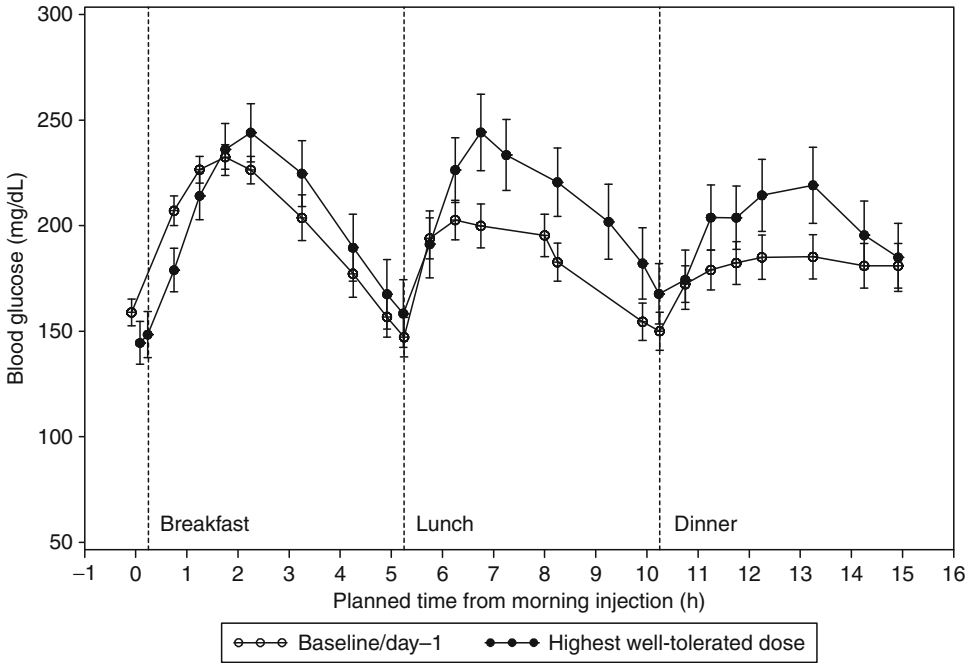

Figure C.2-2

Mean ± SE blood glucose values in the *placebo* group at baseline and at the highest well-tolerated dose (mITT population)

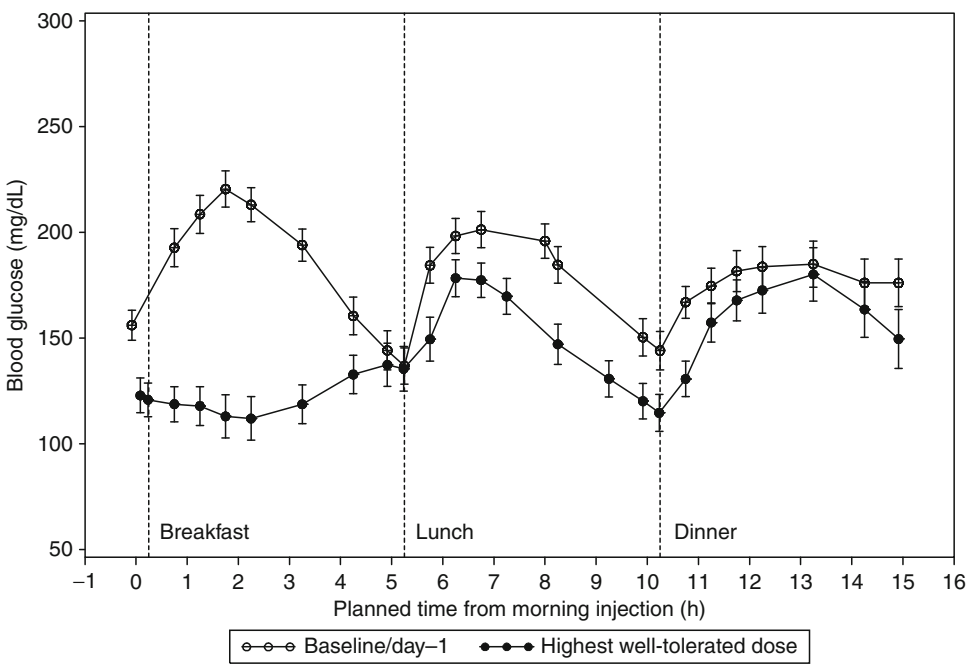

Figure C.2-3

Mean ± SE blood glucose values in the *X002 QD* group at baseline and at the highest well-tolerated dose (mITT population)

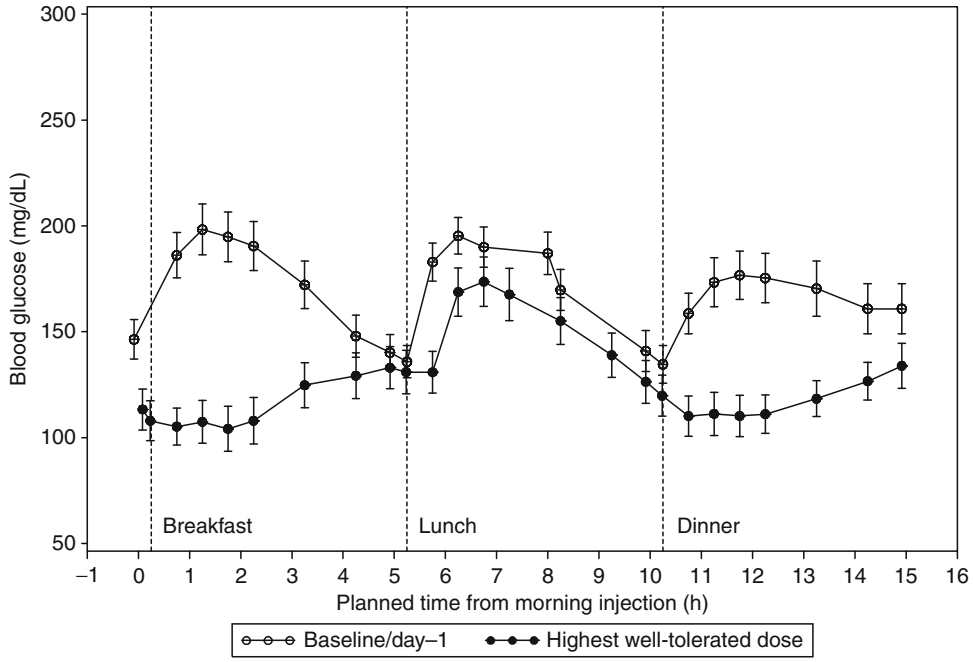

Figure C.2-4

Mean ± SE blood glucose values in the *X002 BID* group at baseline and at the highest well-tolerated dose (mITT population)

MODIFICATIONS OF THE METHOD

Although a solid test meal is close to the conditions of daily life and thus in many studies, a viable option there may be situations in which a solid test meal is not appli cable or the preferred measure:

- If a study is intended to use a glucose challenge for screening/diagnosis of type 2 diabetes, the preferred methodology is the standard 75 g glucose tolerance test such as described by the WHO.
- A standardized solid test meal could become a chal lenge for international studies with different ethnici ties and eating habits. In this setting, a commercially available liquid meal test (e.g., Sustacal) could be an option to standardize across the studies.
- If a study is intended to investigate the effects directly on insulin secretion, the oral glucose challenge should be replaced by an intravenous glucose tolerance test to limit the inter and intraindividual variability related to the intestinal glucose absorption. An overview of the methodology is provided by Fehse (Fehse et al. 2005).

The methodology of the 13C octanoic acid breath test for determination of gastric emptying time should be considered an exploratory test. Due to the easiness of sample collection, it can be implemented in most of the studies without significantly impacting the schedule. The reference methodology, however, is the radionuclide study by ingesting a test meal containing the radiolabel (usually based on technetium). The methodology is by far more demanding with the need of a monitoring of gastric emptying by gamma camera for several hours. Under those conditions, an impact on additional study related procedures is likely. Therefore, scintigraphic assessment should be limited to those studies with a strong need of gold standard data for gastric emptying. An overview of the methodology is given elsewhere (Donohoe et al. 2004).

Although in the presented study, the slow dose titra tion turned out to improve tolerability and the pharma codynamic effect was clearly demonstrated, the selection of the dose regimen for the confirmatory dose range trial based on the 4 week trial alone became a challenge. A dose titration in steps of 2.5 µg every fifth day up to 20 µg was not applicable for an ambulatory study in patients. Once and twice daily were equally effective, and the highest dose was administered for only 4 days. This led eventually to the need of doing a dose range finding study with a large number of dose regimens to avoid missing the dose regi men with the best benefit/risk ratio. Therefore, whenever

possible, the dose regimen in a PDY study serving proof of concept should be as close as possible to Phase IIb requirements.

XOO2 was shown to lower HbA1c as early as 4 weeks. It should be kept in mind that for compounds with later onset of action close to the pharmacodynamic profile of metformin or glitazones, the detection of effects on HbA1c would require at least a 3 month study. Alternatively, one should consider to use fructosamine instead of HbA1c, reflecting the metabolic status of the last 2 3 weeks.

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C.3 Pharmacodynamic Drug–Drug Interactions

Markus Hinder

C.3.1 General Considerations

Pharmacodynamic drug drug interactions occur when one drug (A) alters the effects of another drug (B) without affecting its pharmacokinetics.

Thus, pharmacodynamic drug drug interactions dif fer from pharmacokinetic drug drug Interactions in that they do not interfere with liberation, absorption, distribu tion, metabolism, or excretion (LADME) processes. Pharmacodynamic drug drug interactions can occur (a) at the receptor, (b) signaling (e.g., second messenger), or (c) effector levels (*Fig. C.3 1a d*). They can lead to both enhanced (additivity/synergism) or diminished (infra additivity/antagonism) drug responses. The investigation of pharmacodynamic drug drug interactions is of partic ular interest in situations when drugs likely to be co administered have similar mode of actions or have similar or opposing pharmacodynamic effects.

Pharmacodynamic drug drug interactions are usually being observed under the conditions of polypharmacy, typically in a multimorbid elderly population receiving multiple medications, but also under highly controlled conditions like general anesthesia where traditionally sev eral drugs are being combined (Seymour and Routledege 1998; Bentué Ferrer et al. 2003; Trujillo and Nolan 2000; Scheen 2005; FDA 2006; EMEA 1997). In contrast to the typical pharmacokinetic interaction programs, pharma codynamic interactions are less frequently studied during drug development. Pharmacodynamic drug drug interac tions can be both potentially useful (as shown and utilized in the treatment of infective, cardiovascular and metabolic diseases and cancer) and harmful. Although they occur in a therapeutic context less frequently as compared to phar macokinetic interaction, they can be of paramount impor tance for the patient and drug therapy in general (Seymour and Routledege 1998; Bentué Ferrer et al. 2003; Trujillo and Nolan 2000; Scheen 2005; FDA 2006; EMEA 1997; Cheitlin et al. 1999; Alain et al. 1998; Minto et al. 2000; Haverkamp et al. 2000).

Starting in the 1930s, in the area of experimental sciences, both in vitro and in vivo, a large number of highly sophisticated conceptual tools to study pharmaco dynamic interaction have been described over the last decades (Loewe and Muischnek 1926a; Pöch and Juan 1985; Tallarida 2001a; Chou 2006; Danhof et al. 2007). However, the application and the transfer of these methods to *clinical investigations* have been very limited. The complexities associated with the investigation of drug combinations in clinical trials have multiple reasons: (1) patient populations bear a high variability due to differences in gender, age, race, stage of disease state, and past treatments; (2) in many instances, it is ethically not defendable to use placebo or suboptimal doses as required to answer the scientific question.

Due to these complexities and limitations, this book chapter will not provide an exhaustive review on the complete armamentarium of all available methods. Techniques exclusively suitable for preclinical analyses will not be presented. Tallarida, Chou and Danhof and coworkers (Tallarida 2001a; Chou 2006; Danhof et al. 2007) provided excellent articles covering the com plete field of presently available tools. Thus, this book chapter will focus on methods that are applicable to clin ical trials:

C.3.2 Dose/Concentration Effect Curve Analysis

PURPOSE AND RATIONALE

The analysis of dose/concentration effect curves (DCEC) is the basic pharmacological tool to determine the prop erties of substance A alone and in the presence of another substance B (Ross and Kenakin 2001).

PROCEDURE

Available dose/concentration data of substance A are being plotted against effect on a linear linear scale. Subse quently, the dose/concentration data are log transformed and a corresponding log linear graph is drawn (*Fig. C.3 2a*). Adding data to these graphs from

H. G. Vogel, J. Maas, A. Gebauer (eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology*, DOI 10.1007/978-3-540-89891-7 C.3,

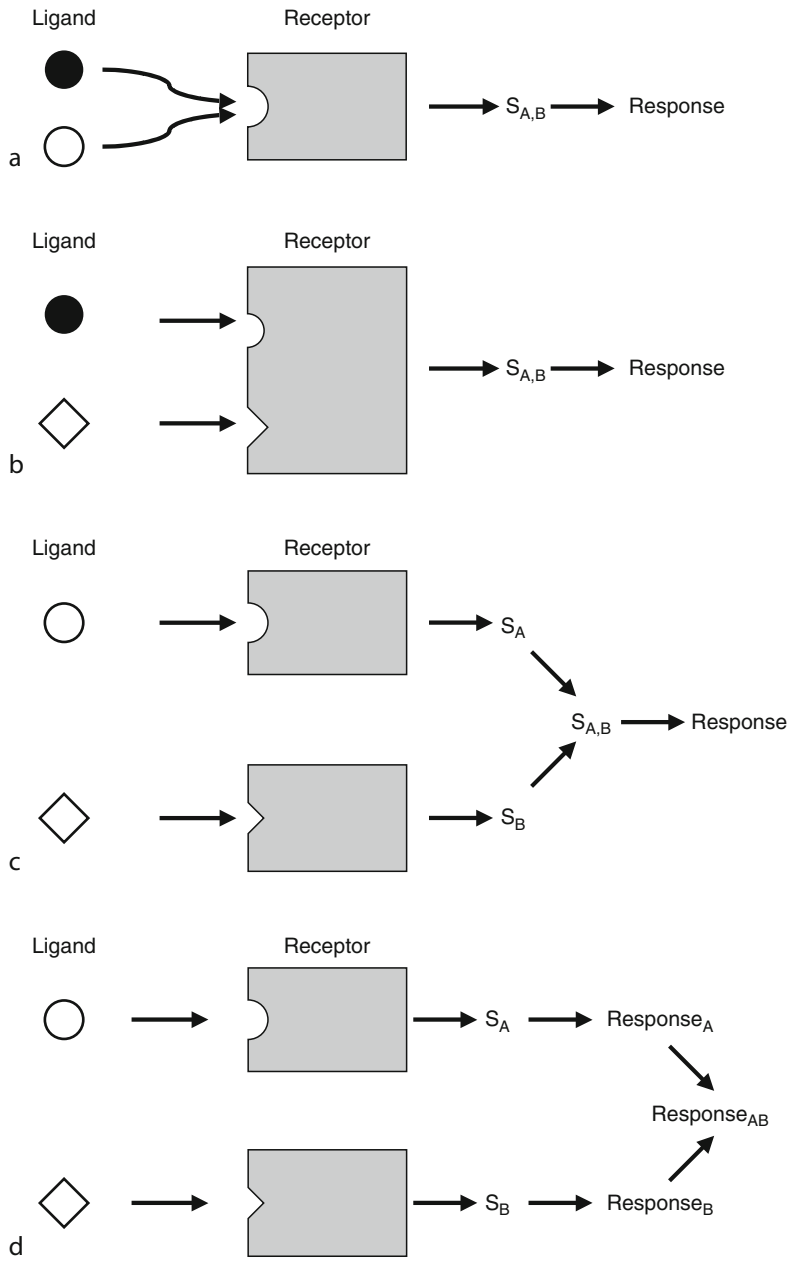

Figure C.3-1
Different mechanisms for pharmacodynamic drug-drug interactions

investigations in the presence of substance B allows exploring the interactions between substances A and B.

EVALUATION

The initial evaluation of the graphs is purely descriptive. The determination of the maximum effect (Emax) and the dose or concentration at which half of the maximum effect is observed (ED50/EC50) allows to principally describing the dose/concentration effect relationship in the presence or absence of substance B. If the maximum effect (Emax) of A in the presence of B is increased, an additive or synergistic effect can be assumed. On the contrary, if the maximum effect (Emax) of A in the presence of B is decreased, a noncompetitive antagonistic effect can be

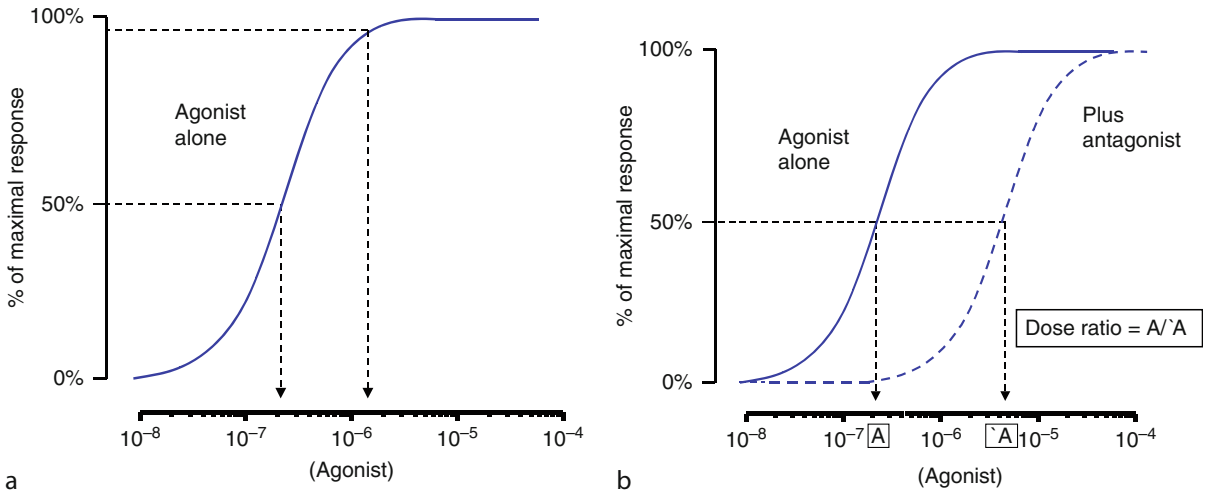

Figure C.3-2
Semilogarithmic dose/concentration effect curves

assumed. The location of the ED50/EC50 value for the combination of A and B, relative to the ED50/EC50 value, provides information if either synergism (leftward shift of the combined dose effect curve) or competitive antagonism (rightward shift of the combined dose effect curve [*Fig. C.3 2b*]) is present.

For further mathematical description of the linear part of the log linear DECE, the Hill equation can be used:

$$E = m \times \log C + b$$

Symbols: *m*: slope of the DECE, *C*: drug concentration, *b*: intercept on the x axis

CRITICAL ASSESSMENT OF THE METHOD

Although the pharmacological principles underlying pharmacodynamic drug drug interactions are well understood and described, their transfer and application in the field of clinical pharmacology poses some problems: Classical pharmacological in vitro and in vivo experiments allow a thorough mechanistic investigation and description of the complete dose/concentration effect relationship, including the maximum effect size (Emax) and doses/concentrations at which half of the maximum effect occurs (ED50/EC50) (*Fig. C.3 2a*). By means of these parameters, mechanistic investigations on drug synergism and antagonism are possible (left or rightward shift of dose response curve or increase or decrease of Emax [*Fig. C.3 2b*]) (Ariens et al. 1956; Berenbaum 1978; Greco et al. 1995).

The use of these methods in the field of clinical pharmacology and therapeutics is often technically limited by the fact that the determination of the full dose/concentration effect relationships is in most of the cases for ethical and safety reasons not possible. Nevertheless, the partial analysis of the DECE provides valuable information, which in conjunction with more complete and mechanistic investigations from preclinical sciences can either confirm or refute previous hypotheses.

C.3.3 Isobolograms

PURPOSE AND RATIONALE

Loewe and Muischnek introduced the concept of the isobologram in 1926 (Loewe and Muischnek 1926b; Loewe 1953). By constructing an isobologram, it is possible to graphically analyze drug drug interactions as being additive, supra additive/synergistic or sub additive/antagonistic. The isobologram is constructed of isoboles (iso = equal, bol = effect) indicating equipotent combinations of different doses of two substances (A and B) revealing the same preset effect size (Tallarida 2001b, 2006; Gessner 1995).

PROCEDURE

Derived from conventional dose/concentration effect curves (DCEC) for both substance A and substance B,

the dose/concentration of the half maximal effect (ED50/EC50) is determined (*Fig. C.3 3a* and *b*). Subsequently, combinations of the two substances A and B are tested and titrated to reveal half maximal effect and the respective dose combinations are recorded.

EVALUATION

Doses/concentrations of substance A and B needed to elicit 50% of response are plotted in a Cartesian graph, as illustrated in *Fig. C.3 3b*. The connecting line for the ED50/EC50 of substance A and B denotes the zero interaction line (or expected additivity) Based on the location of the experimentally determined data points, either lying on, below or above the zero interaction line, additivity (on), supra additivity/synergism (below) or sub additivity/antagonism (above) can be concluded (Tallarida 2001b, 2006; Gessner 1995). *Figure C.3 3c* provides an illustration of possible outcomes of the graphical display.

CRITICAL ASSESSMENT OF THE METHOD

The isobologram in its descriptive form provides hints on interactions and allows for preliminary inferences. Due to the variability inherent to biological measurements, however, there is no general rule how far data points need to be to conclude on non additivity. In that respect, the isobolographic method can, in the statistical sense, neither prove nor disprove an interaction (Tallarida 2001b, 2006).

MODIFICATIONS OF THE METHOD

In order to increase the reliability of the inferences drawn from the isobologram, different authors have used confidence intervals for the description of the data points, allowing for a statistical differentiation of the data points and thereby increasing the precision by which the decision on sub or supra additivity is made (Tallarida 2001b, 2006; Gessner 1995).

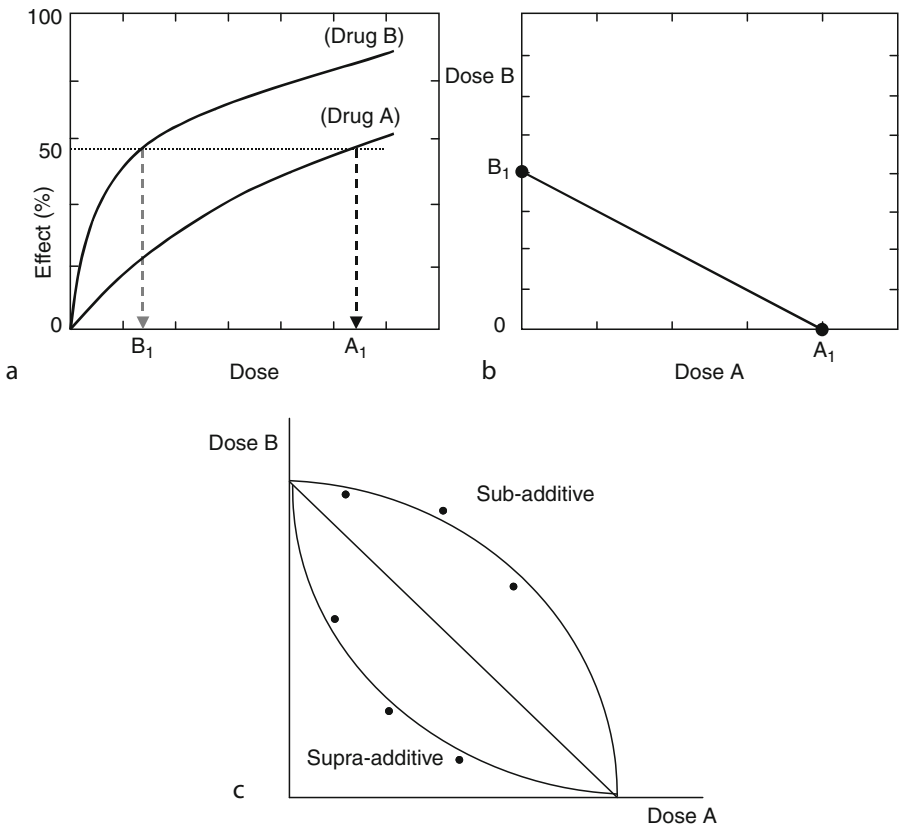

Figure C.3-3
Determination of ED50, zero interaction line and experimental data

Moreover, although usually in experimental set tings, the 50% isobole of the effect is determined and serves as a reference for the conclusions, the construc tion of other isoboles (10%, 90%, etc.) is feasible (Tallarida 2001b, 2006). This can be of special interest for the clinical application when the investigation of only a part of the full dose range is accessible, but an isobolographic analysis is desired. Montes et al. (2000) have used a modification of this principle in postoperative analgesia to demonstrate a synergism between tramadol and metamizol. They allowed postoperative pain patients to titrate their blinded analgesic medication to satisfactory pain control (without pre setting the Edx value) and then determined graphically the position of the different doses required alone and in different mix tures of the combination. Filitz and coworkers were able to demonstrate a supra additive effect of the tramadol acetaminophen combination in human pain models in healthy volunteers for a 12% pain reduction (isobole). A special adaptation with secondary clinical utility comes from the area of anti infectives and chemotherapy. Parsley and coworkers and more recently Nakornchai et al. have tested in vitro the combinations of two antibiotics and the combination of antibiotics and antimalarials, respectively. Although determined in vitro, these data bear great clinical relevance as by comparisons between human pharmacokinetics and in vitro data, first hypoth eses can be built to be further tested in clinical efficacy trials (Parsley et al. 1977; Nakornchai and Konthiang 2006).

C.3.4 Schild Plots

PURPOSE AND RATIONALE

This method uses the phenomenon of the rightward shift of the dose/concentration effect curve (DCEC) of sub stance A in the presence of different doses/concentrations of an antagonist B. The original method stems from experimental pharmacology originally described by Heinz Otto Schild in 1947 (Schild 1947). The method allows to quantitatively characterize the potency of an antagonist by using linear regression (Schild 1947; Arunlakshana and Schild 1959).

PROCEDURE

The antagonist B is being administered in separate exper iments. In addition, increasing doses/concentrations of the agonist A are being administered to determine the ED50 value. The parallel rightward shift of the DCEC in the presence of increasing doses/concentrations of the antagonist is graphically plotted (*Fig. C.3 4a*). The ratio of concentrations needed to elicit the same response in the presence and absence of the antagonist is the dose ratio (DR).

$$\mathrm{DR} = \mathrm{ED50[withAntagonist]/ED50[withoutAntagonist]}.$$

As in principle, the construction of this equation is possible using both concentrations and doses and can be determined at any given effect size; the generic equation is:

$$\mathrm{Ratio} = \mathrm{ECX[withAntagonist]/ECX[withoutAntagonist]}.$$

The Schild plot is constructed by plotting the log of the antagonist concentration (log [antagonist]) on the abscissa versus the log of the dose ratio 1 (log [DR 1]) on the ordinate (insert in *Fig. C.3 4*).

The intercept of the linear regression line to the abscissa denoted the pA2 value and is a measure or the potency of the antagonist.

Mathematically, the pA2 value can be obtained as:

$$\mathrm{pA2} = -\log(\mathrm{Antagonist}) + \log(\mathrm{DR} - 1)$$

EVALUATION

In addition to the estimation of the potency of an antag onist, the determination of pA2 allows some quantitative assessment of the antagonism. If the slope of the regression line is linear and approximately 1, a competitive antago nism can be concluded (*Figure C.3 4b*).

CRITICAL ASSESSMENT OF THE METHOD

Due to ethical and safety/tolerability considerations, clinical pharmacological studies can only deliver data from a limited segment of the dose effect curves, thus frequently missing the maximum response (Emax). DRs in clinical studies are usually determined from doses/ concentrations below the ED50/EC50. Thus, these analyses often have to be performed outside the linear part of the log concentration effect curve. In order to describe the dose/concentration effect relationship, nonlinear fitting techniques are required.

Identification of noncompetitive antagonism usually requires the demonstration of a reduced Emax. Under experimental conditions, when safety/tolerability questions do not impose dose limitations, this can easily be deter mined. In contrast, under clinical conditions, it is only

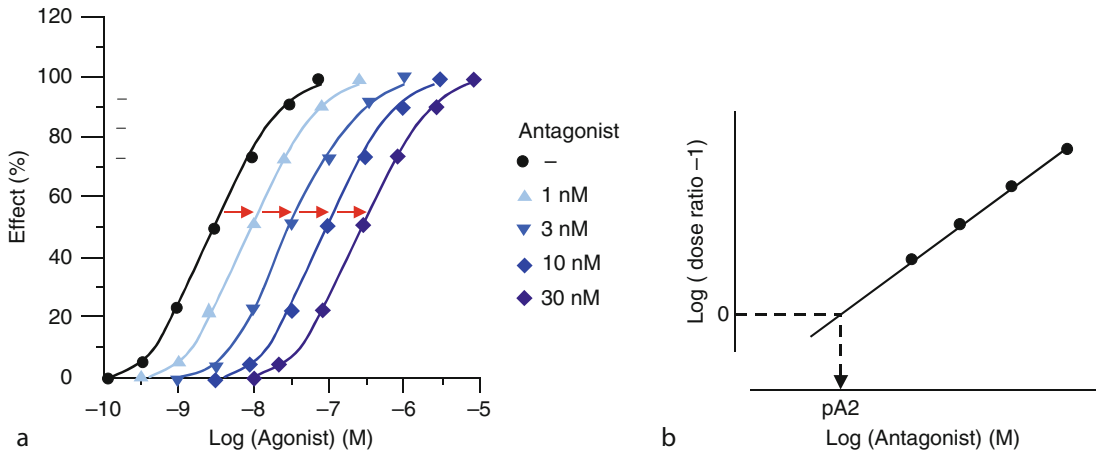

Figure C.3-4
Rightward shift of the DCEC and Schild plot

rarely possible to study maximum agonistic responses. Therefore, the assessment of noncompetitive antagonism based on the decrease of Emax is nearly impossible.

A major advantage of the Schild plot is its indepen dence of confounding factors (counter regulation, physi ological reflexes). Wellstein, Belz, and coworkers have been using this method extensively in the study of new cardio vascular drugs like beta adrenoceptor blockers (Wellstein et al. 1985), angiotensin converting enzyme inhibitors (Wellstein et al. 1987; Essig et al. 1989), and angiotenin II antagonists (Belz et al. 2002). Using the Schild analysis technique allowed differentiation of the duration of action, the derivation of Ki, and subsequently of thera peutic doses.

MODIFICATIONS OF THE METHOD

Theoretically, a construction of dose ratios (DR) and subsequently Schild plots at any given ECx or EDx value are possible.

C.3.5 Factorial Design Trials

PURPOSE AND RATIONALE

Factorial design trials can simultaneously test the influ ence of several variables on one outcome. In the context of clinical pharmacology, the variables often are differ ent treatments or doses and the outcome usually is a clinical parameter (e.g., blood pressure, serum choles terol, myocardial infarction, death, etc.). Factorial design trials are of major importance for clinical trials in which combination treatments, co medications, or fixed combinations are to be assessed. Apart from their scientific merits, factorial designs constitute in many instances a regulatory requirement for testing and the documentation of the rational for combination drugs (EMEA 2004, 2002; FDA 2008, 2006). One of their big advantages is that they can answer two or more questions in one trial with lower sample sizes than a respective parallel group study.

PROCEDURE

In a factorial design trial, participants are allocated to receive one (treatment A) or the other (treatment B) or both (combination of both A and B) or neither interven tion (often placebo or gold standard treatment). *Figure C.3 5a* provides a tabular illustration of the simplest version of a 2 × 2 factorial design. In the case of a 2 × 2 factorial design, the influence of two separate factors that can have two levels/degrees (e.g., present or absent) on one outcome are investigated. In a 2 × 2 design, four different treatment groups can be distinguished: (1) patients receiving both A and B (cell AB), (2) A alone (cell A0), (3) B alone (cell B0), (4) neither A nor B (cell 00). Published example of this design includes the TETAMI study (Cohen et al. 2000, 2003), which tested the effect of a low molecular weight heparin (LMWH) versus a standard heparin (UFH) with and without accompanying platelet inhibition with Tirofiban (TIRO). The possible combinations in this 2 × 2 factorial design are: LMWH + TIRO (1), UFH + TIRO (2), ENOX alone (3), UFH alone (4) (*Fig. C.3 5b*).

a

		Treatment B: Yes	Treatment B: No	Margin
Treatment A	Yes	Both A and B (cell AB)	A alone (cell A0)	All A (cells AB and A0)
	No	B alone (cell B0)	Neither A nor B (cell 00)	All non-A (cells B0 and 00)
Margin		All B (cells AB and B0)	All non-B (cells A0 and 00)	

b

	LMWH	UFH
TIRO	Patients treated with LMWH + TIRO (1)	Patients treated with UFH + TIRO (2)
Ø TIRO	Patients treated with LMWH alone (3)	Patients treated with UFH alone (4)

c

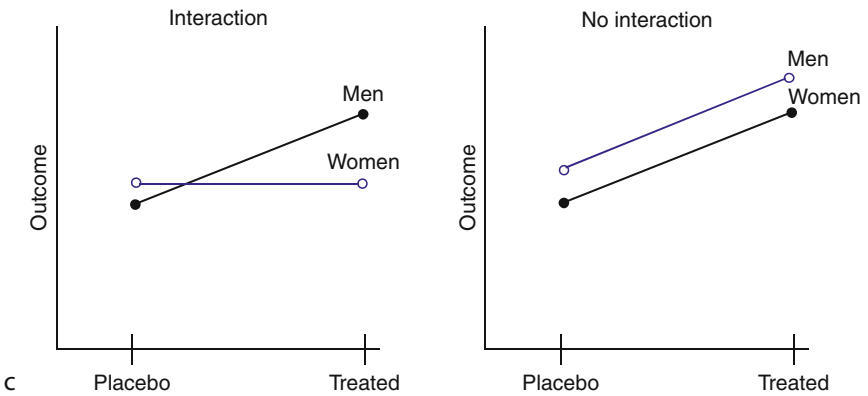

Figure C.3-5
Tables for factorial designs and graphical interaction analysis

EVALUATION

A first graphical evaluation of the arithmetical mean values for the individual cells (EMEA 2002, 2004; FDA 2006, 2008) can be plotted in an interaction plot. *Figure C.3 5c* provides an example for the presence or absence of an intervention in both men and women. If the connecting lines of the mean values are parallel, no interaction is detected; nonparallel lines are indicative of an interaction.

The mathematical analysis treats the factorial design study as two separate trials. All patients receiving A (i.e., cell AB + cell A0) are compared with those who did not

(i.e., cell B0 + cell 00). Likewise, all those given B (i.e., cell AB + cell B0) are compared with those who were not given B (cells A0 and 00). This evaluation taking into account all treatment data generated is called calculation "at the margins" or calculation of the "main effect" (Lubsen and Pocock 1994). In case of supra additive or sub additive interactions, this approach, however, is not adequate (Grant et al. 2005). Here the calculation of the "simple effects"/"inside the table" needs to be carried out (i.e., cell A0 vs. cell 00 and cell B0 vs. cell 00). By determining the ratio and its 95% confidence interval (95% CI) for the respective results of the main effects and the single effects, these data are test for an interaction effect. Margins usually accepted for the conclusion of "no interaction" are, in line with bioequivalence considerations, between 80% and 125% for the 95% CI.

CRITICAL ASSESSMENT OF THE METHOD

Testing the combination of two drugs (A and B) in a parallel arm study would necessitate a study ade quately powered for four parallel treatment groups (placebo of drug A plus drug B; drug A plus placebo of drug; drug B plus placebo of drug A; drug A plus drug B). The advantage of the factorial design is that it uses all participants from both analyses (A and B). Moreover, by use of the single effects and the combined study effects, this design offers additional possibilities of analysis.

Once an interaction is suspected and is the formal aim of the factorial design study, this study needs to be ade quately powered for the interaction analysis, i.e., the indi vidual cells need to be powered to be compared pair wise as single effects/inside the table. Failure to consider this in the planning stage may under power the study to detect an interaction even if it was present (McAlister et al. 2003). The scientific potential of this approach can be enhanced by including different doses of drugs A and B into the trial, i.e., expending the design from a 2×2 to a 3×3 or 4×4 design.

Thus, in conclusion the factorial design bears some more complexity in the design and planning stage, will however compensate for this with the ability to answer several scientific questions within one trial. The large number of clinical trials employing this methodology, in large mortality , epidemiological but also smaller dose finding trials, reflects the potential of this methodology (Grant et al. 2005; Goldberg et al. 2004; Pordy 1994; Scholze et al. 1993; ONTARGET Investigators 2008; Yusuf et al. 2000; Cassidy et al. 2006; Apfel et al. 2004; Robbins et al. 2003; D'Aquila et al. 1996).

MODIFICATIONS OF THE METHOD

Factorial designs can investigate more than two variables and more than two levels. The nomenclature convention determines that the figures denote the number of levels of the independent variable, whereas the number of figures denotes how many independent variables are being investigated. Thus, a $3 \times 2 \times 3$ design investigates three independent variables of which the first and the third have three levels and the second has two levels.

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C.4 Methodologies of Safety Assessment in Clinical Pharmacology

Werner Seiz

C.4.1 Introduction/General Considerations

An important objective of clinical pharmacology is the early and ongoing assessment of the safety and tolerability of a new drug. This is done by assessing the type, frequency, and severity of side effects, assessing in which patient population these side effects may occur at which dose or exposure, for what duration and whether these side effects are reversible. The terminology for the safety assessment of drugs has some specifics that need to be explained right at the beginning of this chapter.

C.4.1.1 Definition of Adverse Events as the Parameter to Assess Safety

The term "side effect" used for marketed drugs is replaced by the term "adverse event" in studies with investigational drugs. An adverse event is defined as any unfavorable and unintended sign, symptom, or disease temporally associated with the use of a drug, whether or not considered related to the drug.

In this chapter the term "adverse event" is explicitly also used for any abnormal laboratory value, as the consequence of abnormal values will be evaluated in the same scheme as for clinical adverse events.

The term "treatment related" is often added as a modifier in order to remove preexisting conditions from consideration. A further term "serious adverse event" is used to describe any untoward medical occurrence that, at any dose, results in death, is life threatening, requires hospitalization or prolongation of an existing hospitalization, results in persistent or significant disability or incapacity, or is a congenital anomaly or birth defect. Serious adverse events have to be reported to the health authorities in an expedited manner, typically.

The severity of an adverse or serious adverse event is classified as either mild, moderate, or severe.

It is important to distinguish between the severity and the seriousness of an adverse event. A severe adverse event is not necessarily serious (e.g., severe abdominal cramps not causing hospitalization), and a serious adverse event is not necessarily of severe intensity (e.g., mild to moderate, prolonged dizziness of an outpatient causing hospitalization) (Herson 2000).

C.4.1.1.1 How to Manage the Safety Assessment of a Drug

One of the most critical steps in the development of a new drug is the first administration of a drug to humans, the first dose escalation, the first multiple dosing, and the first switch from healthy patients to the targeted patient population. In order to acquire the safety data in a responsible way, it is necessary to consider all of the following areas for each clinical study and to plan these items in advance:

- Expect, plan, and manage the occurrence of adverse events. This administrative part of the safety method and includes the selection of the right preclinical animal models for the prediction of the target organ, the definition of the exposure to the drug at the NOAEL, the adequate calculation of the safe starting dose in humans, the decision about the dose escalation and when to stop it, the proper organization of the clinical trial, and the definition of the expected adverse event profile.
- Plan and manage the acquisition of adverse events data. This includes based on the expected adverse event profile the selection of the clinical, technical, and laboratory observations, by which the expected adverse events are to be monitored.
- Plan and manage the interpretation of the adverse events data and their impact on the subsequent development or study conduct. In order to avoid bias the statistical analysis of the safety data obtained has to be predefined, using commonly accepted criteria. For each parameter assessed it should be clear prior to the analysis, which deviation is considered relevant and therefore an adverse event. This is usually done in the statistical analysis plan, which has to be finalized

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prior to closing the database of a study and prior to breaking the randomization code. In clinical pharmacology studies, data monitoring committees (DMC) are typically not included; however, in studies with adaptive designs DMCs might be installed very early in clinical development.

C.4.1.2 Case Study

The importance of an adequate selection of animal models, assessing the significance of the preclinical data obtained for humans, and planning adequately the study conduct in the first in human study has recently (March 2006) been shown quite dramatically. The first dose step in the first in man study with the biotherapeutic TGN1412, a humanized agonistic anti CD28 IgG4 monoclonal antibody (present on regulatory T cells), induced a cytokine release syndrome in all six active treated healthy volunteers, all of which suffered from life threatening, acute shock and subsequent multiorgan failure. At least in one of the participants of the TGN1412 first in man study several fingers and toes were to be amputated finally. Obviously this severe and serious adverse events were not predicted by the animal studies conducted prior to human studies, the dose administered was obviously above the minimum active biological effect level (MABEL) for humans, and all volunteers were already dosed before the first dosed person suffered from the symptoms of the upcoming cytokine release syndrome, that is, within less than 90 min. Although a complete explanation of the event was never unanimously accepted (http://www.circare.org/foia5/clinicaltrialsuspension interimreport.pdf), at least it appears that the drug was given too fast to each subject (3 6 min infusion time) and to too many subjects within too short a time (every 10 min the next subject was dosed) (Horvath and Milton 2009). As a consequence of this event with TGN1412 the regulators worldwide have changed several processes so that this should not happen again.

It is self evident that the evaluation and interpretation of the safety data obtained as a whole is of utmost importance to a drug development program; however, here in this chapter the topic will be the technical description of the most often used clinical, technical, and laboratory methods to acquire safety data and how this will influence decisions on dose escalation or termination of a study. No more thoughts are given to analyze the safety data as a whole and in the context of the already accumulated clinical safety data.

C.4.2 Categorization of Adverse Events for Decision Making

PURPOSE AND RATIONALE

Adverse events should be categorized in the same way across studies so that the decision based on these categories are consistent within a development program and across programs.

PROCEDURE

Each adverse event or finding is classified into one of four categories, whereas grade 1 indicates a deviation from the norm without an obvious relevance for the subject, grade 2 indicates an interference with daily activities for the subject but without need for treatment except non opioid analgetics. Occurence of events of grade 2 have to be seen as an alert on reaching doses, where tolerability to the test compound decreases. Grade 3 indicates that the event or finding requires medical or other treatment or prevents daily activities of the subject. Grade 4 is reserved for definitely inacceptable adverse events, which typically, if not occuring in the placebo group, leads to a termination of the study at least for the dose, where the grade 4 event has been observed (e.g., rhabdomyolysis, angioedema). If there is a rapid change in a parameter, this also might lead to an increase in grading. For laboratory parameters (chemistry, ECG) the grading is done based on the likelihood for further consequences or risks according to the categories above. In order to categorize laboratory values as abnormal they have to be different from the normal range, which is specific to each laboratory. The numbers given here are suggestions and are based on published normal ranges (Kratz et al. 2004).

EVALUATION

For each subject the maximal adverse events' grading can easily be assessed. For gradings of 3 or 4, unblinding is recommended. An individual should not be further dosed if on active drug and grading 3 occurs. If only placebo treated subjects suffer from an adverse event and if this event is not study procedure related, it has no impact on further study conduct. If placebo and active treated patients suffer from grading 3, but less than 50% of active treated subjects, doses should be adapted (=lowered), the number of subjects treated at a time need to be reduced, and the time interval between subjects should be increased, in order to minimize the risk for treated subjects. If the dose step is well tolerated, additional subjects could be treated at the dose with the grade 3 events. Finally, if more than 50% of active treated patients suffer from grade 3 adverse

events at a given dose, the dose below is qualified as the maximal tolerated dose.

CRITICAL ASSESSMENT OF THE METHOD

The categorization of information leads to a loss of information and therefore has to be used with care. Everyone using this method needs to be aware that the full picture and information needs to be taken into account and not just the results from the categorization. The grading system suggested and described here is modified from (Sibille et al. 2010) and not approved by any authority but should be seen as way to consistently aggregate and interpret information. Grading adverse events is in use in oncology and vaccine studies allready (Cancer therapy evaluation program 2009; FDA Guidance 2007). These systems use four or five gradings, where grade 5 is always "death" and grade 4 is mostly of life threatening adverse events, which is not in contradiction with the grading used here.

MODIFICATIONS OF THE METHOD

An alternative to formal categorization of adverse events for subsequent decision making on dose escalation or study progress is the repetitive assessment of the uncategorized clinical and laboratory data by the investigator, the sponsor, and additional experts to achieve a common understanding on how to proceed. The pre categorization of events as described here in this chapter, however, does not prevent such an approach and has the advantage to provide a consistent assessment of the information across dose steps, studies, and compounds.

C.4.3 Decision Making on Dose Increase and to Stop the Study

PURPOSE AND RATIONALE

The decision to stop dose escalation should be based on the observation of adverse events no longer tolerable (by frequency or severity) and by the observed exposure information.

PROCEDURE

The grading of the adverse events and their frequency need to be assessed. As long as no adverse events are observed and the exposure is not above the exposure in the most sensitive species, dose escalation should go on as planned in the protocol. If the exposure is above the NOAEL exposure, careful further dose escalation maybe reasonable to define the maximal tolerable dose. If the severity of adverse events is below three and the exposure of the NOAEL is not reached, dose escalation can proceed. No further dose escalation should be considered, if more than 50% of active treated subjects suffer from adverse events of grade 3.

EVALUATION

In case grade 3 or 4 adverse events do occur, treatment of ongoing subjects should be stopped immediately.

C.4.4 Clinical Adverse Events Monitoring (Report by Subjects)

PURPOSE AND RATIONALE

Most drug related adverse events are based on the spontaneous reporting of clinical signs by the clinical trial participants. Subjects participating in a clinical trial can realize these adverse events at any time.

PROCEDURE

The subjects are asked to report any events, signs, or abnormal observations and feelings to the study personnel immediately. In addition, subjects should be asked direct questions from time to time, such as "Did you make any disagreeable or unexpected observations since you took the drug?" The information obtained need to be documented without interpretation at first. The (preliminary) diagnosis and decision about next steps (physical, and if indicated additional laboratory or technical examinations) will be based on the interpretation by the responsible MD on this report.

EVALUATION

Categorization of adverse events reported by the subjects is to be done by experienced medical personnel taking into acocunt possible differential diagnosis and their time course. The reference http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074775.htm gives an example of recommendations by the FDA.

C.4.5 Clinical Adverse Events Monitoring (Physical Examination by the Clinical Investigator)

PURPOSE AND RATIONALE

Physical examination based on spontaneous reporting of adverse events will be conducted as needed.

PROCEDURE
Physical examination can include auscultation, investiga tion of reflexes, or orientation, etc.

EVALUATION
The investigator needs to decide on the classification of physical findings based on changes to baseline and their relevance.

CRITICAL ASSESSMENT OF THE METHOD
Typically there will not be many clinical findings on physical examination. If there are some, this indicates already quite substantial effects (e.g., angioedema, rashes, ankle edema). Exceptions are findings in the vital signs of heart rate or blood pressure (see below), where frequently effects are seen.

C.4.6 Timing of Monitoring

PURPOSE AND RATIONALE
Timing of clinical and laboratory assessments need to be in line with the timecourse of drug concentration over time.

PROCEDURE
Standard monitoring needs to be done at baseline and repetitively after drug administration. For orally adminis tered drugs this is typically at baseline before drug admin istration, 30 min, 1, 2, 4, 8, 12, and 24 h after dosing for once daily drugs. Timing has to be tailored to the specific profile of a drug.

C.4.7 Vital Signs

C.4.7.1 Heart Rate

PURPOSE AND RATIONALE
The heart rate is influenced by the sympathic and parasympathic system, which can be affected by drugs directly or indirectly. Heart rate as a vital parameter has to be quite stable as heart rate effects in patients with ischemic heart disease could lead to angina pectoris. In phase I studies heart rate typically is most affected by increased vagal tone and subsequent bradycardia and occassional fainting.

PROCEDURE
Continuous ECG monitoring is the method of choice to observe effects on heart rate.

EVALUATION
Normal range: 50 80/min in supine position. Grade 3 definition: <45/min for bradycardia. Grade 1 definition: 100 115/min. Grade 2 definition: 116 130/min. Grade 3 definition: >130/min.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

MODIFICATIONS OF THE METHOD
Holter monitoring allows continous 24 h assessment of heart rate including analysis of cardiac arrhythmias. Holter monitoring should be used whenever there is eveidence of pro arrhythmic potential of a drug.

C.4.8 Vital Signs

C.4.8.1 Blood Pressure

PURPOSE AND RATIONALE
Blood pressure is dependent on stroke volume, heart rate (stroke volume × heart rate = cardiac output), and peripheral resistance. Decrease in cardiac output and/or resistance decreases blood pressure and vice versa. A decrease in blood pressure is most often a result of either vasodilatation or decrease in heart rate, both of which can occur during increased vagal stimulation.

PROCEDURE
Blood pressure can be measured manually or by a machine, in supine, sitting, or standing positions. For functional assessments the Schellong test is an easy to conduct procedure to measure the effect of a physical challenge on heart rate and blood pressure. After 10 min in supine position the subject is asked to take a standing position. Heart rate and blood pressure are measured 2 min after end of supine position. Timepoints of blood pressure measurements need to be adapted based on the observed effects.

EVALUATION
Normal range: 100 140 mmHg systolic in supine posi tion, 50 85 mmHg diastolic in supine position. Grade 1 definition: 140 159 mmHg systolic in supine position and 90 99 mmHg diastolic in supine position for pres sure increase. A quantity of 80 100 mmHg systolic in supine position for pressure decrease. Decrease in systolic blood pressure after 2 min standing by more than 20 mmHg together with increase in heart rate.

Grade 2 definition: 160 179 mmHg systolic in supine position and 100 110 mmHg diastolic in supine posi tion for pressure increase. A quantity of 70 80 mmHg systolic in supine position for pressure decrease. Cannot stay standing after 10 min of supine position for pressure decrease. Grade 3 definition: >180 mmHg systolic in supine position and >110 mmHg diastolic in supine position for pressure increase. Below 70 mmHg systolic in supine position for pressure decrease or syncope dur ing Schellong test.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

MODIFICATIONS OF THE METHOD
Twenty four hour ambulatory blood pressure monitoring (ABPM) is the method of choice for any compound with known or suspected effect of blood pressure as the effect over time can be best followed by continuous monitoring. Blood pressure (and heart rate) will be measured every 15 20 min during day time (defined as 6 a.m. to 10 p.m.). During night time the measurement intervals are 30 min. Full 24 h should be measured, if ABPM is used. ABPM allows to calculate precisely peak and trough effects and duration of effect on blood pressure.

C.4.9 ECG Parameter

C.4.9.1 PR Interval

PURPOSE AND RATIONALE
The PR interval in the ECG is the time during which the electrical excitation is conducted from the atria to he AV node. Prolongation of the PR interval is a potential side effect of drugs affecting repolarization and bears the risk of AV blockade.

PROCEDURE
Evaluators should be trained in ECG anaylsis. Automated analysis is frequent but needs to be validated in order to rely upon it.

EVALUATION
Normal range: 120 200 ms. Grade 1 definition:<0.8 fold LLN or >1.1 fold ULN. Grade 2 definition: >250 ms. Grade 3 definition: AV block 2nd degree or syncope.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.10 ECG Parameter

C.4.10.1 QT Interval

PURPOSE AND RATIONALE
The QT interval in the ECG is the time during which the electrical excitation and repolarization of the ventricles takes place. Prolongation of the QT time and especially the QTc time (QT time corrected for effect of heart rate) is a risk factor for torsades des point, a ventricular arrhyth mia associated with an increased incidence of drug induced sudden cardiac death. QT prolongation of drugs is one of the most frequent reasons for termination of a drug development program.

PROCEDURE
Evaluators should be trained in ECG anaylsis. Automated analysis is frequent but needs to be validated in order to rely upon it.

EVALUATION
Normal range for QTc: 360 425 ms for men, 380 445 for women, increase below 40 ms. Grade 1 definition: Increase above 40 ms and QTc below 475 ms. Grade 2 definition: 476 499 ms and increase below 60 ms. Grade 3 definition: Above 500 ms or increase exceeding 60 ms.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.11 Laboratory Parameter

C.4.11.1 Glucose

PURPOSE AND RATIONALE
A sufficient glucose concentration in blood (>2 mmol/L or >40 mg/dL at minimum) is essential for all life processes. Whenever there are signs of decreased consciousness, this vital parameter has to be assessed immediately.

PROCEDURE
Blood glucose should be measured from capillary or venous blood at predefined timepoints and in addition in cases of suspected hypoglycemia or impaired consiousness.

EVALUATION
Normal range 3.8 6.4 mmol/L (depending on lab). Grade 1 definition for hypoglycemia: 3.5 3.8 mmol/L. Grade 2

definition: 2.2 3.4 mmol/L. Grade 3 definition: 1.7 2.1 mmol/L.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.12 Laboratory Parameter

C.4.12.1 Potassium

PURPOSE AND RATIONALE
Potassium concentration in cell is 25 fold higher than in blood. In all cases were potassium is released into the peripheral blood (e.g., during and after hypoxic events) or a decrease in renal excretion occurs, potassium increases will have the potential for cardiac bradyarrhythmias. Hypokalemia can lead to ventricular tachyarrhythmias. Therefore close monitoring of potas sium concentration in serum is very important in early phases of development as long as the effect on its concen tration in serum is not yet known.

PROCEDURE
Potassium values are measured from serum taken from peripheral veins at predefined timepoints.

EVALUATION
Normal range 3.5 5.0 mmol/L. Grade 1 definition: 3.1 3.4 for hypokalemia and 5.1 6.0 for hyperkalemia. Grade 2 definition: 2.5 3.0 mmol/L for hypokalemia and 6.1 6.5 mmol/L for hyperkalemia. Grade 3 definition: 2.0 2.4 mmol/L for hypokalemia and 6.6 7.0 mmol/L for hyperkalemia.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.13 Laboratory Parameter

C.4.13.1 Alanine Aminotransferase (ALT)

PURPOSE AND RATIONALE
Hepatic damage is one of the most frequent drug related adverse events and needs to be monitored in every clinical pharmacology study. Transaminases (SGPT/ALT and SGOT/AST), alkaline phosphatase, and total and conju gated bilirubin are the serum assays to detect liver damage.

PROCEDURE
ALT, AST alkaline phosphatase, and bilirubin are taken from serum of periphal blood at predetermined timepoints and more frequently, if any increases are seen. If increase of ALT is above threefold ULN, ALT needs to be followed until normalization (below ULN) or until no further decrease of ALT after termination of treatment is observed.

EVALUATION
Any transaminase elevation above the upper limit of normal should be considered as an indicator for hepatic damage. ALT increase is the enzyme specific for liver dam age. Normal range is 0 60 IU/L, strongly dependent on lab. Grade 1 definition: Increase >1.2 fold ULN. Grade 2 defi nition: Increase 2.5 5 fold. Grade 3 definition: >5 10 fold.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment. Increases of ALT are very specific of the liver. Alkaline phosphatase can be increased in other diseases as well, for example, bone disease.

C.4.14 Laboratory Parameter

C.4.14.1 Aspartate Aminotransferase (AST)

EVALUATION
Normal range is 0 40 IU/L, strongly dependent on lab. Grade 1 definition: Increase >1.2 fold ULN. Grade 2 definition: Increase 2.5 5 fold. Grade 3 definition: >5 10 fold.

CRITICAL ASSESSMENT OF THE METHOD
Supporting parameter for ALT analysis.

C.4.15 Laboratory Parameter

C.4.15.1 Phosphatase

EVALUATION
Normal range is 30 120 IU/L, strongly dependent on lab. Grade 1 definition: Increase >1.1 fold ULN. Grade 2 definition: Increase two to threefold. Grade 3 definition: three to tenfold.

CRITICAL ASSESSMENT OF THE METHOD
Supporting parameter for ALT analysis.

C.4.16 Laboratory Parameter

C.4.16.1 Bilirubin

PURPOSE AND RATIONALE
Bilirubin assessment together with ALT measurement is used to identify potential risks of heaptic toxicity.

EVALUATION
Normal range is 5 27 µmol/L. Grade 1 definition: Increase >1.3 fold ULN.

Hy's law (yyy) is a prognostic indicator that a drug induced liver injury leading to jaundice has a case fatality rate of 10 50%. Hy's Law cases have the three following components:

- The drug causes hepatocellular injury, generally shown by more frequent threefold or greater elevations above the ULN of ALT or AST.
- Among subjects showing such ALT/AST elevations, often much greater than 3 × ULN, some subjects also show elevation of serum bilirubin to >2 × ULN, with out initial findings of cholestasis (serum alkaline phos phatase [ALP] activity >2 × ULN).
- No other reason can be found to explain the combi nation of increased transaminases and bilirubin, such as hepatitis A, B, or C, preexisting or acute liver disease, or another drug capable of causing the observed injury.

CRITICAL ASSESSMENT OF THE METHOD
Together with ALT a very powerful parameter to identify true drug related hepatic damage.

C.4.17 Laboratory Parameter

C.4.17.1 Creatinine

PURPOSE AND RATIONALE
Creatinine is solely excreted by the kidney, primarily by glomerular filtration, and therefore is a good marker of renal perfusion and filtration. Drugs affecting renal per fusion or filtration lead to an increase in creatinine. Increases in creatinine only occur if there is already a significant decrease in renal glomerular filtration rate.

PROCEDURE
Creatinine concentration needs to be measured in plasma and urine. Together with the urine production per time (either within 24 h, or time overnight sampling, for exam ple, 1,500 ml excreted between 8 pm and 7 am) the glomerular filtration rate can easily be calculated.

EVALUATION
Normal range: 80 130 µmol/L. Grade 1 definition: >1.1 fold ULN. Grade 2 definition: >1.5 fold ULN. Grade 3 definition: >1.9 3.4 fold ULN.

CRITICAL ASSESSMENT OF THE METHOD
Serum creatinine levels are not very sensitive to large changes in GFR as long as the GFR is still above 60 ml/ min/m^2, but then a rapid increase will be observed. A more sensitive method for renal function is the GFR or the fractional excretion of electrolytes.

C.4.18 Laboratory Parameter

C.4.18.1 Albumin in Urine

PURPOSE AND RATIONALE
Presence of albumin in urine is an indicator of glomerular damage.

PROCEDURE
Albumin is measured from morning urine.

EVALUATION
Normally no albumin is excreted via urine. Any findings of albumin above 300 mg/24 h in urine is indicative of a renal issue that needs to be further evaluated (if prior to treat ment with investigational drug the value was negative).

CRITICAL ASSESSMENT OF THE METHOD
Albumin in urine is always a pathological sign, which needs further analysis.

C.4.19 Laboratory Parameter

C.4.19.1 Creatinphosphokinase (CPK)

PURPOSE AND RATIONALE
CPK is released during damage of skeletal muscle, a frequent side effect of lipid lowering compounds like statins.

PROCEDURE
CPK is taken from serum of periphal blood at predetermined timepoints and more frequently, if any

increases are seen. If increase of CPK is above threefold ULN, CPK needs to be followed until normalization (below ULN) or until no further decrease of CPK after termination of treatment is observed.

EVALUATION
Normal range: 50 400 IU/L. Grade 1 definition: 480 1,000 IU/L. Grade 2 definition: 1,000 2,000 IU/L. Grade 3 definition: 2,000 5,000 IU/L.

C.4.20 Laboratory Parameter

C.4.20.1 Hemoglobin (Male Subjects)

PURPOSE AND RATIONALE
Hemoglobin can be affected by acute bleeding, by chronic suppression of erythrogenesis, or by diluation/concentration due to changes in the intravasal volume.

PROCEDURE
Hemoglobin is assessed from whole blood taken from peripheral veins at predetermined timepoints.

EVALUATION
Normal range for males: 13.5 17.5 g/dL. Grade 1 definition: 12.0 12.5 g/dL and decrease >1.5 g/dL. Grade 2 definition: 10.0 11.9 g/dL. Grade 3 definition: <10.0 g/dL. Normal range for females: 12.5 15.5 g/dL. Grade 1 definition: 11.0 12.0 g/dL and decrease >1.5 g/dL. Grade 2 definition: 9.5 10.9 g/dL. Grade 3 definition: <9.5 g/dL.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.21 Laboratory Parameter

C.4.21.1 Polymorphonuclear Leucocytes (PMN)

PURPOSE AND RATIONALE
Immunotoxic effects of drugs on white bloods cells are not uncommon and need to be detected early on in development.

PROCEDURE
PMN count is assessed from whole blood taken from peripheral veins at predetermined timepoints.

EVALUATION
Normal range: 1.7 7.5 G/L. Grade 1 definition: <0.7 fold LLN or >1.3 fold ULN. Grade 2 definition:1.0 1.3 G/L. Grade 3 definition: <1.0 G/L.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.22 Laboratory Parameter

C.4.22.1 Platelets

PURPOSE AND RATIONALE
Immunotoxic effects of drugs on platelets are not uncom mon and need to be detected early on in development.

PROCEDURE
Platelet count is assessed from whole blood taken from peripheral veins at predetermined timepoints.

EVALUATION
Normal range: 150 350 G/L. Grade 1 definition: <0.85 LLN. Grade 2 definition: 50 125 G/L. Grade 3 definition: <50 G/L.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.23 Coagulation Parameter

C.4.23.1 Activated Partial Thromboplastin Time (aPTT)

PURPOSE AND RATIONALE
Effects on aPPT are seen in cases of decreased hepatic protein synthesis rate.

PROCEDURE
aPTT is assessed from plasma.

EVALUATION
Normal range: 22 43 s. Grade 1 definition: 1.1 1.3 fold ULN. Grade 2 definition: 1.3 1.5 fold ULN. Grade 3 def inition: >1.5 fold ULN until minor bleeds. Grade 4 defintion: Major bleeds.

CRITICAL ASSESSMENT OF THE METHOD
Basic method for safety and tolerability assessment.

C.4.24 Laboratory Parameter

C.4.24.1 Kidney Injury Molecule-1 (KIM-1)

PURPOSE AND RATIONALE
KIM 1 is a rather new biomarker indicating renal toxicity at the tubular level. KIM 1 has been preclinically qualified as an excellent marker for drug related renal toxicity. If there is preclinical evidence for renal toxicity at this region and if KIM 1 has been used in nonclinical toxicity studies, this parameter should be monitored.

PROCEDURE
KIM 1 can be measured using commercially available kits.

EVALUATION
Look for significant changes from baseline and if these occur, stop treatment, follow KIM 1 until normalization.

CRITICAL ASSESSMENT OF THE METHOD
There is limited experience with KIM 1 in healthy subjects and in clinical pharmacology studies. The marker is not well established in its performance in non disease states so far. Therefore descriptive analysis of the marker and anal ysis of traditional parameters such as serum creatinine or BUN together with KIM 1 in order to get more experience with the marker is advised.

MODIFICATIONS OF THE METHOD
There are several additional bimarkers for assessment of renal toxicity like alpha GST or NGAL. They are also well qualified in nonclinical toxicity studies with nephro toxicants. There is only limited information about the normal ranges and the spontaneous variations available currently.

C.4.25 Visual Analogue Scale for Semiquantitatively Assessing Pain and Other Subjective Factors

PURPOSE AND RATIONALE
A visual analogue scale (VAS) is a psychometric response scale, which can be used in questionnaires. It is a measurement instrument for subjective characteristics or attitudes that cannot be directly measured, for example, pain or subjective assessment of the effectiveness of a treatment.

For the quantification of these subjective factors, the VAS is an instrument that tries to measure the severity across a continuum from none to an extreme amount of the characteristic. For example, the spectrum of pain to a subjective suffering from it appears to be continuous and does not take discrete jumps, as the typical categorization of none, mild, moderate, and severe suggests. In order to reflect this idea of an underlying continuum the VAS was introduced.

PROCEDURE
Operationally a VAS is usually a horizontal line, 100 mm in length, anchored by word descriptors at each end, for example, "no pain" and "maximum pain." When responding to a VAS, subjects are asked to indicate their level of agreement to a statement by indicating a position along a continuous line between the two end points men tioned. This continuous (or "analogue") aspect of the scale differentiates it from discrete scales (e.g., "none mild moderate severe" or "A to F").

EVALUATION
The VAS score is determined by measuring in millimeters from the left hand end of the line to the point that the patient marks.

CRITICAL ASSESSMENT OF THE METHOD
As such an assessment is clearly highly subjective, these scales are of most value when looking at change within individuals, and are of less value for comparing across a group of individuals at one time point. It could be argued that a VAS is trying to produce interval/ratio data out of subjective values that are at best ordinal. Thus, some cau tion is required in handling such data. Many researchers prefer to use a method of analysis that is based on the rank ordering of scores rather than their exact values, to avoid reading too much into the precise VAS score.

However, a VAS is extremely simple to use, easy to teach and understand. Therefore, bias introduced by com plexity can be ignored.

For efficacy studies in patients, where pain is a primary or secondary outcome parameter, the VAS is only of lim ited value.

In practice, computer analyzed VAS responses may be measured using discrete values due to the discrete nature of computer displays.

The VAS can be compared to other linear scales such as the Likert scale or Borg scale. The sensitivity and reproducibility of the results are broadly very similar, although the VAS may outperform the other scales in some cases [1].

MODIFICATIONS OF THE METHOD

Due to the limitations mentioned above, several additional tools for pain assessment have been developed and validated, such as the McGill pain questionnaire, where several dimensions of pain are assessed. As for all questionnaires it is very important to have the questionnaire available in the validated version of the native language. Otherwise the outcome of the questionnaires from different languages cannot be compared. These complicated and often patent protected questionnaires do not have a major place in clinical pharmacology studies.

C.4.26 Summary

It should be kept in mind that during the first clinical studies, there is practically no information about the safety and tolerability of a new drug as compared to the knowledge accumulated later on. Nevertheless, only during these initial studies the administration of the drug occurs under such kinds of secure conditions concerning the ability to handle side effects that dose escalation should not be stopped too early. It has to be kept in mind that during phase II and III studies and even more during marketing of a drug, the exposure of the drug to patients might occasionally due to overdose, poor metabolization, or other causes of accumulation be much higher than intended. Especially for these cases the company developing a drug should know, which kind of side effects would be expected.

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C.5 Pharmacodynamic Evaluation: Cardiovascular Methodologies

Christian de Mey · Markus Hinder

C.5.1 Relevance and Constraints of Cardiovascular Investigations in the Early Development of Novel Drug Substances and Medications

Cardiovascular function reflects the overall ability of the two cardiac pumps and their circulation to secure tissular functionality by providing an adjusted supply of oxygen and nutrients while eliminating biological waste products. Failure of this ability causes severe damage and, if protracted and/or extensive, may be lethal. Leading causes of death are cardiovascular (life threatening dys rhythmia, coronary and cerebrovascular atherosclerotic vasculopathies, heart [pump] failure, etc.) and related morbidity is highly incapacitating with important nega tive socio economic implications. It is therefore self evident that cardiovascular morbidity and mortality and its precursor conditions (atherosclerosis) or risk factors (hypertension) are important target indications for drug development.

However, in recent years there has been a clear shift away from the development of new cardiovascular drugs since the cardiovascular market is crowded and since the need for costly long term survival studies has a negative impact on the likelihood of an attractive return on a quite large and risky investment (Garber 2009; Topol 2009). On the other hand, several drugs without target cardiovascu lar indication may affect cardiovascular effectors or the pharmacological modulation thereof; this may result in desired or undesired extension effects and there is justified interest and need to profile such drugs also with regard to their cardiovascular effects, safety, and tolerability. This may relate to general topics such as QT/QTc safety (see below) or to a specific drug or disease related con cern (Drug Administration 2008).

The continuous quest for cost containment ("lean" development) may have led to a well focused effort (only) to satisfy regulatory requirements while avoiding all efforts that do not fit in this framework. Recent exam ples evidence that this carries an important risk since important cardiovascular safety issues may only appear when the drug is already marketed (for example with COX2 selective NSAIDs), whereas these risks might have been anticipated if appropriate pharmacodynamic clinical pharmacology studies had been conducted in the early development of such compounds.

There are several scenarios in which pharmacody namic (PD) studies may have a well established benefit (proof of concept, proof of action, proof of mechanism studies, etc.) since they contribute to a better understand ing of new drugs and their more efficient and safer ther apeutic use. Nevertheless, the lack of positive regulatory sanctioning of such efforts means that these studies, although applauded as highlights of "rational" drug development, are no longer a logical part of the early drug development effort. An important consequence of the overwhelming impact of regulatory "science" on the focus and content of the early development program of new drugs is that increasingly fewer PD human phar macology studies are being conducted and that the human pharmacological characterization of new drugs is mostly confined to merely pharmacokinetic (PK) aspects. This only makes sense if the PKPD relationships of effi cacy and/or safety relevant ("surrogate") PD criteria were known. Unfortunately, this is rarely the case. If conducted at all, such PD and PKPD studies are often placed outside of the clinical documentation of the application for mar keting authorization, for instance as part of a well targeted marketing displacement strategy for "me too" agents with otherwise only marginal therapeutic benefits.

Since there is less demand for PD studies, it becomes more and more difficult to develop and maintain the hardware, software, and expertise that is needed to pro vide such services. This has led to the loss of several cardiovascular methods (for instance digital plethysmog raphy). This might have been avoided if such methods could have kept their place in clinical cardiology. However, clinical cardiologists often lose interest in less sophisti cated noninvasive cardiovascular research methods (for instance systolic time intervals see below) and prefer invasive methods or more easily automated and more

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economic alternatives that require less analyst time, training, and qualification, but are more fairly remunerated. Furthermore, often there are no provisions for a fair remuneration of such diagnostic procedures in clinical cardiology. Nowadays, the technical quality requirements for such PD methods are so high and the demand is so rare that there is little profit in developing them unless they also have a solid place in clinical cardiology. Several useful methods do not.

C.5.2 Validation of Cardiovascular Test Criteria

Participants in clinical pharmacology studies are subject to discomfort and risk without any likely benefit other than a fair remuneration. Their individual risk benefit relation, therefore, is not favorable. Public sanctioning of the study by ethical review does not discharge the investigator from the responsibility of minimizing the individual risk and optimizing the ethical benefit of the study to the group. This responsibility has implications for the methods chosen. In cardiovascular studies on healthy subjects, noninvasive rather than invasive methods ought to be considered, provided their use does not preclude drawing valid conclusions from the study. A study or method (test) is useful if it provides a sufficient and adequate answer to a relevant question with a reasonable amount of effort and at a reasonable cost. This involves both implicit (i.e., usability) and explicit (regulatory acceptance) quality criteria matter: in applied clinical pharmacology, a test is useful if it is able (implicit "usability") and accepted to yield a tangible contribution to the evaluation of a drug under investigation.

C.5.2.1 Empirical Quality Criteria

The usability of a method can be quantified by a formal assessment of empirical quality criteria based on test theoretical principles:

Objectivity. Objective is the extent of investigator independence in conducting the test, analyzing its results, and interpreting its data. Investigator blinding, strict standardization, and extensive training are likely to improve objectivity. Objectivity is quantified by means of the between observer agreement and within observer consistency.

Reliability and sensitivity. Reliability is the capacity for error free measurement characterized by reproducible findings. It reflects the spontaneous physiological variability of the variate and the methodological variability of its quantification. A test or method is reliable if it is hardly subject to such variability and yields highly consistent results when repeated, although this does not imply that the results are valid (see below). Sensitivity is a direct consequence of reliability because it is the capacity to separate systematic from random effects. Repeated measurements provide a powerful tool for evaluating reliability and sensitivity: the estimates of the intra and intersubject variances of repeated observations permit quantification of the intraclass correlation (ρ) of repeated measurements as a measure of reliability (Lienert & Raatz 1994; Winer 1971; Fleiss 1986); the intrasubject repeatability coefficients (δ_{crit}) or individual sensitivity, defined as the maximum absolute difference between two measurements within the same subject (either within or between study days) reached with 95% probability; the smallest significant average difference (δ_{post}) defined as the smallest average difference detectable with the given sample size at $\alpha = 0.05$ and appropriate power ($\beta = 0.10$ 0.20).

Pharmacosensitivity and pharmacospecificity. It is defined as the capacity to detect drug induced systematic effects, pharmacosensitivity relates to the reliability of the method and the prominence (extent and consistency) and intrasubject repeatability of drug related changes. It depends only partially on the method itself because it also relies on the study architecture and environment. Indeed, medication unrelated time, diurnal, and postprandial effects may confound pharmacosensitivity for cardiovascular criteria in particular (de Mey et al. 1987; de Mey et al. 1988). An appropriate study control can account for confounding environmental effects provided there is no interaction between time effects that are treatment related and those that are not. The ability to separate these two is an argument of specificity.

Economy. A test or method is economical if it can be carried out time and cost efficiently for example, when it permits investigation of several participants in parallel, does not require large study samples, can be executed without a large investment of funds and resources, and does not call for investigators with exceptional qualifications. Test automation improves economy because it reduces dependency on observers and facilitates the capture and transfer of data.

Validity. Methods are valid when they measure what is claimed or intended to be measured. In cardiovascular clinical pharmacology, validity is particularly relevant because most noninvasive methods estimate rather than measure physiologic function and its pharmacologic alterations. Proof of the validity of a method lies in its agreement with (valid) invasive measures ("gold" standards).

In most noninvasive methods, validity is inherently limited. Indeed, such methods are based on simplified physiologic assumptions and algorithms that are likely to be affected by method*subject*effect interaction, that is, their accuracy in measuring function varies among subjects and also depends on the nature of the investigational changes that are to be detected.

Assessment of agreement. The assessment of agreement among observers is important in evaluating test objectivity; agreement of repeated observations under identical study conditions provides a measure of intrasubject repeatability and sensitivity and intersubject reproducibility; agreement of the measurements by means of a noninvasive method with those of an invasive gold standard method demonstrate relative validity or lack thereof. All such analyses are based on repetitions of observations and can be analyzed in a similar fashion. Correlation and regression analyses are of limited value in this regard (Altman & Bland 1983; Bland & Altman 1986); other methods should be used instead (Chinn 1990; Bland & Altman 1999; Bland & Altman 2007; Hamilton & Lewis 2010).

C.5.2.2 Issues with the Validity of Cardiovascular Methods

Only heart rate (HR) is a directly measured criterion; however, it only rarely reflects direct drug effects. HR is not well suited to characterize PKPD interrelations of direct drug effects unless reflex changes in the adrenergic and cholinergic modulation of HR are taken into account (de Mey 1997; de Mey 1994).

Several cardiovascular criteria are approximate estimates (for instance: blood pressure [BP], cardiac output [CO]), others are assumptive criteria derived from an algorithmic interpretation of the cardiovascular circulation (for instance: contractility, vascular compliance, preload, afterload, HR corrected QT, etc.); the former are method specific, the latter depend on the model used to explain and understand the cardiovascular system and the changes thereof. Accordingly, most cardiovascular criteria have a limited validity.

The complexity of method specific approximations is well illustrated by "systemic blood pressure." Indeed, the pulse wave and its systolic peak and diastolic nadir change with time and increasing distance from the cardiac pump; accordingly, blood pressure depends on the vascular segment from which it is derived; such pressure can be measured invasively, but this approach is hardly practicable. Instead, blood pressure is "measured" noninvasively. This means that systolic (SBP) and diastolic blood pressure (DBP) are the pressures in a proximal, initially occluding forearm cuff coinciding with a specific change of an acoustic or oscillometric signal generated by the vascular wall movements when the cuff is deflated; accordingly, noninvasive SBP and DBP are not instantaneous, but are offset by the speed of deflation. Noninvasive SBP/DBP estimates depend on the anatomic site of measurement, cuff size, cuff position, etc. Although there may be a relatively good agreement between such noninvasive estimates and direct invasive measurements at recumbent rest, there may be substantial disagreement when noninvasive methods are used to quantify interventional BP changes (see below).

The complexity of algorithmic, i.e., interpretative, criteria is well illustrated by the concept of "contractility"; contractility is a concept initially derived from experiments on isolated papillary (i.e., longitudinal) muscle strips reflecting the intrinsic ability of the heart to contract independent of preload and afterload. By analogy, contractility may be understood as the contractile force of the cardiac pump independent of heart rate, preload, and afterload. Only during the isovolumetric contraction and relaxation phases of the cardiac cycle, there is a short period, during which the heart pumps independent of its afterload. Nevertheless, even during this phase, systolic performance depends on filling (preload) and HR. The latter explains why the duration of the HR corrected preejection period can be used as a measure of the combined impact of cardiac filling and contractility; in the absence of changes in venous capacity, venous return, and duration of the diastole, this might be understood as an index of contractility. Such HR corrections are adopted for several further cardiovascular criteria since hardly any electrophysiological or contractile phenomenon of the circulation is HR independent. The outcome of any such HR correction depends on the algorithm used. Such corrections may help to understand to which extent an observed change results from a direct or indirect, i.e., HR related effect. However, their value is doubtful: if prolonged repolarization is arrhythmogenic, then the safety risk of certain medications might relate to their QT prolonging effect irrespective of whether and to which extent this is (also) associated with a QTc prolongation.

Accordingly, most cardiovascular effect criteria are estimates that are method specific and that are likely to be subject to method*subject*effect interaction. Such an interaction may appear as a method specific overestimation of drug related changes of cardiovascular function, which may be useful in detecting and

differentiating drug effects but has limited value for their mechanistic interpretation. The method specificity of the estimates should be appropriately accounted for by precise method descriptions and standardization. Furthermore, a combination of data generated by different methods should be avoided (de Mey & Erb 1997).

Although the validity (the capacity to measure what is intended or claimed to be measured) of several cardiovascular criteria may be limited, this by itself does not limit their usefulness provided they are accepted to be adequate and sufficient to characterize the cardiovascular system and the drug related changes thereof. Their relevance ought to be seen in a scientific/theoretical context: non invasive estimates of drug related changes of cardiovascular function build cognitive constructions that fit the detectable reflections of physio pharmacologic reactions without necessarily matching them. The ultimate value of these constructions lies in the (regulatory) acceptance of their usefulness in predicting drug efficacy and safety relevant effects beyond the limited scope of the original experiment.

C.5.3 Electrocardiography

C.5.3.1 Standard 12-Lead ECG

PURPOSE AND RATIONALE

The electrocardiogram (ECG or EKG) is the noninvasive amplified superficial signal resulting from the transthoracic spread of the electrical activity of the heart during its depolarization repolarization cycle. First introduced in the early twentieth century based on the work of Alexander Muirhead and Augustus Waller in the late 19th century, electrocardiography now is widely used as a core method to investigate the electrophysiological functionality and integrity (Cooper 1986). ECG analysis provides information on rhythmicity, ectopism, intra atrial conduction, atrial depolarization/repolarization, atrio ventricular conduction, intra and transventricular conduction, ventricular depolarization and repolarization, myocardial mass, myocardial energy balance, etc., and changes thereof either by disease or by investigational intervention (medications, stress testing, etc.)

PROCEDURE

Still relying on the pioneer work of Willem Einthoven (Einthoven 1901; Rivera Ruiz et al. 2008), modern ECG diagnostics now involve digital recording, analysis, and archiving of the ECG tracings and related data (for instance: P wave duration, PQ interval, QRS duration, QT interval, P wave, QRS wave, and T wave vector amplitude and angle (Hurst 1998)). The highest precision is achieved by recording the signals from the bipolar Einthoven leads (I, II, III), amplified unipolar Goldberger leads (aVR, aVL, aVF), and unipolar precordial Wilson leads (V_1 V_6) simultaneously for a sufficiently long time (10 s at least) and at a sufficiently high writing speed (25 50 mm/s).

EVALUATION

Modern electrocardiography is no longer confined to the "reading" of ECG tracings recorded on paper and the measurement of relevant time sections (intervals, segments, durations) and amplitudes by means of an ECG ruler. It now usually consists of a sequence of finely tuned electronic data processing steps: capturing the ECG lead signals; obtaining a digital representation of each recorded ECG channel by analog digital conversion and a special data acquisition software or a digital signal processing chip; processing the resulting digital signal by a series of specialized algorithms, which first condition the signal by removing noise, base level variation, etc.; mathematical analysis of the clean signals to identify and measure selected time segments and amplitudes (features) for interpretation and diagnosis; secondary processing such as Fourier analysis and wavelet transform decomposition with vector feature extraction (Matsuyama et al. 2007; Al Fahoum & Howitt 1999) to provide input to pattern recognition based programs; logical processing and pattern recognition, using rule based expert systems (Kundu et al. 1998), probabilistic Bayesian analysis or fuzzy logics algorithms, cluster analysis (Lagerholm et al. 2000), artificial neural networks (Sabbatini 1996), genetic (Tu et al. 2005) or evolutionary optimization algorithms (Dumont Ast et al. 2010), and other techniques to derive conclusions, interpretation, and diagnosis; reporting of the tracings, the data, and the conclusions drawn from the analysis with a proper sourcing of the information and the analysis steps.

CRITICAL ASSESSMENT OF THE METHOD

Although the basic principles of electrocardiography are well known, there is an obvious need for standardization. Guidance has been provided by several professional organizations (Meek & Morris 2002; Myerburg et al. 2008; Drew et al. 2004; Kligfield et al. 2007; Mason et al. 2007; Surawicz et al. 2009; Rautaharju et al. 2009;

Hancock et al. 2009; Wagner et al. 2009). However, such information usually relates to the conventional recording and interpretation of the ECG signals. In contrast, there is little guidance with regard to the complex electronic data processing that is now inherent to state of the art electrocardiography.

Most ECG devices also print a single or multichannel signal record on paper. Only such (signed) hardcopy record may be accepted as reliable source documentation. However, caution is indicated since many devices use thermopaper, which generally rapidly fades (Anonymous 1990). The date/time stamp of such devices is usually not reliable since it can be easily accessed by the operator and/or is not automatically synchronized with a reliable time server.

Also, most modern ECG devices provide for an automated analysis of relevant ECG intervals (RR, PQ, QRS, QT) usually based on the averaged signals of a 10 s recording (Cain et al. 1996; Graham & Handelsman 1998). Such analyses are often judged to be less reliable. This prejudice is unjustified in healthy subjects with mostly normal ECGs (Fosser et al. 2009; Hoon 1996; De Mey & Gatchev 2005): there is generally good agreement between automated and manual analyses; possibly gross differences between automated and manual analyses in healthy subjects mostly relate to either artificial or electrophysiological signal distortions (such as U waves (Pérez Riera et al. 2008)) that can be easily identified if the tracings are appropriately reviewed by an experienced analyst.

Most analyses, whether automated or manual, are subject to the constraint that it may prove difficult to identify the start of the Q wave; for this reason, the atrioventricular conduction interval is often reported as PR instead of the PQ interval; the PR interval does not extend from the start of the P wave to the R peak, but to the intersection of the isoelectricity ("zero") line with the upstroke of the R wave. The "PR" interval thus represents a simplification of the "PQ" interval whenever the start of the Q wave is not expressed or cannot be measured reliably. This simplification is highly convenient since it is far more easily standardized and/or automated. It is noteworthy that such a simplification is not also generally adopted for the QT interval: the QT interval represents the sum of the ventricular depolarization and repolarization, of which the former is relatively constant, less subject to drug effects, and less likely to be of arrhythmogenic relevance; the measurement of the QT interval relies on two fiduciary points: the start of the Q wave and the "end" of the T wave; both are not sharply expressed; the precision of the estimated repolarization duration could be improved by measuring the "RT" interval, i.e., from the peak of the R wave to the end of the T wave; the former fiduciary point is more easily detected, standardized, and/or automated. Efforts to quantify and qualify the QT/QTc related arrhythmogenic risk might be equally reliable and valid, but far more efficient and economic if more easily automated depolarization surrogates (Laguna et al. 1990; Porta et al. 1998) would be accepted.

Automated ECG analysis usually also reports a clinical "diagnosis" of the condition reflected by the ECG based on the rhythmicity and contour of the ECG cycles using either medical or stochastic algorithms (Clifford et al. 2006; Rajendra Acharya et al. 2007). The ECG contour is stereotypic and deviations from a "normal" morphology may indeed reflect a more or less specific anomaly of cardiac rhythmicity and ectopism, sinus node pacemaker autonomy and function, intra atrial, atrioventricular, intra and transventricular signal spread, myocardial mass, myocardial depolarization and repolarization, myocardial energy balance, etc. Nevertheless, no automated diagnosis should be accepted unless reviewed, confirmed, and/or amended by an experienced electrocardiographist.

Relevant electrocardiographic time intervals and signal durations are affected by heart rate (HR) variations (Malik et al. 2008): the AV nodal conduction time and the PQ/PR interval shorten with increasing heart rate (Danter & Carruthers 1990) and this fluctuation may be used as an index of autonomic function (Leffler et al. 1994). The HR dependency of the QT interval is well known and has resulted in several approaches to "correct" the QT interval for HR below or above 60 bpm: according to Bazett (Bazett 1920), Fridericia (Fridericia 1920), Framingham's regression (Sagie et al. 1992); however, these corrections apply a population mean correction factor for all subjects while there is convincing evidence for significant interindividual variability in the HR–QT relationship (Malik et al. 2002; Batchvarov et al. 2002) implying that the best HR correction for QT should be estimated for each individual (Anonymous 1990; Piotrovsky 2005). This is hardly feasible since it requires a number of "normal" QT measurements at varying HR for each subject (Couderc et al. 2005); normograms have been proposed to solve this problem (Chan et al. 2007). However, rather than to "correct" for HR variations, there might be interest in investigating the disparity of the RR–QT relationship as a more sensitive index of arrhythmogenic risk (Fossa 2008). Data from the International Long QT Syndrome Registry indicate that the probabilistic risk of developing malignant arrhythmias in patients with QT

prolongation is exponentially related to the length of the QTc interval (Moss 1993), but it remains unclear whether a QT prolongation predominantly related to HR (i.e., with normal QTc) would be without risk.

The time course of experimental ECG criteria reflects time effects both related and unrelated to the investiga tional medication. Assuming an additive response model, this results in two important steps in the management of such data: to consider the data both untransformed (U) and as arithmetic changes (Δ) from predose baseline and to match the courses of these U and Δ data for the time course of the respective criteria during a medication free control day ("time matching"). Such time matching using an extra control day within each treatment (placebo, therapeutic dose, supratherapeutic dose, active control) is costly and the need thereof is controversial (Zhang et al. 2009).

In the setting of the ICH E14 Guideline (FDA 2005), an investigational medication is accepted to be without QT/QTc effect if the upper bound of the one sided 95% confidence interval for the largest time matched mean effect (i.e., of the changes from predose baseline relative to placebo) of the drug on the QTc interval excludes (i.e., is smaller than) 10 ms; the study is normally conducted in healthy volunteers investigating both a therapeutic and a (widely) supratherapeutic dose rel ative to a positive (active) (Yan et al. 2010) and a negative (placebo) control in an experimental setting stringently powered to exclude an effect on the QTc inter val exceeding 5 10 ms (Malik et al. 2010; Darpo 2010). This has been subject to extensive critique also because of well founded biostatistical concerns (Hutmacher et al. 2008; Patterson et al. 2005; Tsong et al. 2008) and since a possible effect compartmentalization is not accounted for (Russell et al. 2008). When the largest time matched difference exceeds this threshold, the study is termed "positive." A positive study does not imply that the drug is pro arrhythmic, but influences the evaluations that need to carried out during the further stages of drug development.

Most ECG systems operate as closed "black boxes" with device specific file formats and often nonpublic analysis algorithms. There have been several efforts to develop unified, platform and device independent solu tions: the Standard Communications Protocol for Com puter Assisted Electrocardiography (SCP ECG) (ENV 1064), proposed in 1993 by the Technical Committee of Comité Européen de Normalisation; PhysioNet (Goldberger et al. 2000) providing an online forum for dissemination and exchange of biomedical signals includ ing ECG data, stored in the waveform database format; Digital Imaging and Communications in Medicine (DICOM) 3.0 Supplement 30 data interchange formats (DICOM 1999); and now more recently, the FDA favored use of annotated XML files (aECG) in accordance with Health Level 7 (HL7) standards (Brown & Fabio Badilini 2005; http://www.hl7.org/V3AnnECG/foundationdocuments/welcome/index.htm) that can be viewed by means of an appropriate FDA XML VIEWER (http://www.amps llc.com/Downloads.htm); this initiative involves Cooperative Research and Devel opment Agreements (CRADA) between the FDA and ECG service providers such as Mortara Instruments (dig ital ECG warehouse) or AMPS llc (XMLVIEWER); these collaborations are of interest since they might explain some of the extraordinary requirements resulting from the ICH E14 Guideline requirements (FDA 2005) for "definite" or "thorough" QT/QTc trials. The provision of the viewer is no longer free of cost and is held under the tight control of AMPS llc. Therefore, there is interest in further initiatives that might result in truly public domain solutions (for instance the Open ECG Project (http://www.open ecg project.org/tiki read article.php?articleId=1)).

C.5.3.2 Ambulatory ECG (HOLTER)

PURPOSE AND RATIONALE

The 12 lead ECG only provides a snapshot of the electro physiological activity and integrity of the heart. Continu ous ECG recording captures far more information on several thousand cardiac cycles also under less artificial (ambulatory) conditions. In contrast to conventional ECG, mostly not all leads are recorded and the recordings are more affected by artifacts.

PROCEDURE

The procedure consists of the continuous or event triggered ("event monitoring") digital recording of a 1 , 3 , 6 , or 12 lead ECG either by telemetry of by means of a portable device, often referred as "Holter" monitoring referring to the pioneer work of Norman J Holter who invented telemetric cardiac monitoring in the late 1940s (Holter & Gengerelli 1949).

Continuous telemetric and ambulatory ECG monitor ing are well established diagnostic tools in clinical cardi ology. Various tools and electronic data processing steps are used to capture and store the signals, to analyze them by means of an analyst steered and surveyed automated routine with relevant feature extraction and

a diagnostic/prognostic interpretation of the information (Kowey & Kocovic 2003; Heilbron 2002; Frantz 1998; Brandes & Bethge 2008; Enseleit & Duru 2006; Arya et al. 2006).

EVALUATION

Guidance has been provided for the continuous ECG monitoring in several clinical settings (Kadish et al. 2001; Crawford et al. 1999; Gibson et al. 2007).

Originally, Holter ECG analysis was mainly focused on rhythmicity (sino atrial dysfunction, ectopism, atrial fibrillation, atrial flutter, paroxysmal tachycardia, acceler ated rhythms with normal or aberrant configuration), atrio ventricular conduction delays and blockade, intermittent changes in QRS morphology (parasystoles, ectopic rhythms), etc. (Rao et al. 2007; Eveloy et al. 2006; Crimin et al. 2010). Improved algorithms also now pro vide for the analysis of changes of the QT wave, ST T wave (Bjerregaard et al. 2003; Jager et al. 1998).

CRITICAL ASSESSMENT OF THE METHOD

One of the important advantages of continuous ECG recordings is that it collects a vast amount of data under real life conditions; this permits beat to beat analysis for a far more precise and valid interpretation of the QT related arrhythmogenic risk: indeed, dynamic beat to beat QT interval analysis compares the QT interval to individual cardiac cycles from all normal autonomic states at similar RR intervals, thus eliminating the need for correction functions; in this way, beats with QT intervals exceeding a critical (subject specific) limit can be flagged as outlier beats for further arrhythmia vulnerability assessment (Barta et al. 2010). Furthermore; such beat to beat techniques can also be used to assess the QT TQ interval relationship known as ECG restitution (Fossa & Zhou 2010).

Further procedures allow evaluation of highly sensitive prognostic criteria (Jager et al. 1998; McLaughlin & Zimetbaum 2006), such as QT dispersion, heart rate var iability (Huikuri et al. 2009; Bilchick & Berger 2006; Mäkikallio et al. 2002; Stein 2002; Huikuri et al. 1999; Omerbegovic 2009), and heart rate turbulence (Jurek et al. 2007; Guzik & Schmidt 2002); other methods spe cifically conceived to quantify arrhythmogenic risk are under development (Cain et al. 2003).

Additionally, continuous ambulatory electrocardiog raphy provides for a better characterization of the diurnal variability and the implications thereof for the timing of drug administration (Smolensky & Portaluppi 1999).

C.5.4 Blood Pressure

C.5.4.1 Timed Blood Pressure

PURPOSE AND RATIONALE

Arterial (i.e., systemic) blood pressure (BP) is the pres sure exerted by circulating blood on the wall of the arterial blood vessels. It is the pressure equivalent of the pulsatile ejection of blood out of the left ventricle during the systole, the segmental modulation thereof during the progression of the pulse wave through the distribution and resistance vessels, and the backward reflections thereof from the further circulatory periphery (Kenner 1988). Due to the transformation of the pulse wave, the maximum ("systolic" BP [SBP]) and minimum ("diastolic" BP [DBP]) blood pressure are reflections, but no surrogates of the central hemodynamics. Since blood pressure declines almost exponentially over the diastole, bradycardia has a direct DBP reducing effect unrelated to central pump function and peripheral vascu lar resistance; this is often associated with a relatively higher SBP due to a higher preload while longer filling phase.

For more complex analyses, there is need to calculate the mean BP (MBP); with auscultatory methods, MBP is usually calculated from SBP and DBP as either MBP = DBP + 1/3 × (SBP DBP) or as MBP = DBP + 0.43 × (SBP DBP) (Wezler & Böger 1939). With oscillometric methods, MBP is selected as the minimum cuff pressure at maximum cuff oscillation and thus is separate from the estimates of the SBP and DBP (Safar & Smulyan 2008). Mean blood pressure is a better pressure marker of the overall circulation while relating both to the cardiac pump (heart rate [HR] and stroke volume [SV]) and the vascular periphery (total peripheral vascular resistance [TPR] since MBP = HR × SV × TPR. This can best be illustrated by the complex changes of the pressure homeostasis on postural challenge: when standing up, gravity → venous return ↓ → ventricular diastolic filling ↓ → SV↓ → MBP↓ → baroreflex → efferent adrenergic tone ↑ and efferent cho linergic tone ↓ → beta adrenergic + anti cholinergic HR↑ & beta adrenergic inotropic SV↑ & alpha adrenergic con striction of the arteriolar resistance vessels (TPR↑) and venous capacity vessels (venous return ↑) → MBP↑. This also explains the vulnerability resulting from alpha adrenoceptor antagonists and other vasodilatators: loss of compensation by arteriolar and venous vasoconstric tion makes erect blood pressure rely entirely on HR; how ever, exaggerated postural increases in HR may shorten the diastole, hence compromising ventricular filling to

the extent of inducing paradoxical reflectory vagotonia. The predominance of blood pressure homeostasis also explains why most depressor drugs do not reduce blood pressure in normotensives, but raise HR instead. On the other hand, this also implies that depressor drugs can only be effective as anti hypertensives if the blood pressure homeostasis and/or baroreceptor sensitivity are reset.

Although blood pressure is a physiologically poor marker of the cardiovascular system (Sabovic et al. 2009) (see below), it is a well accepted prognostic surrogate of relevant cardio and cerebrovascular morbidity and mor tality and the modification thereof by treatment either at rest (Rutan et al. 1988; Tikhonoff et al. 2009; Mancia 2009; Raphael et al. 2009), during exercise or controlled stress (Le et al. 2008; Miller 2008) or measured in ambulatory fashion (Staessen et al. 2001; Bastos et al. 2010; Clement et al. 2003).

PROCEDURE

Blood pressure is still mostly measured noninvasively according to the principle of Riva Rocci, i.e., by inflating a cuff around the upper arm up to arterial compressive occlusion and then slowly deflating the cuff while the pressure in the cuff is measured (Riva Rocci 1896). Orig inally, the cuff pressure was measured by means of a mercury sphygmomanometer; most present devices use an aneroid manometer or electronic pressure transducer; nevertheless, blood pressure is still generally reported in millimeters mercury (mmHg).

Vascular signals coinciding with systolic and diastolic blood pressure can be derived by palpation, auscultation, or oscillometry from a suitable vessel site distant from the cuff. Palpatory (systolic) blood pressure is now only used for emergency evaluations; manually operated aus cultatory blood pressure with an appropriately adjusted cuff (Mattoo 2002; Akpolat 2010) has long been consid ered to be the method of choice for clinical practice (using aneroid manometers) and clinical trials (using a random zero mercury sphygmomanometer (de Gaudemaris et al. 1985; Valler Jones & Wedgbury 2005)). In spite of some reservations (Waugh et al. 2002; Pickering 2003; O'Brien 2002), mercury sphygmomanometers are now rarely used also because of relevant ecological concerns (Markandu et al. 2000); blood pressure is now mostly measured by means of oscillometric devices with automated inflation and deflation (Wedgbury & Valler Jones 2008; O'Brien 2003; Braam & Thien 2003); the increasingly frequent use of the automated devices has the implication that many clinicians and nurses are no longer sufficiently well acquainted with the manual auscultatory methods (Safar & Smulyan 2008), which rely on cautious highly observer dependent auscultation of the Korotkoff I sound (first appearance of a clear tapping sound that gradually increases in intensity) for SBP and the Korotkoff IV (sound muffling [DBP_{KIV}]) or Korotkoff V auscultatory criterion (sound disappearance [DBP_{KV}]) for DBP (Ged des et al. 1966; Ettinger 1907; Laher & O'Brien 1982). On the other hand, modern technology has introduced newer robust devices that can be self operated by patients and trial subjects also with devices that measure from the wrist (Braam et al. 2003).

EVALUATION

The shift from manual (mercury based) auscultatory to automated oscillometric methods has been the subject of controversy (Graves et al. 2006), which has only partly been resolved by imposing standardized (cross) valida tion procedures (Stergiou et al. 2010).

Automated systems are highly robust and economic since they do not rely on an experienced analyst. This may result in less well standardized conditions of mea surement by lack of experience and discipline (choice of cuff, position of cuff, position of the microphone or oscillometric sensor, inflation speed, deflation speed, adjusted deflation speed when the pulse rate is low or irregular, posture of the patient, resting time, etc.): in a large survey, a physician's mean BP obtained during a routine visit (146/87 mmHg) was higher than the same physician's mean BP reading taken for research purposes (140/83 mmHg), or when BP was measured by a research nurse (137/78 mmHg) in accordance with strict proce dural guidelines (Myers et al. 1995).

On the other hand, this makes measurements made primarily for safety surveillance more reliable and useful also for further (efficacy based or pharmacodynamic) assessments.

CRITICAL ASSESSMENT OF THE METHOD

Blood pressure devices do not measure but estimate blood pressure. These estimates are subject to method effects; the resulting limitations may not be evident when comparing devices and techniques at recumbent rest. For instance, DBP estimates by means of auscultatory KIV and KV criteria agree quite well at rest (Lichten stein et al. 1986; Folsom et al. 1984), but far larger differ ences may exist when estimating interventional changes in blood pressure (Kaijser 1987; Gould et al. 1985; Burton 1967).

This is particularly evident when comparing acoustic criteria (auscultation or graphical analysis of the

microphone signals) when investigating the hemodynamic effects of inodilatory interventions (i.e., eating (de Mey et al. 1987), isoprenaline (de Mey & Enterling 1992; de Mey et al. 1992a), PDE III inhibition (de Mey et al. 1991; BeIz et al. 1988), and high doses of celiprolol (de Mey et al. 1993)). In the presence of such inodilatory changes, DBP_{KV} and DBP_{KIV} estimates were found to disagree substantially, the former (easier yes/no categorical criterion) estimating far larger DBP reductions and less efficient BP homeostasis (on the basis of MBP) than the latter (de Mey 1995). DBP_{KIV} estimates of the effects of isoprenaline have been shown to agree well with invasively measured BP responses, whereas DBP_{KV} overestimated them (Dietz & BeIz 1991). The bias thus primarily affects DBP_{KV} (rather than DBP_{KIV}) with a more protracted audibility of vascular wall (motion) sound in the presence of inodilatory hemodynamic changes, which is not primarily due to BP itself.

Accordingly, indirect blood pressure measurements are method specific estimates of blood pressure that should not be combined across methods; agreement between different methods for observations at rest does not preclude substantial disagreement in estimating interventional blood pressure changes; auscultatory estimates of DBP based on the Korotkoff V criterion (disappearance of vascular wall motion sound) yield highly biased estimates of inodilatory reductions of diastolic blood pressure, which they grossly overestimate, so that estimates based on the Korotkoff IV criterion ("muffling" of vascular wall motion sound) should be used instead. On the other hand, the Korotkoff V measurements might be useful as highly sensitive (albeit) nonspecific indices of inodilatory cardiovascular changes; there is value in evaluating both Korotkoff V and Korotkoff IV DBP provided their different meanings are understood and highlighted.

Although the auscultatory KIV criterion may be conceptually more valid and less confounded, it is hardly suitable for routine use since such auscultation is observer dependent and tedious. In the same experiment (Kaijser 1987), there was good agreement between DBP_{KIV} and oscillometric DBP, whereas the latter only slightly underestimated the inodilatory reduction in DBP and increase in SBP. Since oscillometric methods are robust, reliable, and highly economic, there is no reason to prefer tedious KIV auscultation; however, although more accurate in estimating drug related BP changes than KV auscultation, oscillometric BP methods have limited conceptual validity, while method specific and possibly affected by method*effect interaction.

C.5.4.2 Ambulatory Blood Pressure Monitoring (ABPM)

PURPOSE AND RATIONALE

Timed blood pressure measurement is only a snapshot of the blood pressure pattern; it fails to detect blood pressure fluctuations due to autonomic modulation, physical and emotional stress, and the modification thereof by therapeutic intervention (Smolensky & Portaluppi 1996; Hermida 2007). There is now good agreement that the diagnosis of hypertension (and the resulting decision to treat this condition with antihypertensive medication chronically) should not rely on an isolated office BP reading.

Similarly, the appropriateness and efficacy of antihypertensive interventions should take ambulatory blood pressure measurement/monitoring (ABPM) data into account (Myers et al. 2010) also with regard to their chronobiological fluctuations (Hermida et al. 2007). Indeed, numerous larger scale outcome studies have shown that ambulatory blood pressure measurement/monitoring (ABPM) yields better predictors of cardiovascular events when compared to timed manual BP readings in the physician's office or at home, even when the latter are taken carefully and in strict adherence with pertinent guidelines (Parati et al. 2009; Verdecchia et al. 2009). Furthermore, when patients were referred by their general practitioner for ABPM recordings, the mean BP taken in the office of the patient's own physician (152/87 mmHg) was significantly higher than the manual BP taken by the technician in the ABPM unit (140/80 mmHg) and the subsequent mean awake ABPM (134/77 mmHg) (Myers et al. 2009).

PROCEDURE

The first device for noninvasive ambulatory BP (ABP) monitoring (ABPM) was developed in 1962 and subsequently modified by Sokolow et al. in 1966 (Sokolow et al. 1966). It used a microphone taped over the brachial artery, a cuff inflated by the patient, and a magnetic tape recorder for storing cuff pressure. Presently, most devices are automated and rely on the oscillometric analysis of the vascular sound.

A large variety of devices for ambulatory measurement are available. These devices generally provide for both event triggered and automated oscillometric measurements according to a preset protocol of regular intervals (that may be set differently for the day and night measurements) (Myers et al. 1999). It is important to use a device that has been validated independently

(Friedman et al. 2008) for instance according to the pro tocol of the British Hypertension Society (O'Brien et al. 1993) or that of the US Association for the Advancement of Medical Instrumentation (Association for the Advance ment of Medical Instrumentation 1993) or both.

EVALUATION

The interpretation of ABPM data should be based on standardized criteria (O'Brien et al. 2000; Verdecchia et al. 2004).

An average day time ABP <135 mmHg systolic and 85 mmHg diastolic is generally considered normal for adults; levels <130/80 mmHg may be considered optimal. Subjects with day time systolic average ABP values <130 mmHg can be considered to be at only minimal cardiovascular risk even if the reading in the physician's office was higher (exclusion of white coat hypertension).

In hypertension management, it is important to ana lyze both day and night time readings (Littler et al. 1978; Mancia et al. 1983), although the latter may have to be set at broader intervals in order not to disturb sleeping rest. The day night time fluctuations are generally used to calculate the BP dip (= $(1 - (SBP_{sleeping}/SBP_{day\text{-}time})) \times 100$), with categories such as non dipper (0 10%), dipper (10 20%), extreme dipper (>20%), and reverse dipper (<0%) (O'Brien et al. 1988).

Further important criteria are the overall BP variability and early morning surges (Stergiou et al. 2008; Li et al. 2010; Stolarz Skrzypek et al. 2010), and the pulse pressure (SBP DBP) (Verdecchia et al. 2001). Since most systems also report pulse rate, ABPM data can also be used to assess pulse rate variability.

CRITICAL ASSESSMENT OF THE METHOD

There is little doubt that snapshot BP diagnostics are incomplete and may be misleading with regard to the diagnosis of hypertension and the need for treatment thereof; similarly, the evaluation of the efficacy of antihy pertensive medication and/or of the cardiovascular (BP) safety of noncardiovascular medications ought to take diurnal fluctuations into account. Accordingly, ABPM is a very important method in the clinical pharmacological evaluation of cardiovascular effects.

C.5.5 Plethysmographic Methods

An important characteristic of physiological cardiovascu lar function is the adaptation of the vascular tone and cardiac output to varying central and peripheral tissue demands. Plethysmography is the method of measuring volume changes. It quantifies the proximal inflow, out flow, and heart function through registration of distal volume changes.

C.5.5.1 Finger Pulse Plethysmography

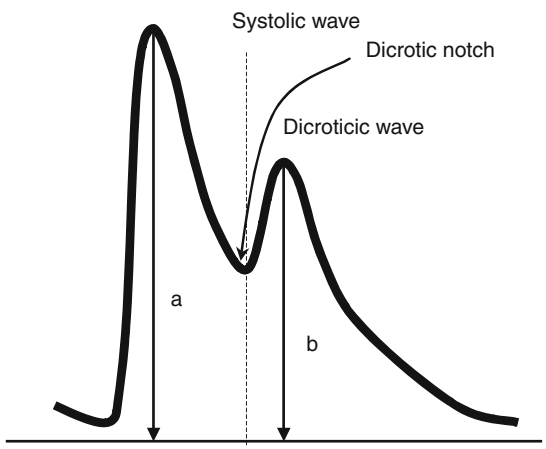
PURPOSE AND RATIONALE

Finger pulse plethysmography (FPP), also called digital pulse plethysmography, can be used to quantify both the effect of age and disease as well as pharmacological inter ventions on the venous and arterial vasculature (Murray & Foster 1996; Morikawa 1967; Imhof et al. 1980; Schinz et al. 1982; Buschmann et al. 1993; Lund 1986; Imhof et al. 1982; Wiegand et al. 1992; Millasseau et al. 2002; Millasseau et al. 2003; Takazawa et al. 1998; Jähnchen et al. 1997). Each individual peripheral pulse wave has a characteristic form (graph 1) consisting of a systolic (part a) and a diastolic part (dicrotic wave, part b), which is separated by the dicrotic notch. While the systolic part of the pulse wave is mainly determined by left ventricular function and the compliance of the arterial vessels, the dicrotic wave represents the secondary reflec tion of the pulse pressure from the periphery and the aortic valves.

In general, one can say that the longer it takes for the pulse wave to be reflected and the smaller the reflection phenomenon is, i.e., the smaller b is relative to a, the lower the overall vascular tone.

PROCEDURE

After a 5 10 min habituation period in the supine position, a cuff of the pulse oscillograph (Infraton



Pulsoszillograf, OS 20/4, Boucke, Tübingen, Germany) is placed at the distal end of the index finger and inflated to ~30 mmHg. The measurements should be performed in a quiet room with standardized room temperature (20 22°C) and humidity (40 60%) and preferably by the same investigator. The pulse wave signal is digitized by means of an analog/digital converter and sent to a commercially available personal computer. After regis tration of 10 subsequent pulse waves, a specialized software (Arterienpulswelle, PharmaData International, Wiesbaden, Germany) is used to calculate for each pulse wave recorded the a/b ratio (graph 1) and the mean a/b value for all 10 subsequent pulse waves.

EVALUATION

Visual inspection of the a and b marks on the pulse waves by the investigator and the possibility to correct the marks manually in the software program ensures the quality of the automated evaluation. The arithmetic mean of 10 subsequent valid pulse waves is used as a marker of vascular tone per subject and time point.

CRITICAL ASSESSMENT OF THE METHOD

FPP is being extensively used to quantify the vascular state correlated to demographic factors like age, vascular func tion, or diseases. In addition, FPP can be used to charac terize the vascular effects of pharmacological therapies and interventions both in healthy volunteers and patients.

The most frequently studied class of compounds are organic nitrates for which consistently a characteristic increase of the a/b ratio has been described (Morikawa 1967; Imhof et al. 1980; Schinz et al. 1982; Buschmann et al. 1993; Lund 1986; Imhof et al. 1982; Wiegand et al. 1992; Millasseau et al. 2002). It has been for a long time a debate if the increase in a/b is due to changes in left ventricular afterload (arterial) or left ventricular preload. Stengele et al. (Stengele et al. 1996a) demonstrated in a direct comparison to the direct vasodilatator nifedipine the characteristic changes of the a/b ratio under nitrate therapy are a sign of left ventricular preload changes, also known as venous pooling. In the area of the nitrates, the a/b ratio can be regarded as a validated biomarker of the anti ischemic effect of nitrates (Jähnchen et al. 1997; Stengele et al. 1996a; Stengele et al. 1996b). Another well documented drug often used as a positive control is salbutamol, which leads via its beta2 agonistic properties to nitric oxide NO dependent vasodilatation and thus increases the a/b ratio. This effect can be antago nized by co administration of the NO synthase inhibi tor, *N* monomethyl L arginine (L NMMA) (Chowienczyk et al. 1999). Thus, the above mentioned substances are well suited as positive or negative controls for the internal validation of studies on investigational vasoactive substances.

In addition to drug effects, a number of patient related factors have been described that also influence the a/b ratio. These include reduced prominence of the dicrotic wave with increasing age and in the presence of arterial hypertension (Millasseau et al. 2002; Millasseau et al. 2003). Additionally, a decreased response to gylcerol trinitrate and salbutamol has been described in hyperten sive and diabetic patients, respectively (Millasseau et al. 2002; Millasseau et al. 2003; Chowienczyk et al. 1999).

MODIFICATIONS OF THE METHOD

a/b ratios can also be determined by means of photoplethysmography (e.g., Hellige, Freiburg, Germany). Instead of a cuff, infrared light can be used to determine the pulse wave form. After analog digital conversion of the signal, the measurement algorithm and the handling of the data is identical to the method described above.

C.5.5.2 Venous Occlusion Plethysmography

PURPOSE AND RATIONALE

Venous occlusion plethysmography (VOP) is based on the principle that a cuff on one or several limbs (arms or legs) is inflated to a pressure above the pressure in the veins but below the pressure in the arteries. This setting allows further arterial influx into the limbs whereas the venous return is stopped; at the same time, volume detectors distal to the cuff measure the volume changes of the respective limb. VOP can provide data on arterial basal influx, reactive hyperemia, venous capacity, and after deflation of the cuffs also on venous return. These parameters can be deter mined both in the presence or absence of vaso active phar macological substances (Whitney 1953; Wilkinson & Webb 2001; Panza et al. 1991; Benjamin et al. 1995; Christ et al. 2000; Barac et al. 2007; Lind et al. 2002). In addition to being a research tool, VOP is used in clinical practice as a standard tool both for the diagnosis and monitoring of therapy of peripheral vascular diseases (Compactus Manual, Compactus Medizintechnik, Eurasburg, Ger many; McNally et al. 1993; Warwick et al. 1994).

PROCEDURE

Subjects need to be investigated in a quiet room and under standardized room conditions regarding room

temperature and humidity. They need to lie on their back with their legs and ankles above the heart level, supported by foam blocks. A pneumatic occlusion cuff is being placed around each limb and connected with an automatic inflator (Compactus, Gutmann Medizine lektronik, Eurasburg, Germany). Mercury strain gauges are placed on the maximum circumference, largest part of the limb, to detect the minimal swelling of the limb (and thus the volume changes) following occlusion. The distal parts of the limbs (i.e., hand or feet) are also being isolated with inflatable cuffs. After calibration, the Compactus device follows an automated schedule: Base line (arterial) blood flow: Inflation of the upper limb cuff for 6 s to 60 mmHg leads to venous occlusion. This is followed by a subsequent rapid deflation for 54 s. Three consecutive 6:54 s cycles are performed. Mean arterial inflow is determined within the initial 4 s of each infla tion cycle.

Determination of venous capacity is performed by inflating the upper limb cuff in 1 min intervals to 40, 60, and 80 mmHg, respectively. After rapid deflation, venous return can be determined.

Reactive hyperemia: The cuff at the distal part of the limb is inflated to suprasystolic pressure to exclude it from systemic circulation. The upper limb cuff is inflated to suprasystolic pressure (standard: 180 mmHg, in hyperten sives: 50 mmHg above systolic blood pressure) for 3 min. Thereafter, the limb cuff is rapidly deflated. Arterial flow reserve is measured immediately thereafter by 5 cycles of inflation to 60 mmHg for 5 s followed by rapid deflation for 5 s. Peak flow is the highest arterial inflow determined.

EVALUATION

The measured flow rates are expressed in units of ml/100 ml tissue/min. Depending on the scientific focus, either maximum/peak values for the respective flow parameters (e.g., peak postischemic flow) or the cumulative total flow determined as area under the plethysmographic curve (AUC) can be determined. Depending on the statistical design of the study, the necessary statistical procedures need to be adapted. This applies for the between group comparisons or the assumptions regarding normal distri bution of the data.

CRITICAL ASSESSMENT OF THE METHOD

The method and apparatus described above is one possi bility for performing VOP. In the scientific literature, a number of other devices have been described that, how ever, all are based on the same physiological principle (Hokanson Inc, Bellevue, WA, USA, Fitrass, DOMED Medizintechnik, Krailling, Germany, Medimatic, Copen hagen, Denmark). The method described here has the advantage that it uses a ready to use device, which is robust enough for daily clinical use. Moreover, its use as a diagnostic tool shows that the pure measurements can be performed by specially trained technical staff which does not necessarily need to be medically qualified, allowing the measurement of the parameters over longer periods of time for research purposes.

Knowing that environmental factors like temperature, psychological factors including mental stress but also circadian rhythm are affecting blood flow makes the standardization of these factors a key factor for standard ization during the measurements (Panza et al. 1991; Pedrinelli et al. 1989).

Regarding the study design under which the measured parameters can best be compared and statistically analyzed is the cross over design. Here, the interindividual variabil ity is eliminated and differences in the parameters are limited to environmental factors or the factor, e.g., drug under investigation.

MODIFICATIONS OF THE METHOD

The method of plethysmography has undergone multiple modifications since its first use more than 90 years ago (Benjamin et al. 1995). The use of mercury strain gauges facilitated and therefore broadened its use tremendously. Nowadays, the method is either used in the clinical setting to diagnose or monitor peripheral vascular dis ease or for research purposes. It becomes an especially powerful cardiovascular research tool once it is combined with intravascular administration of vaso active drugs, which can be used to positively or negatively control and validate the measurements. In this setting, it has been and still is being used to determine the effects of new drug candidates or to investigate physiological mechanisms of vascular control. Classical drugs used as positive controls include acetylcholine, which via a nitric oxide (NO) dependent pathway leads to vasodilatation or NO synthase inhibitors like *N* monomethyl L arginine (L NMMA) or norepinephrine (Pedrinelli et al. 1989; Martens et al. 2006; Sundberg & Lehtonen 2000; Hermann et al. 2006). The sensitivity of the method to interventions is demonstrated by its ability to mirror changes induced by a large variety of both pharmacolog ical and nutritional interventions (Pedrinelli et al. 1989; Martens et al. 2006; Sundberg & Lehtonen 2000; Hermann et al. 2006; Abbink Zandbergen et al. 1999; Anumba et al. 1999; Butler et al. 2001; Gori et al. 2001; Wilkinson et al. 2001a; McCall et al. 2009; Morgan et al. 2006; Hesse et al. 2005).

C.5.5.3 Pulse Wave Analysis

PURPOSE AND RATIONALE

Pulse wave analysis (PWA) encompasses a range of differ ent methods for the description and analysis of the pulse wave form. This in turn allows to draw inferences on blood flow and on vascular function in the body (Murray & Foster 1996; Wilkinson et al. 1998a; O'Rourke & Gallagher 1996). Although a whole range of methods have been described in the past (Laurent et al. 2006), the two dom inant procedures used today are the determination of the pulse wave velocity (PWV) and the augmentation index (AIx) (Wilkinson et al. 1998a). Both methods allow the quantification of the elastic properties of the large elastic conduit vessels including the aorta. Characteristic changes of PWV and AIx have been described for a number of cardiovascular risk factors and diseases, like aging, hyper tension renal impairment, peripheral artery disease, etc. (Laurent et al. 2006; London & Cohn 2002; Safar et al. 2002; Wilkinson et al. 2002a; Weber et al. 2004; Benjo et al. 2007; Protogerou et al. 2006; Sutton Tyrrell et al. 2005; London et al. 1994; Mattace Raso et al. 2006; Brewer et al. 2007). Likewise, interventions known to decrease cardio vascular risk have been shown to positively influence PWV and AIx (Breithaupt Grögler et al. 1996; Breithaupt Grögler et al. 1997; Raison et al. 2002; Smith et al. 2002; Orr et al. 2009; Williams et al. 2009; Williams et al. 2006; Manisty et al. 2009; Madden et al. 2009; Hall et al. 2001).

PROCEDURE

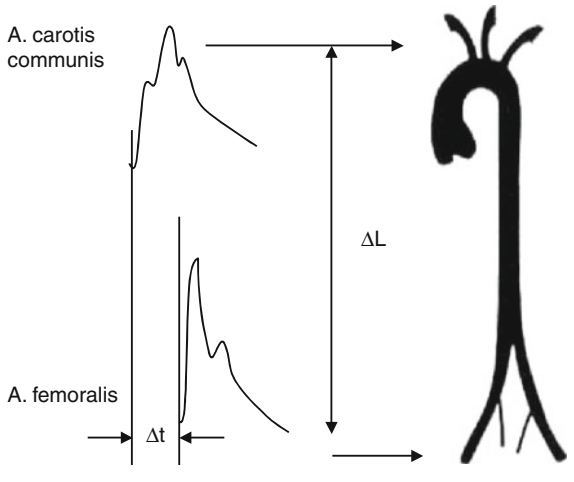
All PWA measurements should be performed in a stan dardized body position (sitting or supine) in a quiet, temperature controlled room (22 ± 1°C) after a brief period (at least 5 min) of rest.

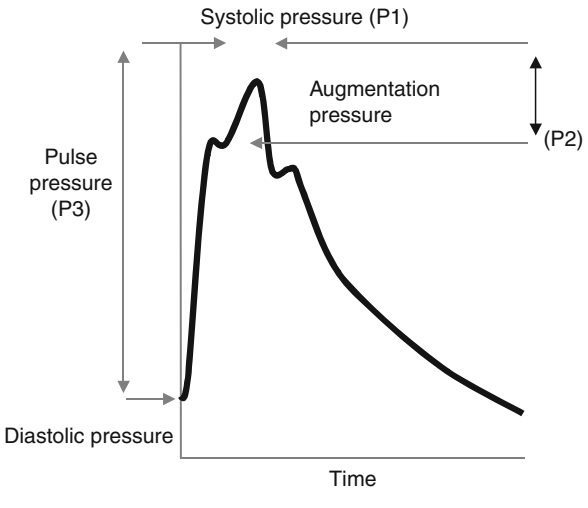
Pulse Wave Velocity (PWV)

The pulse wave velocity is defined as the time it takes for the pulse wave to travel from a proximal site to a distal place (relative to the heart) divided by the distance (PWV = Δdistance/Δtime [m/s.]) (graph 1). To this end, the foot of the arterial pulse wave is being recorded by applanation tonometry using commercially available transcutaneous, hand held devices (e.g., Complior®, France; SphygmoCor®, AtCor, Australia) at a proximal (carotid artery) and a distal (femoral artery) site of the body with simultaneous or R gated determination of the time interval between the two measurement points. The distance between the two measurement points is measured on the body surface either as the direct distance between the radial and femoral artery or the fossa jugularis/jugulum and the symphysis (Laurent et al. 2006; Oliver & Webb 2003).

Augmentation Index (AIx)

Radial artery pressure pulse waveforms are recorded using a transcutaneous, hand held high fidelity applanation tonometer (e.g., SphygmoCor®, AtCor, Australia). Under optimal conditions, this noninvasive measurement reveals virtually identical pressure waves as an intra arterial trans ducer as demonstrated by Sato and co workers (Sato et al. 1993). To avoid confounding differences in hold down pressure of the tonometer, the peripheral waveforms are calibrated with a pressure value determined by a mercury sphygmomanometer at the brachial artery (Kelly et al. 1989). An averaged radial pressure waveform derived





from a 10 s recording is subsequently converted by the commercially available software module (SphygmoCor®, AtCor, Australia) converts this peripheral arterial wave into a corresponding central waveform by using a validated transfer function (Wilkinson et al. 1998a; Takazawa et al. 1996; Segers et al. 2001; Cameron et al. 1998; Chen et al. 1996; Chen et al. 1997).

EVALUATION

Pulse Wave Velocity (PWV)

PWV is calculated from the reading by dividing the distance between the proximal and distal measurement point divided by the measured time.

Mathematical formula:

$$\mathrm{PWV} = \Delta\mathrm{distance}/\Delta\mathrm{time}(\mathrm{m/sec.})\ (\mathrm{graph\ 1}).$$

Augmentation Index (AIx)

The augmentation (pressure) is defined as the (pressure) difference between the second and first systolic peak of the central pressure waveform (P2 P1, graph 2). The augmentation index (AIx) as a measure of systemic arterial stiffness, is defined as the augmentation pressure as a proportion (expressed in percent) of the pulse pressure (P1 P3, graph 2).

Mathematical formula:

$$\mathrm{AIx}[\%] = (\mathrm{P2} - \mathrm{P1})/(\mathrm{P1} - \mathrm{P3}) \times 100 \text{ or } \mathrm{AIx}[\%] = (\Delta\mathrm{P}/\mathrm{PP}) \times 100$$

where ΔP is augmentation pressure and PP is pulse pressure

Dependant on the timing and magnitude of the augmentation, AIx can be positive, zero, or negative. If due to stiff arteries the reflected central wave occurs early, augmentation occurs before peak pressure (i.e., before systolic ejection) and AIx is positive. If due to highly elastic vessels, the reflected wave occurs late relative to peak pressure (young healthy persons), AIx is negative. Thus, AIx is a parameter of the relative contribution of augmentation to the arterial pressure waveform.

CRITICAL ASSESSMENT OF THE METHOD

AIx is sensitive to differences in mean arterial pressure, heart rate, and height (Wilkinson et al. 1998b; Wilkinson et al. 2000; Wilkinson et al. 2002b; Wilkinson et al. 2001b; Gatzka et al. 2001; Smulyan et al. 1998). For a reference heart rate of 60 bpm, the amplification increases by approx. 1%. Therefore, the above described system generates additionally an AIx value for a simulated 75 bpm heart rate (AIx@75) automatically. Other variables need to be analyzed separately; first by descriptive statistics, i.e., within or between group comparisons and if applicable by multiple logistical regression.

For the application of the methods, Liang and co workers have provided an excellent overview on trial design and sample size considerations when using the two above methods (Liang et al. 1998). Koivistoinen et al. only recently provided, after decades of research, the first reference ranges for PWV (Koivistoinen et al. 2007).

MODIFICATIONS OF THE METHOD

As described under purpose and rationale, there are multiple other devices and therefore modifications of the methods described here. At the same time, they follow the identical physiological phenomena and mainly differ in their mathematical output (Laurent et al. 2006; Mackenzie et al. 2002).

C.5.6 Systolic Performance

The systole extends from the end of the late diastolic filling (closure of the mitral valve) to the start of the next isovolumetric systolic relaxation phase (closure of the aortic valve); therefore, it includes: the isovolumetric contraction phase (until opening of the aortic valve) and the ejection phase(s); the right ventricle contracts first, then shortly followed by the left ventricle.

Performance and energy requirements of the heart muscle and heart pump depend on preload (ventricular filling), heart rate, afterload (the "load" that the heart must eject blood against ≈ aortic input impedance as defined by total peripheral resistance, arterial conductivity and distensibility, and wave reflections (London et al. 2004)) and inotropy (load and heart rate independent performance) (Bowditch 1871; Frank 1895; Patterson et al. 1914; Zimmer 2002; dos Remedios 2007; Solaro 2007; Ashrafian et al. 2008).

C.5.6.1 Systolic Time Intervals

PURPOSE AND RATIONALE

Systolic time intervals (STI) are the time equivalents of the electromechanical systolic (forward) pump performance (Blumberger 1940; Blumberger 1942; Katz & Feil 1923).

PROCEDURE

Relevant segments can be derived from the simultaneous high speed (Spodick et al. 1978) registration of

the electrocardiogram (ECG), phonocardiogram (PCG), carotid pulse mechano cardiogram, impedance cardio gram (ZCG) (Li & Beiz 1993; Stern et al. 1985), or by echocardiography. Although there is some delay between central events and their peripheral reflection (Lewis et al. 1974), this has relatively little impact on the accuracy of the estimation of the timing of central events (Metzger et al. 1970; Buch et al. 1970; Van de Werf et al. 1975). The pre ejection period (PEP) corresponds to the duration of the isovolumetric contraction phase from the start of the ECG Q wave up to the start of the ejection (opening of the aortic valve, between the first and second component of the first PCG heart sound); the left ventricular ejection time (LVET) from the start of the systolic ejection (end of PEP) up to the end of the ejection (closure of the aortic valve, between the first and second component of the second PCG heart sound, nadir of the carotid pulse wave, nadir of the dZ/dt curve by ZCG, etc.); the total electromechanical systole (QS2) then corresponds to the sum of PEP and LVET.

An increase in HR shortens STIs, LVET, and QS2 in particular, whereas the PEP is less HR dependent (Spodick et al. 1994; Joubert & Beiz 1987; Rousson et al. 1987). Accordingly, there are numerous attempts to "cor rect" STI for HR (STI_c) (Li & Beiz 1993; Harris & WS 1969; Weissler & Garrard 1971).

EVALUATION

The PEP reflects the isovolumetric contraction time (ICT); the PEP is shortened by an increase in HR, an increase in preload (ventricular filling), a decrease in afterload, and by a positive inotropic stimulation. Accord ingly, the PEP is particularly sensitive to medications that induce inotropic stimulation and vasodilatation ("inodilators") provided there is no restriction of venous return. Inotropic stimulation increases the VET only slightly; accordingly, the shortening of the $QS2_c$ and the reduction of the PEP/VET ratio ("Weissler Index"), which are often propagated as "contractility indices" are predominantly defined by the shortening of the PEP. A reduction in afterload shortens the PEP, prolongs the HR corrected VET with a reduction of the PEP/VET ratio, whereas the $QS2_c$ is hardly changed. Vasodilatation induced changes in STI are hardly changed by concomi tant beta adrenoceptor blockade and atropine; therefore, PEP and VETc can be assumed to be (also) highly afterload dependent, whereas the $QS2_c$ is not (Stern et al. 1984; Nakamura et al. 1983).

Normally, the electrocardiographic QT interval is shorter than the QS2. Adrenergic stimulation and other forms of inotropic stimulation prolong the QT interval relatively to the shortening of the QS2. Accordingly, the shortening of the QS2/QT ratio has been propagated as one of the many "contractility indices" (Boudoulas et al. 1981a; Boudoulas et al. 1981b). There have been some early appli cations in clinical cardiology (Boudoulas et al. 1982), but no application in cardiovascular clinical pharmacology.

CRITICAL ASSESSMENT OF THE METHOD

HR corrections of STI are based on historic linear regres sions in quite small samples. It is doubtful that these equations are stable and universal. Indeed, it is hardly likely nor can it be verified that they can be extrapolated to further subjects (Sr et al. 1988) and different experi mental conditions (Wolf et al. 1978; Mäntysaari & Länsimes 1992). Furthermore, these HR corrected STIs are meaningless mechanically since HR is an intrinsic determinant of pump action, performance, and effi ciency. A shortening of the PEP or QS2 should only then be accepted as an index of enhanced "contrac tility" if a simultaneous change of vascular load can be excluded.

The value of STI in cardiovascular clinical pharmacol ogy relates particularly to their excellent reproducibility (Levi et al. 1982; Sundberg 1993; Scott et al. 1989) and high pharmacosensitivity (Hassan & Turner 1983; Lewis 1983; Imhof et al. 1987; Boudoulas 1990): STI have been used in clinical cardiology to monitor progressing pump dysfunction (Price et al. 1991; Hamada et al. 1991; Utsunomiya et al. 1990; Chenard et al. 1988) including iatrogenic cardiomyopathies (Hutchinson et al. 1978; Lenzhofer et al. 1983; Chaudron et al. 1982); in cardio vascular clinical pharmacology, STI have been used to characterize cardiotonics (Harris et al. 1967; Carliner et al. 1974; Belz et al. 1978; Belz et al. 1979; Johnson et al. 1981; Belz et al. 1981; Alken & Belz 1984; Vemuri et al. 1992), negative inotropics (Fieldman et al. 1977), reduction in preload (Harris et al. 1973; Belz et al. 1984; Belz et al. 1985; de Mey & Enterling 1986; de Mey & Enterling 1987), and stress interventions (Vitolo et al. 1991; Groza et al. 1983).

STI have been very important in the late 1980s and throughout the 1990s for the noninvasive characteriza tion of drug effects on systolic performance. Now, such methods appear antiquated also since there are no mod ern state of the art devices to measure and analyze STI. Their displacement by more sophisticated echocardio graphic methods in clinical cardiology have led to a loss of interest and acceptance of such methods, although the echocardiographic methods themselves have failed to gain wide acceptance in the experimental evaluation of cardiac effects.

C.5.6.2 Myocardial Performance Index (Tei)

PURPOSE AND RATIONALE

The echocardiographic Myocardial Performance or "Tei" Index (MPI) is the modern analog of the STI.

PROCEDURE

MPI is based on the estimates of the isovolumetric contraction and relaxation time (ICT and IRT) and ejection time (ET) obtained by pulsed wave Doppler (PWD) or tissue Doppler echocardiography of the mitral annulus (TDE) (Bruch et al. 2000; Klein et al. 1994).

EVALUATION

Doppler echocardiographic ICT, IRT, ET, and MPI are important tools in clinical cardiology for the noninvasive follow up of patients with myocardial infarction, major cardiac surgery, and after heart transplantation.

CRITICAL ASSESSMENT OF THE METHOD

These methods have the important add on advantage to assess both systolic and diastolic function and to be able to distinguish between left and right ventricular function.

The MPI (= (ICT + IRT)/ET) estimates are method specific and this method specificity should be accounted for: both methods were shown to have high diagnostic accuracy for heart failure, but with distinct and method specific diagnostic cut offs (Gaibazzi et al. 2005; Duzenli et al. 2009). The methods rely on a very high level of analyst expertise: they are observer dependent and not economic; the latter aspects might explain why such methods find little application in the experimental evaluation of cardiac drug effects, in spite of the wealth of information that could be gained.

C.5.6.3 Noninvasive Estimates of Stroke Volume and Cardiac Output

PURPOSE AND RATIONALE

The stroke volume (SV) and cardiac output (CO = HR × SV) are the volume equivalents of the systolic, i.e., forward cardiac pump function.

PROCEDURE

Several noninvasive methods have been investigated and propagated for the experimental investigation of SV and CO:

- Carbon dioxide rebreathing (indirect Fick method) (Russell et al. 1990).
- Transthoracic impedance cardiography (ZCG (Nyboer et al. 1940; Kubicek et al. 1966) see below).
- Diastolic pulse contour analysis ("PCA"), i.e., analysis of noninvasive radial artery pulse wave forms by means of a third order, 4 element modified Windkessel model of the circulation quantifying the Windkessel model criteria: systemic vascular resistance [SVR], large artery "capacitive" compliance [C1], small artery "oscillatory"/"reflective" compliance or "reflectance" [C2], and inductance [L inertance of blood) for instance by means of the CVProfilor by HDI Hypertension Diagnostics (Paeras et al. 2005) This method uses an estimate of SV from the ejection time (ET), heart rate (HR), body surface area (BSA), and age, and all PCA criteria (SVR, C1, C2, and L) rely on this estimate (and the constraints of its algorithmic simplicity).
- Systolic pulse wave analysis ("PWA"): reconstruction of the pulse wave form of the ascending aorta from distant (carotid/brachial/radial) pulse wave contours by means of a validated general transfer function (GTF) (Cameron et al. 1998; Chen et al. 1996; Karamanoglu et al. 1993) deriving the central augmentation index (AIx), the time to wave reflection (Tr as a measure of central aortic compliance), and algorithmic estimates of central hemodynamics for instance by means of the Sphygmocor CPM by PWV Medical/AtCor Medical).
- Echocardiographic techniques: M mode echocardiography (Gibson 1979), two dimensional echocardiography (de Mey 1994; Schiller et al. 1989; Quinones et al. 1981; Erbel et al. 1985; Assmann & Roelandt 1987; American Society of Echocardiography Committee on Standards 1989; Erbel et al. 1988), three dimensional echocardiography (Soliman et al. 2008).
- Transthoracic pulsed wave Doppler echography of the aorta ascendens (Otto et al. 2002; Evans et al. 1989a; Evans et al. 1989b; Ihlen et al. 1984; Haites et al. 1985; Acton & Broom 1990; Phillips et al. 2009), transoesophageal Doppler echography (Dark & Singer 2004; Boulnois & Pechoux 2000), etc.

EVALUATION

The older devices required tedious signal analysis and complex nonautomated signal and data processing, which relied on public algorithms; newer methods are mostly highly automated black boxes with proprietary

algorithms that often are device specific nonpublic "adaptations" of the original algorithms.

CRITICAL ASSESSMENT OF THE METHOD

Invasive measurements of SV and CO are method specific estimates relying on a "black box" analysis of the dilution of a controlled injection of dye or a cooled volume of saline ("thermodilution"). Therefore, invasive SV and/or CO measurements are far less robust "golden standards" than often claimed (Renner et al. 1993; Nishikawa & Dohi 1993; Latson et al. 1993).

In intensive care medicine, newer methods have been introduced that are called "minimally" invasive: they provide for continuous hemodynamic monitoring without repeated central catheter dilution; they monitor systolic function based on wave/contour analysis of (invasive) arterial peripheral pulses with or without calibration with pulmonary artery thermodilution (for instance PiCCOplus system by Pulsion (Godje et al. 2002; Gödje et al. 1998), LiDCO^{TM}plus system by LiDCO (Pearse et al. 2004), FloTrac and Vigileo by Edwards Lifesciences (Manecke 2005), and P.R.A.M. Mostcare System by Vytech Health (Romagnoli et al. 2009)). The surge of "minimally" invasive methods also illustrates 1) the need for reliable methods for continuous monitoring, and 2) the lack of satisfaction with and acceptance of truly noninvasive methods to meet this requirement.

The related constraints are illustrated in the following by the past and present positioning of transthoracic impedance cardiography (ZCG) in the clinical pharmacological characterization of investigational changes in cardiovascular function.

ZCG is based on the observations in the 1930s and 1940s that typical changes occur in transthoracic impedance (Z) to a high frequency low voltage AC current applied through the thoracic cage during the cardiac cycle; these changes were originally primarily seen as the consequence of volume shifts with an increase in volume and decrease in impedance during systole and a decrease in volume and increase in impedance during diastole (Nyboer et al. 1940; Nyboer 1960; Whitehorn & Perl 1949); now it is understood that the contour of the time course of the negative velocity of the transthoracic impedance changes (dZ/dt) is analogous with the blood flow velocity in the central large vessels (Witsoe & Kottke 1967; Kottke et al. 1974) and the differential of the carotid pulse curve (Frey & Siervogel 1981) while also including venous and right ventricular components (Baker 1977). In clinical cardiology, there was little interest in such rheological plethysmographic concepts because of the various invasive methods that became available. The need for noninvasive monitoring methods in the aerospace industry led to the first impedance cardiographic applications (Kubicek et al. 1966; Moskulendo et al. 1962) mainly by Patterson und Kubicek and their co workers (Kubicek et al. 1966; Patterson et al. 1964; Patterson 1965; Kubicek et al. 1969) who were the first to use tape electrodes around the neck and thoracic base and to analyze the negative velocity of the transthoracic impedance changes (dZ/dt, $\Omega.s^{-1}$) rather than ΔZ. Assuming a simplified model (Patterson 1989; Miller & Horvath 1978), they developed an algorithm to estimate stroke volume (SV) from the ejection time (LVET), maximum dZ/dt (dZ/dt_{max}), the baseline impedance ($Z0$), the distance between the input and output electrodes (L), and a blood resistivity factor (ρ): $SV = \rho \times dZ/dt_{max} \times LVET \times (L/Z0)^2$ (Kubicek et al. 1966); in spite of its many constraints, Kubicek's equation still is the core algorithm of ZCG (White et al. 1991; Goldstein et al. 1986; Porter & Swain 1987).

The registration of the ZCG signals is not observer dependent, but the analysis of the signals (delineation of the ejection time and measurement of dZ/dt_{max}) is. Originally, ZCG analyses also included an assessment of STI (see above) and therefore required the simultaneous registration of at least three signals (ECG, ZCG, PCG); the ZCG signal has points of repair to delineate the start and the end of the LVET, albeit that these are more easily and accurately identified if the PCG and carotid pulse curve (4 channel method) are recorded as well. In the early 1980s, this approach, which required tedious 3 or 4 channel signal analysis, was frequently used in cardiovascular clinical pharmacology (for instance Cardiodynagraph, Diefenbach GmbH), also since it permitted an almost continuous monitoring (White et al. 1991; Goldstein et al. 1986; Porter & Swain 1987; Mohapatra 1981). In the mid 1980s, an alternative method became popular (NCCOM3 by Bomed Medical Manufacturing); it was particularly attractive since it used less inconvenient spot rather than adhesive tape electrodes (Gotshall & Sexson 1994), was fully automated, and relied only on the ECG and ZCG; furthermore, this method used its own physiologic algorithm and equations to estimate SV (Sramek et al. 1983; Bernstein 1986), the results of which disagree grossly with those according to the conventional equation by Kubicek applied on the same signals (de Mey & Enterling 1988); furthermore, the lack of support information (PCG and/or carotid pulse curve) makes the method less accurate in estimating LVET and, accordingly, SV (de Mey & Enterling 1993). Today, the Bomed device and devices for the conventional ZCG method are no longer available. To our knowledge, only one device now offers a ZCG methodology (TaskForce

Monitor [TFM] by CNSystems): like the Bomed device, the TFM is a black box for the automated analysis of ECG and ZCG signals from point rather than tape electrodes and using its own nonpublic equation to estimate SV; like the Bomed device, the absence of either PCG or carotid pulse and the fully automated signal analysis make it difficult to measure the LVET accurately since the fiduciary delineation of the LVET by the ZCG alone is not generally sufficient. The TFM also includes a finger pulse plethysmographic (DPG) method for the continuous parallel monitoring of blood pressure; these DPG are calibrated intermittently with noninvasive oscillometric SBP/DBP measurements. Such an approach is reliable only when the relationship between digital and forearm blood pressure is stable and is not altered by the intervention that is being investigated. Conventional ZCG is well reliable (de Mey et al. 1992b) and highly sensitive for drug effects, inodilatory effects in particular; they may agree with other invasive (O'Brien 2002; White et al. 1991; Mohapatra 1981; Fuller 1992; Pickett & Buell 1992) and noninvasive methods (Miles et al. 1993; McKinney et al. 1984; Milsom et al. 1982), but often appear to overestimate SV (Mohapatra 1981; Lamberts et al. 1984; Aust et al. 1982) and the changes thereof (Pickett & Buell 1992). The alternative methods have a similarly high reproducibility and are sensitive, but may be less accurate in estimating LVET and, accordingly, SV. However, all three have limited validity since they yield method and device specific estimates of SV (McKinney et al. 1984; Milsom et al. 1982; Lamberts et al. 1984) that are not unlikely to be affected by substantial method*subject*effect interaction.

The fate of ZCG is exemplary for most noninvasive cardiovascular methods: they are method and device specific estimates that may be very reproducible and sensitive, for drug effects in particular; they have a limited validity since they do not generally agree well with the established golden standards; this per se does not preclude their usefulness provided this limitation is understood and accounted for, also since the golden standards may prove impractical or impossible to use in similar collectives. However, in order to be useful, these methods need to be accepted as such. In drug development, this means that data generated with such methods need to be useful and acceptable for regulatory purposes. However, with the exception of ICH E14, there is no regulatory need or benefit in pursuing cardiovascular endpoints in early development studies. In the framework of "lean" drug development, this means that there is little demand for such studies. Accordingly, it has become difficult to improve their hard and software to meet present day quality standards and to keep the required operational expertise. Due to these latter constraints, it has become even more difficult to satisfy regulatory requirements. In consequence, several of these methods, although evidenced to be highly informative, are no longer available. Newer methods, especially those related to pulse wave velocity and pulse wave contour analysis or Doppler echocardiography may find a similar fate unless they find high acceptance in clinical cardiology.

C.5.7 Diastolic Performance

PURPOSE AND RATIONALE

The diastole extends from the end of the systolic ejection (closure of the aortic valve) to the start of the next isovolumetric systolic contraction phase (closure of the mitral valve); therefore, it includes: the isovolumetric relaxation phase (until opening of the mitral valve); the rapid filling phase, which begins when LV pressure falls below left atrial pressure and the opening of the mitral valve and involves interaction between LV suction (= active relaxation) and visco elastic properties of the myocardium (= compliance); diastasis, i.e., when left atrial and left ventricular pressures are almost equal and left ventricular filling is essentially maintained by the flow coming from pulmonary veins using the left atrium as a passive conduit; atrial systole, which corresponds to left atrial contraction and ends with the closure of the mitral valve (Angeja & Grossman 2003; Yellin & Meisner 2000; Zile 1989). The diastole is far more dependent on the HR than the systole and the diastolic filling lasts longer when the HR is slower.

According to the European Cardiology Society, establishment of the diagnosis of diastolic heart failure requires: (1) the presence of a clinical syndrome of heart failure (dyspnoea or fatigue at rest or with exertion, fluid overload, pulmonary vascular congestion on examination, or X ray); (2) demonstration of an ejection fraction $\geq$50%; and (3) demonstration of diastolic dysfunction (The European Study Group on Diastolic Heart Failure 1998). Others prefer the term "heart failure with a normal ejection fraction" (HFNEF), characterized by elevated ventricular filling pressures and abnormal filling patterns (Maurer et al. 2004) to allow for a better distinction between active and passive components, emphasizing that HFNEF may occur with or without impairment of the isovolumetric relaxation (active dysfunction).

Removal of calcium from the myofilaments and uncoupling of actin myosin cross bridge bonds govern the rate of myocardial relaxation and thus the rate of ventricular pressure decline. This active component of diastole is typically characterized by the time constant of relaxation (τ), determined by fitting a mono exponential curve to the isovolumetric section of the ventricular pressure curve (Yellin et al. 1990). Subsequently, the mechanical properties of the ventricle are determined by passive factors, such as the degree of myocellular hypertrophy (myocardial mass), cytoskeletal, and extracellular matrix properties and chamber geometry; this is reflected by the end diastolic pressure volume relationship (EDPVR) and the features derived from it: ventricular chamber stiffness (i.e., slope of EDPVR at a given volume [dP/dV]) and compliance (the mathematical reciprocal of stiffness). Both are load dependent and are no measures of load independent diastolic function (lusitropy). In consequence, diastolic dysfunction may involve either or both active or passive ventricular properties. With an increased τ (which is typically observed with all forms of hypertrophy, and with aging), a higher mean left atrial pressure may be required to achieve normal filling volumes, especially at high heart rates. However, an increased τ is not ubiquitously associated with elevated mean left atrial pressure and heart failure. Instead, shifts of the EDPVR have been suggested to be a predominant factor of the hemodynamic and symptomatic abnormalities of heart failure in HFNEF: A leftward/upward shifted EDPVR is indicative of decreased chamber capacitance, whereas a rightward/downward shifted EDPVR (increased ventricular capacitance) occurs in all forms of dilated cardiomyopathy (remodeling). Accordingly, there are various conditions with distinctly different properties of the passive and/or active diastolic components that may result in HFNEF (The European Study Group on Diastolic Heart Failure 1998).

PROCEDURE

An in depth analysis of diastolic function requires invasive investigations to assess the pressure volume relation along the overall cardiac cycle, which permits to derive τ, end diastolic stiffness, etc. (Zile 1989).

Noninvasively, Doppler Ultrasound recordings of transmitral and pulmonary venous flow velocities and time intervals are useful alternatives (Rokey et al. 1985), and Doppler echocardiography has become the primary tool for identifying and grading the severity of diastolic dysfunction in patients demonstrating elevated ventricular filling pressures and abnormal filling patterns (Rokey et al. 1985; Galderisi 2005; Stoddard et al. 1989).

This involves the determination of the early diastolic velocity (E), atrial velocity (A), deceleration time of E velocity (DT), and the isovolumetric relaxation time (IVRT) from the transmitral Doppler signals. Complementary evaluation of pulmonary venous flow might be of interest (Masuyama et al. 1995); further methods rely on Tissue Doppler technology (Nagueh et al. 1997) and color M mode derived flow propagation rate (Garcia et al. 2000). These investigations are carried out at rest with controlled maneuvers (Valsalva (Nishimura & Tajik 1997), leg lifting (Pozzoli et al. 1997)).

EVALUATION

In contrast to inotropic changes, lusitropic changes of diastolic function are not regularly investigated and characterized except in patients with postmyocardial dysfunction and other forms of heart failure. Furthermore, there are no drugs that are targeted specifically on improving diastolic function, albeit that ancillary positive lusitropic properties have been demonstrated for some medications.

Investigation of diastolic properties might be of interest in differentiating responsiveness and lack there of in the evaluation of treatments of heart failure, but is only rarely used in this context.

In hypertension, diastolic function is also of interest since diastolic dysfunction is inherent to concentric left ventricular remodeling that is commonly seen in hypertensives.

CRITICAL ASSESSMENT OF THE METHOD

Load independent diastolic function (lusitropy) suffers from the same conceptual validity constraints as inotropy (load and heart rate independent systolic function). The lack of distinction between true lusitropy and passive components of diastolic performance is obvious. However, this does not preclude that the procedures to characterize ventricular relaxation and filling (even if composite and ambiguous criteria) provide a better understanding of the overall cardiac function.

C.5.8 Endothelial Function Testing

C.5.8.1 Background

Healthy arteries usually react upon defined physiological and pharmacological stimuli by endothelial dependent vasodilatation, a phenomenon named endothelial function. The interest in endothelial function testing (EFT) comes from observations in coronary angiography where atherosclerotic coronary arteries did not respond

to the physiological vasodilator acetylcholine (ACh) with physiological vasodilatation but with a paradoxical vasoconstriction (Ludmer et al. 1986). From today's understanding, impairment of endothelial function ("endothelial dysfunction") constitutes one of the earliest phenomena of asymptomatic atherosclerosis (Ludmer et al. 1986; Nabel et al. 1990; Celermajer et al. 1992; Heitzer et al. 2001; Cohn et al. 2004). On the one hand, endothelial dysfunction (ED) is a precursor in patients at risk of cardiovascular disease, and correlates during later stages of atherosclerosis with the severity of the disease; on the other hand, ED has been validated to be a predictor of future cardiovascular morbidity and mortality in patients with and without manifest cardiovascular disease (Heitzer et al. 2001; Cohn et al. 2004; Schächinger et al. 2000; Suwaidi et al. 2000; Halcox et al. 2002; Targonski et al. 2003; von Mering et al. 2004; Neunteufl et al. 2000; Perticone et al. 2001; Gokce et al. 2003; Gokce et al. 2002; Shechter et al. 2009; Suzuki et al. 2008; Brevetti et al. 2003; Murakami & Ohsato 2003; Chan et al. 2003; Fichtlscherer et al. 2004).

From a methodological standpoint, the assessment of EF can be distinguished according to a) the vascular bed investigated, b) the stimulus used to elicit EF, and c) the method used to quantify EF (Barac et al. 2007; Lind et al. 2002; Ebrahim et al. 1999). *Table C.5 1* provides a summary on the different possible methodological combinations. A methodology that stands out in this spectrum is the quantification of intima media thickness (IMT). IMT is not a functional test and does not need a vasomotor stimulus. It represents rather a method to characterize and quantify the structural changes occurring in the vessel wall, which proceed or accompany functional or symptomatic atherosclerosis (Corretti et al. 2002).

Coronary artery flow can be quantified by determining the coronary artery diameter with and without endothelium dependent (acetylcholine (ACh)) or independent vasodilators (nitroglycerine) by means of quantitative coronary angiography (Schächinger et al. 2000). As this method is highly invasive and requires a dedicated catheter laboratory, it is not considered as part of the classical clinical pharmacology methods; armamentarium is therefore not further discussed here.

Historically, all three endothelial function tests had a predominant role as pathophysiological and later on as diagnostic research tools. Meanwhile, a number of published data are also available where these methods have been used to quantify pharmacological and therapeutic effects of drugs, nutritional supplements, physical exercise, and food. Review of the available literature suggests that IMT has mostly been used in the quantification of the beneficial effects of statins. The other two methods, have widely been used across the cardiovascular treatment spectrum: drugs acting on the renin angiotensin system (ACE inhibitors, angiotensin II antagonists, calcium channel blockers (Mancini et al. 1996; O'Driscoll et al. 1997; O'Driscoll et al. 1999; Anderson et al. 2000; Zanchetti et al. 2009)) and lipid lowering drugs (statins, ezetimibe, niacin, thiazolidinediones, fibrates (Spieker et al. 2002; Vogel 1999; Westphal et al. 2008; Ostad et al. 2009; Taylor et al. 2004; Tack et al. 1998; Pistrosch et al. 2004; Evans et al. 2000), smoking cessation (Celermajer et al. 1993), folate/folic acid (Doshi et al. 2001; Mangoni et al. 2002), physical exercise (Hambrecht et al. 2000; Magyari et al. 2004; Goto et al. 2003; Moyna & Thompson 2004; Niebauer & Cooke 1996), and Mediterranean diet (Rallidis et al. 2009; Fuentes et al. 2001)).

Table C.5-1
EFT settings and methods involved

Vascular bed investigated	Vasomotion induction method	Vasomotion detection method
Coronary artery	ACh and SNP	Angiography
	FMD, EDV, RH	Ultrasound
	or	or
Brachial artery	Intra-arterial ACh & SNP	VOP or ultrasound
	or	or
	Albuterol and nitrate	AIx
Carotid artery	None	US = IMT

FMD flow mediated vasodilatation, *EDV* endothelium dependent vasodilatation, *RH* reactive hyperemia

C.5.8.2 Flow-Mediated Vasodilatation (FMD), Endothelium-Dependent Vasodilatation (EDV), Reactive Hyperemia

PURPOSE AND RATIONALE

Shear stress in the (arterial) vessel wall leads to nitric oxide (NO) release from the vascular endothelium, which subsequently leads to vasodilatation, a phenomenon named flow mediated vasodilatation (FMD). As this process is endothelium dependent, it is also called endothelium dependent vasodilatation (EDV); reactive hyperemia (RH) is a synonym.

PROCEDURE

To induce endothelium dependent flow mediated vaso dilatation, the subject is placed in a quiet room with a 5 10 min of rest in the supine position in a warm temperature controlled room. The brachial artery is imaged using B mode ultrasound in the longitudinal plane. A pneumatic cuff is placed around the forearm and inflated to a pressure of 50 mmHg above the subject's systolic blood pressure to stop blood flow. After 5 min of ischemia, the pressure is suddenly released and the increased blood flow detected is measured as the change in artery diameter by B mode ultrasound imaging. In order to avoid influence of the pulsatile changes in the vessel wall, it is advisable to use ECG synchronized images.

EVALUATION

Images acquired at baseline, and after 1 min of cuff release (time of maximum blood flow) are used to quantify the artery diameter. Ultrasound images are evaluated using the m line between media and adventitia for ECG synchronized images during diastole. FMD is the change in artery diameter as a function of the baseline diame ter, usually expressed as percent change of the baseline diameter.

Mathematical formula:

$$\%\text{FMD} = [(\text{diameter post ischemia} - \text{baseline diameter}) / \text{baseline diameter} \times 100].$$

CRITICAL ASSESSMENT OF THE METHOD

The noninvasiveness and relative simplicity of this tech nique has made it attractive for widespread use, both in clinical research settings and even larger population based studies. The predictive value of FMD for cardio vascular outcomes in patients at risk or with cardiovas cular disease has been extensively documented and made this technique a gold standard biomarker to test phar macological or other therapeutic interventions as a surrogate for cardiovascular outcomes (Shechter et al. 2009).

The lack of uniform equipment across investigational sites, and the interobserver variability and the lack of uniformity of pressure and timing of ischemia has com plicated the comparability of research results across dif ferent studies (Black et al. 2008). Thus, assessment of FMD should preferably be performed by the same investigator, with intensive training in the methodology, applying always the same research protocol (ischemia pressure, time of ischemia, time of readout of maximum dilatation).

Yeboah et al. (Yeboah et al. 2008) have shown that FMD and IMT measurement mirror different stages of the atherosclerotic process, suggesting that FMT and IMT cannot be considered as interchangeable methods. Reactive hyperemia in healthy volunteers in contrast to patients assessed by venous occlusion plethysmography could not be antagonized by means of the NO synthase inhibitor L NMMA, suggesting that in healthy volunteers, FMD seems not to be completely reflected by NO dependent vasodilatation (Nugent et al. 1999).

MODIFICATIONS OF THE METHOD

In addition to FMD as a phenomenon of endothelium dependent vasodilatation, single sublingual doses of glyceroltrinitrate (0.4 mg) can be used to determine the endothelium independent vasodilatation.

Moreover, although used relatively rarely, the increase in blood flow can be quantified by means of venous occlusion plethysmography (VOP) instead of the ultra sound technique (Nugent et al. 1999).

C.5.8.3 Forearm Perfusion Technique

PURPOSE AND RATIONALE

The forearm perfusion technique uses the phenomenon that endothelium dependent (ACh) and independent (sodium nitroprusside) vasodilators can be administered locally into an artery without causing systemic effects. The changes in blood flow are subsequently determined noninvasively by means of venous occlusion plethysmog raphy (VOP) or ultrasound imaging. The most frequently used technique uses increasing concentrations of acetyl choline (ACh, e.g., 7.5, 15, and 30 μg/min) into the bra chial artery and sodium nitroprusside (SNP, e.g., 1, 3, and 10 μg/min), which are infused into the brachial artery. To avoid sequence effects and to ensure blinding of the investigators, the sequence of ACh and SNP infusions should be blinded and randomized. One modification for the noninvasive delivery of nitrate bodies instead of SNP is the sublingual administration of 0.4 mg glyceroltrinitrate.

PROCEDURE

Investigations are performed in a quiet, temperature controlled room with participants in the supine position. A catheter is inserted into the brachial artery through which drugs can be infused at low concentrations. Baseline blood flow is determined during infusion of physiological saline. To elicit endothelium dependent vasodilatation, 5 min infusions of ascending doses of

acetylcholine (e.g., 25 and 50 µg/min, Clinalfa, Läufelingen, Switzerland) are delivered through the cath eter. Endothelium independent vasodilatation is induced by infusion of sodium nitroprusside (SNP, e.g., 1.6, and 3.2 µg/min). Before, during, and after infusion, forearm blood flow is determined by venous occlusion plethys mography (VOP) as described in the chapter "plethysmo graphic methods." Alternatively, but less frequently applied, blood flow can be determined by ultrasound imaging as described in the chapter on flow mediated vasodilatation (Pistrosch et al. 2004; Vogel et al. 1997).

EVALUATION

Results can be presented in a Cartesian graphical showing flow rates (usually expressed as (ml/min/100 ml)) as a function of the respective ascending dose intervals (usu ally expressed as either expressed as time or cumulative dose given). Adding data of different agonists and antag onists to the same graph allows for a direct graphical comparison and distinction of the magnitude of the dif ferential effects. As with the flow mediated vasodilatation, maximum, stimulated, or antagonized flow rates can also be presented as percentage of the baseline flow rate.

Mathematical formula:

$$\%\text{flow} = \%[(\text{flow post intervention} - \text{baseline flow})/\text{baseline flow} \times 100].$$

CRITICAL ASSESSMENT OF THE METHOD

Initially, the forearm perfusion technique has been used to characterize the impaired endothelial function in hypertensive patients (Targonski et al. 2003; von Mering et al. 2004). Later on, endothelial dysfunction could also be detected in other cardiovascular disorders like coronary or peripheral artery disease, smokers, patients with hypercholesterolemia, and diabetes patients (Lind et al. 2002; Ebrahim et al. 1999; Corretti et al. 2002; Mancini et al. 1996; O'Driscoll et al. 1997; O'Driscoll et al. 1999; Anderson et al. 2000). Due to the possibility of infusion of specific pharmacological agonists and antagonists, the method allows to mechanistically investi gate vascular pathophysiology and characterize the vascu lar effects of new molecules, and to establish dose response relationships, respectively. A disadvantage of the forearm perfusion technique is its invasiveness, which makes it unsuitable to be used in clinical trials involving large populations and multiple clinical centers.

MODIFICATIONS OF THE METHOD

Stenborg and coworkers (Stenborg et al. 2007) have intro duced the "endothelial function index," which is determined as a ratio of the ACh induced vasodilation divided by the SNP induced vasodilation at a predefined dose of each drug.

C.5.8.4 Pulse Wave Analysis/ Augmentation Index (Aix)

PURPOSE AND RATIONALE

Similar to the invasive forearm technique, where local administration of acetylcholine elicits endothelium dependent vasodilation, beta 2 adrenoceptor agonist have been demonstrated to decrease the augmentation index via a nitric oxide (NO) dependent mechanism. Thus, AIx together with agonists or antagonists of the NO system can be used to characterize and quantify vas cular responses both in healthy volunteers and in patients at risk or with manifest cardiovascular disease.

PROCEDURE

The procedure for the assessment of the augmentation index AIx is described in the chapter on plethysmographic methods.

EVALUATION

Please refer to the description of Aix in the chapter on plethysmographic methods.

CRITICAL ASSESSMENT OF THE METHOD

Wilkinson et al. (Wilkinson et al. 2002c) demonstrated that inhalation of the beta 2 adrenoceptor agonist albute rol and sublingual glyceroltrinitrate (GTN) led to charac teristic and reproducible changes of the central aortic pulse wave and therefore in the derived parameters aug mentation index (AIx). Both compounds diminish the reflected part (P2, i.e., the second systolic peak) of the pulse wave and thereby reduce the augmentation index. The effect of albuterol could be antagonized by infusion of the NO synthase inhibitor NG monomethyl L arginine (LNMMA), whereas the effect of GTN remained unaf fected. This suggests that the albuterol induced AIx reduction is comparable to the endothelium dependent vasodilation in the forearm endothelium and NO dependent. Moreover, inhibition of basal NO synthesis was shown to increase AIx (Wilkinson et al. 2002d), underlining the sensitivity of the method to indirectly measure effect of NO in the systemic vasculature. Further methodological validation versus the two established methods, flow mediated vasodilator (FMD) and intima media thickness (IMT), were provided by Ravikumar et al. and Lind et al.

(Ravikumar et al. 2002; Lind et al. 2005). They could demonstrate in a direct comparison that all three methods FMD, Aix, and IMT are equally effective in detecting the structural and functional endothelial damage.

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C.6 Pharmacodynamic Evaluation: CNS

Keith A. Wesnes · Helen Brooker

C.6.1 General Introduction

In early phase clinical trials of new medicines, two of the major CNS pharmacodynamic questions that can be directly and widely addressed are first, whether the com pounds have positive or negative effects on aspects of cognitive function and second, whether they will alter various mood states. The use both of tests of cognitive function and of self rating scales of mood, alertness, sleep iness, etc., has a long history in clinical pharmacology, and the methods which have become established in this field will be reviewed. Many if not all CNS disorders have various profiles of alterations to cognitive function and mood, some being characterized by the cognitive deficits, for example, ADHD and dementia, and others the mood changes, for example, depression. Traditionally, cognitive function tests were used in clinical pharmacology to deter mine if the compound was likely to pose safety risks, by disrupting various aspects of cognitive function which are crucial for everyday activities, such as car driving. How ever, in the last 20 years or so, this application has broad ened to seek potential benefits to cognitive function, because the opportunity to correct cognitive dysfunction has become the target of many medicines. Obvious limi tations to what can be addressed in early clinical pharma cology studies are, for example, the complex behavioral problems exhibited in schizophrenia, or whether the mechanism of correcting cognitive dysfunction can be evaluated in healthy volunteers. This review will initially focus on the established techniques that are currently employed in clinical pharmacology for the assessment of human performance and various mood states. The review will then consider various measures of brain activity, starting with electroencephalography (EEG) that has a long history in clinical pharmacology; but also imaging and nuclear medicine techniques as they have started to make valuable contributions to this field and hold great future potential. The review will close by considering models that have been widely used in clinical pharmacol ogy to help assess the therapeutic potential of new medicines.

C.6.2 Cognitive and Behavioral Tests and Test Systems

C.6.2.1 Introduction

Cognitive function can be directly measured by evaluating how well a volunteer or patient can perform a particular task. Literally, tens of thousands of tests of cognitive func tion have been developed, and a large number of these have been used in clinical pharmacology over the decades. As in many fields, automation of such assessments has brought significant advantages to the quality, reliability, and sensitivity of the measurements. However, automa tion can also enable major aspects of function to be assessed which cannot be done with any acceptable degree of precision with nonautomated tests (e.g., aspects of information processing or the speed of retrieval of infor mation from memory). This section will review the range of pharmacodynamic assessments of CNS function which have been successfully employed in clinical pharmacology.

Tests of cognitive function that are to be used as tools to determine whether various treatments may influence mental abilities should fulfill a number of requirements before they can be considered suitable for use in this field. Thousands of tests have been used over the decades, and these can create a bewildering choice for researchers. Sadly there is no central authoritative, independent, and defin itive guide on how to select the appropriate instrument for any particular purpose. For this reason the reader is now provided with one set of criteria that any test or test system should to fulfill before being considered "fit for purpose" for the important purpose of evaluating how compounds may change cognitive abilities and mood states.

None of the requirements listed below are "nice to haves," rather they are all minimum requirements and essential properties of tests. That is to say, it is the absence of established evidence to satisfy any single criterion, which should encourage the potential user to look else where. Some tests are extensively validated, but for exam ple notoriously insensitive to change, or just simply unreliable; and their continued use in clinical trials does

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not facilitate the advancement of knowledge in this field. Unless such decision making becomes widely applied, the proliferation of tests used in clinical pharmacology will continue unabated. One unfortunate consequence of such uncontrolled proliferation will be that reviewers of the effects of individual drugs or drug classes will continue to acknowledge that the wide variety of tests and test variants employed has resulted in the conclusions of the review process being less definitive than is ideal, based more on qualitative generalizations rather than quantitative comparisons.

C.6.2.1.1 Validation

This is central to test selection, and like the other criteria, is a necessary but by no means sufficient requirement in the section process. This is one of the most misunderstood concepts in science, often confused with reliability, sensitivity, widespread use, utility, etc., but which in fact relates to none of these. The fundamental basis of the validity of a test is very simply that it measures what it purports to measure. Thus, for instance, if a test is designed to measure the ability to store and later recall a series of words, it is a valid test of recall if it indeed measures the ability of volunteers to correctly recall the words, and thus construct validity has been established. This example is easy to grasp, but consider, for example, the construct validity of using a test such as Trail making as a measure of "executive function." This is far less easy to establish, both because of the variety of definitions of "executive function," and the numerous aspects of cognitive function involved in the performance of the Trail making task. The probable answer is that Trail making does measure some aspects of executive function, but also several other aspects of cognitive function, and therefore changes in performance of the test may not definitively be interpreted as changes to executive function, because several other aspects of cognitive function may also have been involved. Construct validity is widely recognized as the ultimate demonstration of the validity of a measure, and is often strongly supported by sophisticated statistical methods such as cluster or factor analysis.

Other important aspects of validation that need to be addressed include face validity, criterion validity, and predictive validity. Face validity is not essential, but it can facilitate the compliance of volunteers or patients when asked to perform a task, as they can easily grasp why they are being asked to do so. Criterion validity is showing that the test measures the same aspects of function as other established tests, and correlation measures are widely accepted as methods for achieving this. However, the correlations do need to account for a satisfactory proportion of the variance, simply being statistically significant is not sufficient alone. Predictive validity is also a useful property of a test: either, for example, used to identify an early sign of cognitive deterioration, which will go on to result in clinical pathology, such as Alzheimer's disease; or to determine if an effect identified in an early clinical pharmacology study will actually translate to an effect in a patient population.

C.6.2.1.2 Sensitivity

Simple to understand and easy to determine, researchers should only ever adopt a test to attempt to identify an effect, either positive or negative, provided the test has previously been shown to be able to detect such an effect. If the test is new, it should be used alongside other established tests in the field to assess its future utility.

C.6.2.1.3 Reliability of Tests over Repeated Administration

Again a simple construct to understand and easy to determine; a test is reliable if the scores it yields remain stable when repeatedly administered to volunteers or patients on occasions when there is no reason to expect a change in the particular ability or state which the test is designed to assess. Correlation measures are widely used, but are inadequate alone, as high correlations between repeated administrations will often occur in tests that show large practice or training effects. The ideal demonstration of test retest reliability involves stability of the scores on repeated administration together with a reasonable degree of correlation between the repetitions. It is generally the case that tests which involve complex strategies to perform, and/or a range of aspects of cognitive function, and/or skills which are subject to procedural learning often show training effects. This training effect may be further increased if certain tests do not have parallel forms, and the participant therefore performs better when retested simply due to having, for example, remembered some specific aspects of test performance, for example, the stimuli used in memory testing. The absence of parallel forms for certain tests limits their usefulness in clinical pharmacology.

It has long been recognized that such practice (training) effects exist with many cognitive and other tests, and that these can compromise the ability to reliably identify

effects in clinical trials (McClelland 1987; Wesnes and Pincock 2002). A number of factors contribute to this phenomenon over and above simple "learning effects." These include the subject's full understanding of the test requirements; initial "test anxiety," which fades as familiarity with the test requirements increases; as well as the development of strategies to perform more complex tasks. Pre study training in clinical pharmacology is essential in both volunteers and patients to reduce these effects. In volunteers for many less complex tasks, it has been found that the training effects tend to plateau after four repetitions. This has led to the recommendation that four training sessions on each test should be conducted prior to the first day of dosing in clinical pharmacology (McClelland 1987). In trials with patients, it may not always be feasible to conduct four pre study training test sessions, but generally at least two sessions is a minimum requirement. For self ratings, such as mood scales, it is also good practice for the subject or patient to perform them at least once prior to the study to ensure understanding of the scale requirements, etc.

C.6.2.1.4 Utility

Experienced and properly qualified psychologists are rarely widely available in clinical trials, and when not, tests should ideally be simple to administer, easy for the patient or volunteer to understand, and while demanding, must neither be onerous (e.g., excessively long) nor threatening (e.g., involve negative feedback). The instruments of choice for most clinical pharmacology trials are tests or test systems with well established and easy to administer instructions, both for test administration and scoring.

C.6.3 Cognitive and Behavioral Tests and Test Systems

C.6.3.1 Computerized Cognitive Assessment Systems

PURPOSE AND RATIONALE

Such systems have a long history in clinical pharmacology. Their purpose is to assess a range of aspects of cognitive function, using the computer to present the information, to record and also analyze the responses. Properly validated systems bring much value to clinical trials in terms of utility, reliability, and sensitivity. An early example was the Leeds Psychomotor tester, which was a portable device which assessed choice reaction time (CRT) and critical flicker fusion, which has been used widely in clinical trials (Hindmarch 1980). Other portable and fully computerized systems appeared in the 1980s including the CANTAB, CDR System, and the Everyday Memory Test (Crook et al. 1992). Since then a number of others have appeared, and the following currently available proprietary systems which have been used in clinical pharmacology are: the CANTAB (www.camcog.com); CDR System (www.unitedbiosource.com); CNS Vital Signs (www.cnsvs.com); CNTB (www.i3global.com); CogScreen (www.cogres.com); CogState System (www.cogstate.com); Cogtest (www.cogtest.com); Psychlogix (www.psychologix.com); Vienna Test System (www.schuhfried.at).

PROCEDURE

Information is presented via computer monitors and the responses are generally made using the keyboard, touch screens, response buttons or boxes, and even foot pedals.

EVALUATION

Data is stored automatically on the computers, and data processing can take place immediately or when transferred back to the company. Each system has a range of measures and scores.

CRITICAL ASSESSMENT OF THE METHOD

These systems represent both the present and the future of cognitive testing in clinical trials. Some can be administered in group settings but others require one to one testing. Their standardization allows data to be contrasted between various studies using the same system (e.g., Hindmarch 2009). Many of the aspects of cognitive function assessed cannot be measured either definitively or not at all noncomputerized tests. The systems are by no means interchangeable and the reader is advised to examine the relative suitability of the different systems for the specific requirements of the study.

MODIFICATIONS OF THE METHOD

Most systems are under continual development and refinement, encompassing technological advances. For example, some of these systems have Internet versions to enable patients to be tested remotely. Other important developments for remote testing have included conducting cognitive testing over standard phone lines

(see e.g., Mundt et al. 2006; Wesnes 2002; www.healthtechsys.com) or via mobile phones (see e.g., www.penscreen.com).

A development which enables pencil and paper tests to be performed in the traditional manner but captured electronically comes from Digital Pen and Paper technology (see e.g., www.manentia.co.uk; www.penscreen.com). Not only does this automate the marking and scoring of the tests, but it also has the possibility of enabling all aspects of performance to be captured in the task, overcoming many of the limitations of traditional pencil and paper tests, which will be identified in subsequent sections. Once such technology is combined with the computer based systems described here, a far wider range of tasks and assessments of cognitive function will be available for use in clinical pharmacology.

C.6.3.2 Noncomputerized Test Batteries

PURPOSE AND RATIONALE

The purpose of these batteries is to assess a range of core aspects of cognitive function in order to identify problems in differing clinical conditions, and also to determine the effects of medicines and drugs. There are two systems that are currently widely in use in clinical pharmacology which will serve as examples of their class.

- Repeatable Battery for the Assessment of Neuropsychological Status (RBANS; Gold et al. 1999)
- MATRICS Consensus Cognitive Battery (MCCB)

Both have been extensively validated and comprise a range of traditional neuropsychological tests.

PROCEDURE

The tests are administered by trained specialists, with set of instructions and forms.

The RBANS takes around 30 min to administer and consists of a 10 word list learning task, administered four times, a brief story that is presented for two recall trials, a 10 item confrontation naming test, category fluency, complex figure copying, a judgment of line orientation task, digit span test, coding task, delayed recall of the story, complex figure, and word list, and finally a 20 item word recognition test.

The MCCB takes around an hour and consists of: Symbol Coding, Category Fluency (Animal Naming), Trail Making Test: Part A, Continuous Performance Test Identical Pairs, WMS® III Spatial Span, and Letter Number Span, Hopkins Verbal Learning Test Revised, Brief Visuospatial Memory Test Revised, Neuropsychological Assessment Battery® NABs Mazes, and the Mayer Salovey Caruso Emotional Intelligence Test (MSCEIT™): Managing Emotions.

EVALUATION

The RBANS consists of a Total Scale Index and five domain specific Index Scores: the Immediate Memory Index, Visuospatial/Constructional Index, Language Index, Attention Index, and Delayed Memory Index.

The MCCB yields a Global Index and seven domain scores: Speed of Processing, Attention/Vigilance, Working Memory, Verbal Memory, Visual Memory, Reasoning and Problem Solving, and Social Cognition.

CRITICAL ASSESSMENT OF THE METHOD

Both procedures require trained and appropriately qualified administrators, and also one on one testing. The RBANS has established sensitivity, has two parallel forms, and over 15 language versions. The MCCB has yet to show sensitivity to treatment effects, and of the nine nonautomated tests in the battery, only three have alternate forms (the Hopkins Verbal Learning Test and the Brief Visuospatial Memory Test have six each, and NABs Mazes test has two). There are currently four or so language versions of the MCCB but more are being developed. Early trials have experienced quite large training effects on the MCCB (e.g., Freedman et al. 2008; Marx et al. 2009).

MODIFICATIONS OF THE METHOD

The individual tasks or tasks from individual domains can be used separately.

C.6.4 Attention Tests

There are a wide variety of these procedures and the major test types used widely in clinical pharmacology will be described. Some divided attention tests have also been used (see e.g., Schmitt et al. 2000).

C.6.4.1 Simple and Choice Reaction Time

PURPOSE AND RATIONALE

The purpose of these tests is to assess how quickly a volunteer can correctly identify either a single predetermined stimulus (simple reaction time) or chose between a number of alternatives (CRT). Donders (1868) is credited with setting up the first laboratory

demonstration, showing that a simple reaction time (SRT) is shorter than a recognition reaction time, and that the CRT is longest of all (Kosinski 2008). These tests have always been automated, and the computer is the ideal instrument for this. These tests measure crucial aspects of focused attention, and when properly implemented, can differentiate between early stages of attention and the cognitive processing of information. Such testing can be performed even in highly cognitively disturbed populations such as Alzheimer's patients or patients with schizophrenia. Any comprehensive computerized system requires such tests. Such tests are widely used in early clinical pharmacology trials (e.g., O'Neill et al. 1995; Silber et al. 2006), and have also been shown to detect hallmark cognitive impairments which can differentiate different types of dementia (Ballard et al. 2002), and which have led to the identification of attentional deficits to be central to the cognitive disruption in consensus criteria in particular dementias (e.g., Parkinson's disease dementia Emre 2003; Dementia with Lewy Bodies (McKeith et al. 2004), as well as ideal tools to use in pivotal therapeutic trials in these populations (e.g., Emre et al. 2004).

PROCEDURE

The implementation is simple, in SRT a predefined auditory stimulus (e.g., tone) or visual stimulus (e.g., light or word) is presented, and the time taken for the volunteer to react is recorded, ideally to the nearest millisecond. These reaction times can be as short as 180 ms. In CRT, the subject is anticipating two or more possible stimuli (e.g., high or low pitched tone, or different visual stimuli), and must make an appropriate response to each, usually by pressing different response buttons. The tests involve 50 or more trials, are short, usually 2 3 min for SRT and CRT, and for SRT measures of speed are recorded, while with CRT, both accuracy and speed are measured. Thousands of different procedures have been adopted, the crucial similarities between the various procedures being repeated presentations of the stimuli, and the averaging over the responses to identify SRT and CRT.

EVALUATION

Well developed tests involve the same responses for SRT and CRT, so that the extra processing time required for deciding which stimuli can be estimated by subtracting SRT from CRT, enabling cognitive processing speed to be measured independently of the motor response times. The four core measures are the average time taken to respond on SRT and CRT, accuracy in CRT, cognitive processing time, and the variability of the various reaction times. Little concordance has been reached on the methods for calculating speed, some tests using the mean RT, others the median RT, and some systems removing outlying long responses from the calculations and terming them, for example, "gaps," signifying that they represent lapses in the ability to sustain attention.

CRITICAL ASSESSMENT OF THE METHOD

When properly applied these tests are a hugely valuable technique in any clinical pharmacology study.

MODIFICATIONS OF THE METHOD

Too numerous to mention, care needs to be taken when selecting appropriate versions.

C.6.4.2 Vigilance Tasks

PURPOSE AND RATIONALE

One of the pioneers of the study of vigilance was Norman Mackworth (1948) who developed the still widely used Mackworth Clock. This instrument simulated a radar screen, the original device has a large black pointer on a large circular background like a clock, the pointer made one revolution per minute, and clicked forward every second. The task was to detect infrequent double clicks, and initially volunteers were required to perform the task continuously for 2 h. This was shown to be sensitive to the effects of amphetamine by his daughter (Mackworth 1965), and other variants on the task have been used extensively (e.g., Wesnes 1977), including computer based versions (e.g., Riedel et al. 2005). Numerous other tests of vigilance have been developed and widely used in clinical pharmacology and other areas (for review, see Canisius and Penzel 2007). However, many such shorter computer based vigilance tests have been developed and used extensively, showing sensitivity to drug effects (e.g., Psychomotor Vigilance Tasks (PVTs) see next section) some even over a vigil of only 3 min (e.g., Digit Vigilance Task, Wesnes et al. 1988).

PROCEDURE

The tests are always automated, the stimuli easy to detect initially, and good tests assess accuracy and response latency. They can be long, up to 2 h, but such procedures are rarely practical in clinical pharmacology, and shorter versions like the Walter Reed test are often preferred.

EVALUATION

The core measures in vigilance tasks are the proportion of targets actually detected, the number of false positive responses (false alarms), and the speed of responses.

These can be assessed for the entire duration of the test, or broken down, even minute by minute. Signal detection theory measures are often calculated from the responses, providing indices of the actual ability to detect the targets, and the response bias, that is, the willingness to make a positive response, the attitude to errors, etc. Speed of response is an essential part of the profile of the test and should always be recorded.

CRITICAL ASSESSMENT OF THE METHOD

The ability to sustain attention is a different aspect to that measured typically in short SRT and CRT testing, and vigilance tasks are important in properly detecting the ability of a compound to influence the ability maintain focus, even over minutes. Well designed tests do not show large training effects with repeated use, and for clinical pharmacology the instruments of choice are briefer versions of the Mackworth Clock (e.g., 15 min), or other tests such as the Walter Reid (see next section) or the Digit Vigilance task mentioned above.

MODIFICATIONS OF THE METHOD

The major change has been the demonstration that it does not take 2 h to measure a drop in the level of vigilance, and that these vigilance decrements can be detected by other computerized tests over minutes. Further, the evaluation of speed which was not originally part of the Mackworth Clock brings an important extra dimension to the ability to sustain attention, not just the missing of targets, but the slowing in response speed.

C.6.4.3 Psychomotor Vigilance Tasks (PVTs)

PURPOSE AND RATIONALE

These tests have components of simple reaction time testing incorporated within a test session of up to 15 min. The Walter Reed palm held PVT (Thorne et al. 2005) is based on the commercially available PVT (PVT 192; Dinges and Powell 1985; www.ambulatory monitoring.com) and has similarities to the Wilkinson "unprepared simple reaction time test," which ran on a cassette recorder (Wilkinson and Houghton 1982). The tests are widely used, in sleep research for example, and are very sensitive to the effects of sleep deprivation and also compounds that can help overcome these deficits, e.g., modafinil (Walsh et al. 2004).

PROCEDURE

The subject monitors a screen on a handheld device, or a computer monitor. The task is to press a button as quickly as possible each time a bull's eye appears on the screen, the interstimulus intervals vary unpredictably between 1 and 5 s, and the task is performed continuously, for vigils as short as 5 or as long as 15 min.

EVALUATION

The data collected include reaction time, minor lapses (reaction times longer than 500 ms), and major lapses (reaction times longer than 3,000 ms).

CRITICAL ASSESSMENT OF THE METHOD

Simplicity and ease of administration of the test, even in field conditions are strong features. The test is highly sensitive to a variety of stressors and medications. Major lapses reflect inattention in a variety of situations. The main limitation is that the test simply assesses simple reaction time, and as mentioned earlier, other valuable information on attentional processes, such as cognitive processing speed, is not gathered if CRT is not also assessed.

MODIFICATIONS OF THE METHOD

Both the PVT 192 and the Walter Reed palm held psychomotor devices allow the user to adjust a wide variety of the test parameters, and thus comparability between different studies can be hard to establish. Test duration is also crucial for sensitivity yet there is little agreement on the optimal duration over which PVTs should be run (e.g., Loh et al. 2004). PVTs can be administered in on a variety of devices, and novel platforms are being continually developed.

C.6.4.4 Rapid Visual Information Processing

PURPOSE AND RATIONALE

This test was developed from the Bakan vigilance task by Wesnes when developing tests sensitive to the enhancements to attention which could be produced by nicotine (Wesnes 1979). In the Bakan task digits were presented via headphones at the rate of 1 per second, and the task was to detect and report sequences of three consecutive odd digits (e.g., 3, 5, 7). To make the test more demanding and thus more sensitive to change, the digits were displayed on a computer screen, the rate was increased to 100 digits per minute, the targets were set to be either three consecutive odd digits or three consecutive even digits, and the density was eight targets per minute, to enable minute by minute changes to be assessed. The computerization also enabled the speed of responding to

be assessed, a critical improvement to the sensitivity of the task. The test thus measures sustained attention/vigilance, information processing, and the ability to hold information in working memory. The early findings that nicotine improved performance on the task even in nonsmokers (Wesnes and Warburton 1978, 1984a) confirmed the sensitivity of the test to detecting enhancements and these early findings have now been extensively replicated in laboratories worldwide (e.g., Parrott and Winder 1989), and have been replicated in both Alzheimer's patients (Sahakian et al. 1989) and schizophrenia (Hong et al. 2009). Nicotinic agonists are now in development for both of these indications (e.g., Freedman et al. 2008). The test is available on two computerized systems (CDR and CANTAB), and dozens of variants have been developed are in extensive use (Google currently reports 2,260,000 entries for the task).

PROCEDURE

The digits are displayed one at a time on a computer screen at the rate of 100/min. The targets are either three consecutive odd digits or three consecutive even ones. Each time the task is performed, a different sequence of digits is displayed, and there are eight targets every minute. The targets are reported by the pressing of a button box.

EVALUATION

As in vigilance tasks, the three core measures are the proportion of targets actually detected, the number of false positive responses, and the speed of responses. Millisecond resolution is essential. Signal detection theory parameters of sensitivity and response bias can be applied. The performance can be measured over any interval, even minute by minute.

CRITICAL ASSESSMENT OF THE METHOD

The widespread use of the system attests to its utility and sensitivity. It is highly sensitive to practice effects, and it is essential that pre study training occurs, ideally on four occasions. It should not be used in isolation otherwise any changes identified will be confounded between sustained attention, information processing, and working memory. The use of other vigilance and attention tests should also be conducted in order to determine and help identify whether attention and/or working memory has been affected.

MODIFICATIONS OF THE METHOD

The test has been used in conjunction with EEG assessment, allowing the P300 to the targets to be measured during the performance of the task (Edwards et al. 1985), and it has also been used while subjects are undergoing positron emission tomography (PET) scanning (Coull et al. 1996). The test is highly flexible and can be administered for just a few minutes or even an hour. The speed of presentation of the digits has also been varied, and in some versions, the speed of the digits varies during the task according to the quality of performance.

C.6.4.5 Continuous Performance Tasks

PURPOSE AND RATIONALE

Continuous Performance (CPT) tasks are variants on vigilance, RVIP, SRT, and CRT tasks, having features in common with all of them. They warrant their own section as they are widely used (see review Riccio et al. 2001). While there are literally hundreds of versions of these tasks (Google reports 9,610,000 entries), the most commonly used is the Conner's CPT II (http://www.devdis.com/conners2.html), which is widely employed to study attention deficit/hyperactivity syndrome (ADHD), and contains measures of impulsivity (false alarms) which are an important part of the disorder. Other proprietary tests are the Test of Variables of Attention (T.O.V.A.® http://www.tovatest.com), the Gordon Diagnostic System (GDS http://www.gsi add.com/gordondiagnosticsystem.htm), and the IVA Plus (www.braintrain.com). There is a simpler version of the Conners CPT, the identical pairs version (CPT IP; Cornblatt et al. 1989). The Conner's CPT II is the most widely used of the tests and will be described below to illustrate the methodology of these types of tests.

PROCEDURE

To perform the Conner's CPT II, the subject is required to respond to letters that appear on the screen, by pressing the left mouse button as quickly as possible for every letter that appeared on the screen apart from the letter "X." The interstimulus intervals (ISIs) are 1, 2, and 4 s with a display time of 250 ms. The task takes 14 min to complete. The test is suitable for children aged 6 and over and of course adults.

EVALUATION

The test produces a large number of variables, and needs expert interpretation. The measures are:

- Omissions
- Commissions
- Hit Reaction Time
- Hit Reaction Time Standard Error
- Variability of Standard Error
- Detectability/Attentiveness

- Perseverations
- Hit Reaction Time Block Change
- Hit Standard Error Block Change
- Hit Reaction Time ISI Change
- Hit Standard Error ISI Change
- Response Style

Some of these are signal detection measures mentioned previously (detectability and response Style), commis sions (false alarms) being a measure of impulsivity.

CRITICAL ASSESSMENT OF THE METHOD

The CPT II is extensively validated and widely used in ADHD to check the suitability of treatments, and is also employed in many clinical pharmacology trials. The test is sensitive to the effects of stimulants and also drugs that impair performance. There are practice effects which can be overcome by appropriate pre study training. The test is time consuming, and shorter vigilance or attention tests may be more appropriate in trials where there is limited time available for making assessments.

MODIFICATIONS OF THE METHOD

As stated above, there are numerous versions, and the user is recommended to employ a standardized one such as the CPT II to enable comparisons to be made between studies.

C.6.4.6 Pencil-and-Paper Tests of Attention

PURPOSE AND RATIONALE

There are numerous versions of such tests available, and four widely used versions will be used to illustrate these procedures, the Digit Symbol Substitution Test (DSST), Letter Cancellation Test (LCT), the Symbol Digit Modal ities Test (SDMT), and the d2 test of attention (http://www.hogrefe.co.uk/?/test/show/52/). The aim of the tests is to detect changes in attention, information processing, perceptual speed, motor speed, visual scanning, and work ing memory. The DSST is part of the Wechsler Adult Intelligence Scale (WAIS) developed by David Wechsler in 1939. The DSST has been widely used in clinical research over the last 60 or more years, possibly being the most widely used test of its type. It is highly sensitive to cognitive disturbances in a wide variety of clinical conditions (e.g., hypertension Kalra et al. 1994; schizophrenia Dickinson et al. 2007), as well as to the effects of a wide variety of drugs (e.g., Brumback 2007).

PROCEDURE

Three tests are all traditionally administered in a one to one fashion, although a number of people can be tested at one time in some situations. A stopwatch is used to make the timings.

C.6.4.6.1 Letter Cancellation Test (LCT)

In one common version of this test, the cover page is shown to the subject with the instructions: "This proce dure is referred to as a cancellation test. We will be working with both capital and lower case letters just like these." The first page is then presented with the instructions: "In this portion of the test, you are to look for the capital letters and mark each one with a slash (/). Go across the lines, from left to right, one after the other, marking the capital letters as quickly as you can. Try not to miss any." The subject is instructed to begin and the stopwatch started, 60 s later the subject is instructed to stop. The next sheet is then presented, with these instructions: "This time, you are to look for the double spaces, and mark the letter that precedes and the one that follows the double space. Go across the lines, from left to right, one after the other; go as quickly as you can". Again this is timed for 60 s. Other conditions are also performed.

C.6.4.6.2 Symbol Digit Modalities Test (SDMT)

These are the standardized instructions for one commonly used version of this test (Keefe et al. 1994). The SDMT requires the subject to substitute a number for its corresponding geometric figure. There are nine figures, each corresponding to the numbers one through nine. On the record form, there are a series of rows containing geometric figures in the top half, but the bottom half is left blank. The subject completes the task by writing the num ber that corresponds to the geometric figure in the box below each figure. The key with the geometric figures and corresponding numbers appears at the top of the page. Before administration of the test, subjects complete ten practice responses. Any errors are noted immediately by the tester and corrected by the subject. When it is clear that the subject understands the task, he or she is told to fill in the remaining boxes as quickly as possible, complet ing one box at a time, one row at a time, before proceeding to the next. Skipping from box to box with the same geometric figure is not permitted. There is a 90 s time limit. The test can be administered by having the subject

write out the correct response or by having the subject report the correct answer (i.e., number) aloud. The test can be administered in its written format individually or in groups.

C.6.4.6.3 The Digit Symbol Substitution Test (DSST)

This test is similar to the SDMT, except that the volunteer is required to copy the geometric figures into the rows of blank boxes according to which digit is on the top of each box. The test is also timed by a stopwatch, and 90 s is allowed for the subject to complete as many of the boxes as possible. The subjects are monitored, and must work from left to right across each row. There is no auditory version of this task.

C.6.4.6.4 d2 Test of Attention

The test is presented in a landscape layout of 14 test lines with 47 characters in each line. Each character consists of a letter "d" or "p" marked with one, two, three, or four small dashes. The respondent is required to scan the lines and cross out all occurrences of the letter "d" with two dashes while ignoring all other characters.

EVALUATION

C.6.4.6.5 Letter Cancellation Test (LCT)

The scores are the number of correctly identified items, the number of missed items, and the number of commission errors, that is, marks which are made inappropriately.

C.6.4.6.6 Symbol Digit Modalities Test (SDMT)

Subjects receive one point for each correctly completed box. The total score is the total number of correctly com pleted boxes in the time allowed. The practice items are not counted in the scoring.

C.6.4.6.7 The Digit Symbol Substitution Test (DSST)

As in the SDMT, the subjects receive one point for each correctly completed box.

C.6.4.6.8 d2 Test of Attention

Two scoring keys are provided; one for identifying errors of omission (missing characters that should have been crossed out) and one for identifying errors of commission (crossing out characters that should not have been crossed out). Total number of items processed is the sum of all items processed whether correctly or incorrectly. It is a highly reliable measure of processing speed. Per centage of errors measures the qualitative aspects of performance. It represents the proportion of errors made across all items processed. Total number of items pro cessed minus errors (TN E) provides an indication of the implications of the combined speed and accuracy scores for attentional and inhibitory control. There is also a measure of fluctuation rate.

CRITICAL ASSESSMENT OF THE METHOD

It is easy to see the widespread appeal of these tests as they are short, easily administered, and sensitive to impairments in disease states and also to drug effects. The d2 test is well standardized as is the ideal one for use in clinical pharmacology. Unfortunately, for the others, as with CPTs, there are numerous different versions of each of these tests and little standardization between methods, though commercial versions of SDMT, for example, are available, which helps mitigate this problem a little.

Further, with their varying requirements, the tests do not measure the same aspects of cognitive function (e.g., Morgan and Wheelock 1992). This difficulty with the wide range of cognitive processes involved in these tests means that it is not possible to be certain which facet of cognitive function has altered if scores change from one condition to another, for example, it could be due to changes to all or any of the various processes, for example, attention, motor speed, information processing, or working memory. These tests thus are best used in conjunction with other tests, which yield more precise evaluations of attention, for example, to enable the findings to be properly interpreted. A further major problem for the use of the DSST in clinical pharmacology studies is that there are no agreed criteria for deciding if the geometric shapes have been properly recreated. It is easy to spot the mistakes, but if a volunteer wishes to rush, the shapes can be written quite poorly, but will still be marked as correct, and thus the total score will be inflated. This problem with speed accuracy trade offs is a major limitation of the DSST in trials of cognition enhancers.

However, the major drawbacks for these procedures are the absence, in many cases, of parallel forms, and

this contributes at least in part to the large training effects, which occur with this type of test (Westhoff and Dewald 1990). For example, the d2, the best standard ized of all pencil and paper tests of attention, shows significant training effects in volunteers of test repeti tion over 30 min or 1 2 days with effect sizes of around 1.5 on all major parameters, and in patients with similar effect sizes over intervals of 1 18 days (d2 Manual, www.hogrefe. co.uk). Such dramatic effects greatly limit the utility and sensitivity of such proce dures in studies where test repetition is necessary, par ticularly in studies seeking to identify cognition enhancement, where the potential effect sizes of such enhancement are much smaller than the magnitudes of the training effects.

However, some versions of the LCT can show equiva lent sensitivity to brief computerized vigilance tasks (e.g., Parrott et al. 1996), whereas computerized tests of atten tion and information processing can show superior sensi tivity to the DSST, for example, in detecting impairment in clinical pharmacology studies (e.g., Brooke et al. 1998; Wesnes and Warburton 1984b).

The strengths of these tests are their widespread availability and long history of use, but they should be used sparingly in future, and always as part of a battery of tests, ideally alongside computer based tests. Also the requirement for close monitoring of the tests often makes them unsuitable for busy clinical pharmacology safety studies where staff numbers can be limited. However, as noted earlier, Digital Pen Tech nology could help many of these problems to be overcome.

MODIFICATIONS OF THE METHOD

Some versions of the tests, for example, the LCT, measure the time taken to complete a sheet as opposed to the number of cancellations made. Numerous versions of the tests have been developed, making it hard to generalize findings. For example, some versions of cancellation tests use digits, one actually being called a Digit Vigilance test (Kelland and Lewis 1996). Computerized versions of DSST and SDMT have been developed (e.g., Mattila et al. 1994), and the administration of the tests can also be computer facilitated, that is, the instructions appearing on the screen, and the timing of the test performed by the computer, although the volunteer still performs the tests using a pen and paper. The d2 is available in a computerized form.

A major innovation in this field is the use of www. penscreen.co.uk

C.6.4.7 The Stroop Test

PURPOSE AND RATIONALE

This classic test has been used extensively in worldwide research since Stroop (1935) first published his demon stration of the phenomenon now called the Stroop Effect. Basically, it entails measuring the time taken to read a series color names (e.g., black, red, green) when printed in black ink, and comparing that time to the longer time it takes when the color names are written in nonassociated colors, for example, the word red printed in blue ink. It is a very powerful and reproducible effect, and the test has been used in hundreds and possibly thousands of different forms during the last 70 years.

Performance of the test is shown to involve activation of the frontal lobes, and it is thus often used to measure aspects of "executive function." However, it also measures selective attention, cognitive flexibility, and processing speed. It is highly sensitive to drug effects, and, for exam ple, early work on nicotine and scopolamine, interpreted the opposite changes produced on the Stroop effect by the two drugs in terms of changes to selective attention and information processing (Wesnes 1979).

PROCEDURE

There is no widely adopted standardized procedure for the Stroop test; this is taken from one of a number of commercially available methods (http://www.mhs.com/ product.aspx?gr=cli&prod=stroop cw&id=overview)

The Stroop Color and Word Test consists of a Word Page with color words printed in black ink, a Color Page with "Xs" printed in color, and a color Word Page with words from the first page printed in colors from the second page (the color and the word do not match). The respondent goes down each sheet reading words or nam ing the ink colors as quickly as possible within a time limit. A stopwatch is required to administer each test.

Testing usually takes around 5 min.

EVALUATION

Errors can be recorded, but are not always, and the prin cipal measures most commonly reported are the times taken to read the various sheets, or the number of items read in a prespecified time limit. The interference effect is often reported separately, for example, the difference in the time taken to read color names printed in black ink, and the time taken to read color names printed in different colored inks.

CRITICAL ASSESSMENT OF THE METHOD

The brevity of the test, its sensitivity to drug effects, and its widespread use make it an excellent component of a cognitive evaluation. It can be computerized, and the frontal lobe activation during the task often encourages researchers to use it to test executive function. The huge variety of different methodologies employed mean that differing studies can only be compared in a qualitative method.

MODIFICATIONS OF THE METHOD

Too numerous to mention, and computerized versions have also been developed.

C.6.5 Working Memory

PURPOSE AND RATIONALE

Interest in clinical pharmacology in working memory started with the publication of Baddeley's (1986) seminal book on the topic and the adaptation of the Sternberg test in psychopharmacology studies (e.g., Sherwood et al. 1992). Working memory referred to the temporary ability to hold information on line, and had two "subsystems": the "articulatory loop" and the "visuospatial scratchpad." The articulatory loop was the mechanism we use to keep information temporarily available, like repeating a telephone number or a person's name. The visuospatial scratchpad is our temporary memory of where things are around us, is quite independent to the articulatory loop (e.g., Vicari et al. 2003; Ellis et al. 2005), and is well assessed by computerized tests (e.g., McGurk et al. 2005). Another more recently recognized component of working memory is the short term store we use to help encode information into episodic memory, which is assessed with tests like immediate word recall.

PROCEDURE

The Sternberg technique involved presenting series of digits of differing lengths, and then asking the volunteer to press a key to signal whether or not a probe digit was the in the original series. The original Sternberg memory scanning procedure was too lengthy for practical use in clinical pharmacology, and adaptations were made, for example, showing five different digits, requiring the vol unteer to hold on to them in memory, and then showing 30 digits one at a time and requiring yes/no button presses according to whether each digit was one of the original five (Ellis et al. 2003). This measures the articulatory loop with more sensitivity, precision, and reliability than the classic digit span test, which though widely used is notoriously insensitive to drug effects.

The N back group of tasks also measure the articula tory loop, being like a CPT, but the responses for each bit of information have to be based on whether the informa tion was presented previously, the "N" referring to differ ing levels of difficultly which can be created by specifying how far back the information was presented, the easiest obviously being one back. This test is used widely (e.g., Ashford 2008; Owen et al. 2005).

The visuospatial scratchpad is our temporary memory of where things are around us is quite independent to the articulatory loop (e.g., Vicari et al. 2003; Ellis et al. 2003) and is well assessed by computerized tests (e.g., McGurk et al. 2005).

EVALUATION

In all tests both accuracy of responding and the speed of response need to be recorded, millisecond resolution is essential. Signal detection theory parameters of sensitivity and response bias should be applied in order that false positive responses are identified and a true measure of the ability of the subject to retain the information in working memory be identified. In N back tasks, the differing inter vals defined by "N" are analyzed separately.

CRITICAL ASSESSMENT OF THE METHOD

The methods are very useful in identifying cognitive dys function, clarifying whether articulatory and or spatial working memory is disrupted, and helping to assess the effects of medications and drugs. The numerous unstandardized versions of the tests make comparisons difficult, though versions of all of the tests have been standardized and used widely and should be preferred.

MODIFICATIONS OF THE METHOD

The tests have been used in conjunction with both EEG assessment and MRI (e.g., Owen et al. 2005). There are many variants, and this will only proliferate going forward.

C.6.6 Episodic Memory

C.6.6.1 Word Recall and Recognition

PURPOSE AND RATIONALE

The ability to learn and retrieve verbal information has been assessed for generations. The object is to assess how

well verbal information can be encoded and stored, and subsequently recalled or recognized. Working memory is generally believed to last for a few minutes, and any information retrieved say after 10 min is widely accepted to have been stored in episodic memory (e.g., Tulving 1984) which is one of the major types of declarative memory.

PROCEDURE

There are literally hundreds of different variants of these procedures, but the general methodology will be described and some ideally used procedures mentioned.

A series of words, generally 12 15, are either read or shown to the subject at a controlled rate, say 1 every 2 s. The subject is then immediately given say a minute to recall the words, by speaking them aloud or writing them down. There are three major variants on the subse quent procedure:

1. There is now a gap of say 10 or 15 min, filled with other tasks, before the volunteer is asked again to recall the words.
2. The list is re presented, and the volunteer again asked to recall them. This procedure can be repeated up to five times, after which a gap is introduced as in 1 (e.g., Hopkins Verbal Learning Test, Rey Auditory Verbal Learning Task).
3. Only the words not recalled on the previous recall session are re presented, and the volunteer asked to recall the entire list (e.g., Buschke Selective Reminding).

Then "delayed recall" takes place, without further prompting, generally followed by recognition testing, in which the original words mixed with new words (distractors), are re presented and the subject must iden tify whether or not the words were in the original list.

A variation is the paired association test, in which pairs of words are presented, some associated, for exam ple, knife and fork, and others not, and later, just the first word is presented, for example, knife, and the task is to recall the word originally paired.

EVALUATION

Words correctly recalled are scored, and words recalled falsely are noted. Signal detection theory measures can be applied to recognition, in order that simply saying "yes" to everything is not misinterpreted as good perfor mance. Ideally, speed of recognition is assessed. Rates of learning can be assessed for techniques that repeatedly present words.

CRITICAL ASSESSMENT OF THE METHOD

This forms an essential part of any comprehensive evalu ation of cognitive function. The choice of method should be driven by the experimental questions being addressed, as well as the general environment of the study. In early safety studies, the one on one nature of administration is unsuitable, and the verbal presentation or responses can be troublesome in group testing or in "hospital ward" situations. Many tests only have limited validated parallel forms, Or different language versions making them unsuitable for studies where repeated testing is performed, or international trials. Measurement of speed of recogni tion is not possible unless the tests are computerized, yet this is an important aspect of memory to assess and may be impaired in early stages of diseases, such as mild cognitive impairment (Nicholl et al. 1995). Some com mercially available computerized systems have numerous (over 60) parallel versions and language versions (e.g., the CDR System), and also assess speed of recognition. Stan dardized tests like the RAVLT, HVLT, or CVLT also facili tate comparisons of findings between populations or different compounds.

MODIFICATIONS OF THE METHOD

Many verbal memory tests have been computerized, and can be performed in association with EEG or imaging techniques. Recall and recognition testing can also be conducted at extended delay intervals, even days. The advantage of this methodology is that it permits drugs believed to improve retrieval and/or storage processes to be evaluated. This is achieved by administering the drug after the information has been presented and encoded, and thus any changes in recall or recognition can most reasonably attributed to changes to storage and retrieval mechanisms; though it is important to assess attention at the time of testing, as changes to attention may influence recall and recognition.

C.6.6.2 Picture or Object Recognition

PURPOSE AND RATIONALE

Here, the aim is to identify the ability to store and retrieve nonverbal information, primarily pictures, faces, or objects in episodic memory. There is a huge variety of such procedures available. They are grouped separately as while they involve many similar aspects of brain function to verbal tests, there are several procedural differences.

PROCEDURE

The information is presented visually to the subject, ide ally via a computer screen. Recall and recognition can be assessed in the same way as with verbal tasks. Some tests actually involve presenting real objects to subjects.

EVALUATION

The ideal measures control for errors and false positive responses using signal detection theory measures. Speed of retrieval of information can be assessed with some com puterized tests.

CRITICAL ASSESSMENT OF THE METHOD

Again, the diversity of the available procedures makes comparability between studies difficult, and standardized proprietary traditional tests with clear scoring rules are the recommended choices (e.g., the Brief Visuospatial Mem ory Test Revised) or validated computerized alternatives (e.g., Nicholl et al. 1995).

Parallel forms are important for clinical pharmacology studies to avoid learning the study materials, though gen erally training effects are small or not present on these types of tasks if parallel forms are available.

MODIFICATIONS OF THE METHOD

Computerization of these procedures has long been avail able and is the optimum choice. Virtual reality tests are also becoming available and will enter trials soon.

C.6.7 Problem Solving and Executive Function

PURPOSE AND RATIONALE

The term executive function describes a set of cognitive abilities that control and regulate other abilities and behaviors, see Baddeley (1987). Such "executive func tions" are important for goal directed behavior, and include the ability to initiate and stop actions, to monitor and change behavior as required by the situation, and to plan future actions when faced with novel tasks or situa tions. The abilities to form concepts and think abstractly are also sometimes considered components of executive function.

As the name implies, executive functions are high level aspects of cognitive function that influence or control more basic abilities like attention, memory, and motor skills. They can be thus difficult to assess directly. Many of the tests used to measure other abilities, particularly those that look at more complex aspects of these abilities, can be used to evaluate executive functions. Tests of verbal fluency that require subjects to generate words under time pressure (e.g., words beginning with a particular letter) may also reveal problems with executive function. Execu tive functions are often associated with the frontal lobes (e.g., Shallice and Burgess 1991), and tests that can be show to utilize this area of the brain are often termed tests of executive function.

There is a number of tests used in clinical trials to assess executive function (see Ylikoski and Hänninen 2003).Tests that are commonly used in clinical pharma cology to assess executive function are the Trail Making Test, Word fluency tests, Stroop tests (see separate sec tion), and planning tests like the Tower of London (similar to the Tower of Hanoi and Stockings of Cambridge).

PROCEDURE

C.6.7.1 Trail Making Test – Parts A and B

Part A: The numbers 1 through 25 are presented separately within circles distributed in an apparently random fashion on a sheet of paper. The participants must draw a line connecting the circles numbered 1 through 25 in consec utive order as quickly as possible. The beginning and end points are clearly stated on the sheet. Mistakes must be pointed out by the administrator and corrected by the participants before they can continue with the task.

Part B: The numbers 1 through 13 and the letters A through L are presented separately within circles distrib uted in an apparently random fashion on a sheet of paper. The participants must connect the numbers and letters in alternating sequence (i.e., 1 A 2 B 3 C, etc.) until they reach "L" by drawing a line from one to the next as quickly as possible. The beginning and end points are clearly stated on the sheet. As in Part A, mistakes must be pointed out by the administrator and corrected by the participants. Per formance in both parts is timed with a stopwatch.

C.6.7.2 Word Fluency – Letter and Category Fluency

For letter fluency, the participant is asked to produce orally as many words as possible beginning with a given letter in 1 min. This is generally repeated two further times, the letters most commonly used being F, A, and S.

For category fluency, the participant is asked to pro duce as many names from a given category as possible

within a minute. This task is just done once per testing session. Common categories are animals, food names, kitchen items, clothes, etc.

C.6.7.3 Planning Tests

Tower of London: A common variant of this task consists of two boards with three vertical pegs of descending height and several different colored beads. The examiner places one set of beads in the target fashion, puts beads in a different fashion on the second board, and asks the subject to move the beads between the pegs on the second board to match the beads in as few moves as possible. There are several stages to the test, with increasingly com plex goals, so that a subject has to complete, for example, three tasks, the first requiring two moves to reach the goal, the second four moves, and the third five moves.

EVALUATION

C.6.7.4 Trails Test

The time taken to complete each part is recorded in seconds, together with the number of errors. Part A can be subtracted from Part B (which generally takes longer) to derive a time measure reflecting the extra processing, etc. for the more complex aspects of Part B, removing some of the motor components of the task.

C.6.7.5 Word Fluency

The total words correctly produced in both tests are the main scores. Repeated words or words not in the correct category are not included in the totals.

C.6.7.6 Tower of London

The time taken and the number of moves are the primary measures, though a variety of other measures can be derived.

CRITICAL ASSESSMENT OF THE METHOD

As these tests all involve multiple aspects of cognitive function, the test scores themselves cannot specifically relate to executive function, and other tests of attention, motor control, information processing, and working memory should be used alongside them to help determine the particular aspects of function which may have been affected.

There are generally very limited numbers of parallel forms available, and together with the multiple aspects of function involved, and possible strategies, large training effects occur when these tests are repeated, which limits their utility in clinical pharmacology studies.

There are numerous versions of each test, and the reader is recommended to use proprietary standardized versions of these tests, for example, the Delis Kaplan Executive Function System™ (D KEFS™), contains ver sions of all of these tests plus a Stroop test. Equally, there are standardized proprietary computerized versions of these tests, including the Stockings of Cambridge in the CANTAB System and the Groton Maze in the CogState.

MODIFICATIONS OF THE METHOD

Computerized versions of most of these tests have been developed and have clear advantages, and groups are also working on virtual reality tests (e.g., Zhang et al. 2001; Josman et al. 2008), which are finding their way into clinical pharmacology studies. A colour version of the Trail Making Test has been developed which facilitates the use of the method in countries which do not use the roman alphabet (Colour Trails www.parinc.com).

C.6.8 Car Driving

C.6.8.1 Driving Simulators

PURPOSE AND RATIONALE

Historically, one of the expected risks of many drugs was to compromise road safety. To evaluate the potential risks, one possible but not widely used option was to use driving simulators in clinical pharmacology studies (e.g., Owens and Ramaekers 2009; Sansone and Sansone 2009; Iwamoto et al. 2008). The known limitation of many simulators is that they do not fully simulate the "real driving situation," and that tests of core aspects of cogni tive function such as attention tests could more accurately and precisely identify the risks from novel medications. Nonetheless, some clinical pharmacology units (e.g., Forenap in France, www.forenap.com), have offered a driving simulator as part of their service for many years in early drug development, using the F 230 driving simulator (www.farosindia.com/F230Car.htm). The F 230 is described by the manufacturers as offering high end simulation, incorporating active control of all dashboard features including windshield wiper and complete instru ment cluster; with a high traffic density of up to 50 cars, and including simulation of real life situations like rain

and night. The hardware includes steering wheel with force feedback, and an enclosed driver cabin. Such technology is clearly preferable to many trials that used computer games. There have been recent important developments for clinical pharmacology, for example, the National Advanced Driving Simulator at the University of Iowa (www.nads sc.uiowa.edu/facilities.htm), claimed to be the world's most advanced driving simulator that is now being offered for use in clinical pharmacology studies.

PROCEDURE

The procedures of the various simulators vary widely, but the superior ones involve sitting in a car or an enclosed space resembling a car, and operating the car using normal controls in a variety of traffic conditions for periods as short as 15 min up to several hours.

EVALUATION

Important measures are brake light reaction times, reac tion times to unexpected events, and the standard devia tion of the lateral position of the car on the road. In a recent review, Liguori (2009) concludes: "Outcome Measures: Deviation from lateral position and reaction time have been increased by benzodiazepines and alcohol. Vehicle Speed has been characterized as insensitive to drug effects. Future directions: The standard measures of lane deviation, reaction time, and speed may be sufficient for identification of basic behavioral impairments. Yet other aspects of drug related accidents including manipula tions of weather, time of day, traffic conditions, decision making, risk taking, divided attention, and sleep depriva tion deserve further study."

CRITICAL ASSESSMENT OF THE METHOD

Clearly, the more sophisticated the simulator and the more appropriate the measurements of driving ability the greater the likelihood that any effects will predict to real driving. Computer games should be avoided due to the large learning effects and the lack of authenticity. The clinical relevance of drug induced changes in driving per formance can be established, for example, by comparison of the effects of drugs with those of alcohol measured in a calibration study using the same method.

MODIFICATIONS OF THE METHOD

The National Advanced Driving Simulator of the Univer sity of Iowa represents the current state of the art, and its sensitivity to identifying drug effects of real life driv ing should become apparent as it becomes used in clinical trials.

C.6.8.2 On-Road Car Driving

PURPOSE AND RATIONALE

Clinical pharmacology studies have been conducted in actual driving situations. In one early study, Hindmarch et al. (1977) evaluated a benzodiazepine with the UK's Institute of Advanced Motorists whose officers were responsible for the marshalling of the subjects and the awarding of error scores. The driving tasks assessed involved maneuvering through a slalom, estimating widths through which to navigate the car at a distance, garaging a car, and reverse parking. The Experimental Psychopharmacology Unit, at Maastricht University, has developed on road driving safety test. The highway driv ing test was originally developed during the 1970s for driver fatigue research in the USA. It was standardized for drug screening purposes in 1982 and it has been used in more than 75 separate studies for measuring drug effects on driving performance (Owens and Ramaekers 2009). In the seminal study in the field (O'Hanlon et al. 1982) expert drivers operated an instrumented vehicle in tests over a highway at night after being treated with diazepam (5 and 10 mg), a placebo, and nothing. The volunteers reacted to 10 mg of diazepam with increased lateral position variability.

PROCEDURE

In the highway driving test, subjects drive a specially instrumented car over a 100 km (61 mile) primary high way circuit in normal traffic, accompanied by a licensed driving instructor who has access to dual controls. The subject's task is to drive with a constant speed of 95 km (58 miles) per hour and maintain a steady lateral position between the delineated boundaries of the slower (right) traffic lane. The vehicle's speed and lateral position relative to the left lane delineation are continuously recorded and digitally sampled.

EVALUATION

Several measures are recorded, including the time to speed adaptation, the primary performance parameter being the Standard Deviation of Lateral Position (SDLP, in cm), an index of "weaving."

CRITICAL ASSESSMENT OF THE METHOD

It has been widely used, and is clearly as close to a real life situation as would be ethically permitted in a clinical pharmacology study. As with driving simulation, its clin ical relevance can be benchmarked against the established effects of specified doses of alcohol. The SDLP though

would not be the only parameter which would indicate a real life risk to safety, and for matters such as reacting to an unexpected event for example, a driving simulator may provide a better evaluation of the risk with a particular medication.

MODIFICATIONS OF THE METHOD

Two further tests have been developed at Maastricht, the Car Following Test and the City Driving Test. In the Car Following Test, the subjects drive a specially instrumented car over a secondary highway again accompanied by a licensed driving instructor having access to dual controls. The task is to follow a leading instrumented car (controlled by an experimenter), at a fixed distance. At a speed of 60 or 70 km/h, the leading car executes deceleration or accelera tion maneuvers, and the subjects have to adapt their speed accordingly, as well as react to occasional discrete events such as brake light activation in the leading car. Test dura tion is approximately 30 min. In the City Driving Test, subjects drive a specified route of approximately ±15 km on two lane undivided streets through business and resi dential areas within the City of Maastricht. The primary parameter in this test is driving quality as evaluated by the accompanying licensed driving instructor, using a shortened version of the Royal Dutch Tourist Association Driving Proficiency Test. This test comprises 90 items scored as pass or fail, providing summary scores for five categories of driving skills (vehicle checks, vehicle handling, traffic maneuvers, traffic observation, and special maneu vers). Further, subjects' eye movements at intersections can be monitored by a head mounted eye tracking system. Correct checks for traffic at intersections are scored off line using combined visual recordings of the driving scene and subjects' eye movements.

C.6.9 Psychophysical Thresholds

C.6.9.1 Critical Flicker Fusion (CFF) Threshold

PURPOSE AND RATIONALE

Critical Flicker Fusion (CFF) is a traditional psychophys ical threshold. The threshold is the maximum frequency at which a flickering light source can still be perceived to flicker by the observer. It is highly sensitive to the effects of drugs (e.g., Smith and Misiak 1976). This threshold, known as the Critical Flicker Fusion Threshold (CFF) can be measured using a wide variety of techniques. The general finding is that stimulant drugs increase the frequency at which fusion occurs, while sedative drugs impair it. It is widely used in clinical pharmacology and is widely believed to reflect "alertness" (e.g., Parrot 1982).

PROCEDURE

The procedure generally used is the method of ascending and descending limits, and involves observing a light source such as a light emitting diode. The rate of flicker is initially be set at a low frequency that is clearly identi fiable, and is then increased until the observer reports that the flicker can no longer be perceived. The rate of flicker is then set at a high frequency that appears con tinuous, and then is decreased until the observer reports that the flicker can be identified. Generally, three ascend ing and descending trials are conducted. In some methods, the observer actually alters the rate of flicker by turning a dial.

EVALUATION

The CFF is determined by averaging the frequency recorded for all trials, and is measured in Hz.

CRITICAL ASSESSMENT OF THE METHOD

There are hundreds of different methods that have been used in clinical pharmacology, which makes comparisons between studies difficult. However, the major limitation with the method is that it is not a direct measure of cognitive function, rather a psychological threshold, which can correlate with states like alertness, but can also dissociate due to alterations to sensory processes that are unrelated to cognitive function. It is thus useful for infer ring alertness, and is a sensitive method to detect central actions of drugs, but cannot be used alone to definitively assess cognitive function. It can also be heavily influenced by pupil size (Smith and Misiak 1976); enlarged pupil size increases the frequency at which threshold occurs, while decreased pupil size has the opposite effect. Unfortunately, many widely used procedures do not control for pupil size. The problem here is that many compounds independently influence pupil size, and thus either the possible effects on CFF can be exaggerated or underestimated (e.g., Lawrence et al. 2004). Schmitt et al. (2002) revaluated safety findings from previous work, which had not controlled for pupil size, and concluded: "Mydriasis masked the detrimental effects of both SSRIs on CFF during the acute assessments. Our results raise questions regarding the validity of the assessment of the behavioral toxicity of SSRIs based on CFF measurements without ample control for pupil size,

especially when these concern acute measurements." Another problem in CFF assessments when simply using the method of ascending and descending limits is that response bias, or the willingness to make a positive response, can affect the outcome. MacNab et al. (1985) showed that using "a block up down spatial forced choice method" can overcome this problem.

MODIFICATIONS OF THE METHOD

To overcome the problem of pupil size, CFF assessments should be conducted using an artificially controlled pupil size, generally by viewing the light source monocularly via a small hole 2 or 3 mm in diameter (Lawrence et al. 2004). A further refinement is to use the "block up down spatial forced choice method" recommended by MacNab et al. (1985), which has been successfully applied in clinical pharmacology studies (e.g., Wesnes et al. 1988). Variants of CFF that have been used in clinical pharmacology are an auditory version (see Eysenck and Easterbrook 1960), and the two flash threshold (see Gruzelier and Venables 1974).

C.6.10 Postural Stability

PURPOSE AND RATIONALE

The intention here is to determine whether drugs can affect the sway that occurs when we attempt to stand still. McClelland (1989) reviewed the literature and even 20 years ago identified a large number of techniques that were in use at the time. A wide variety of drugs increase body sway, though none in McClelland's extensive review decreased it. One direct technique for measuring postural stability is the Wright ataxiameter (Wright 1971), and versions of this simple elegant technique have been used extensively in clinical pharmacology (e.g., Andrews et al. 2007; Strougo et al. 2008; Wesnes et al. 2000). To assess postural stability, a cord from the ataxiameter is attached to the waist of the participant who is required to stand as still as possible with feet apart and eyes closed for 1 min.

The most commonly used indirect techniques are force platforms (balance platforms) (e.g., Korttila et al. 1981), and can be either static or dynamic (e.g., Browne and O'Hare 2000). In static techniques, volunteers stand on a platform, and postural stability is inferred by sensors that measure the displacement of the point of pressure on the platform. Dynamic methods involve the platform titling in particular directions, requiring the subject to maintain balance. There are numerous suppliers (e.g., www.amti.biz; www.adinstruments.com; www.lafayetteinstrumenteurope.com), but no single system appears to be widely adopted in clinical pharmacology.

PROCEDURE

The most easily used technique in clinical pharmacology studies are methods based on the Wright ataxiameter. A cord from the ataxiameter is attached to the participant who is required to stand as still as possible with feet apart and eyes closed for 1 min. The simple mechanical device yields an index of sway, which is essentially a measure of total sway path in one dimension (sagittal sway only).

EVALUATION

There is a single unit of measurement, units of one third degree of arc of sway.

CRITICAL ASSESSMENT OF THE METHOD

The simplicity of the technique, its established drug sensitivity, and the single outcome score account for the widespread use of the ataxiameter. Instrumented force platforms can yield more sophisticated evaluations, but are generally expensive and the assessments vary greatly between different manufacturers. There is also a safety concern with dynamic platforms, limiting their use in early phase studies or trials with the elderly.

MODIFICATIONS OF THE METHOD

The ultrasonic measurement of postural stability is possible, and has been discussed, but is not yet in widespread use.

C.6.11 Saccadic Eye Movements

PURPOSE AND RATIONALE

Saccadic eye movements are rapid steplike conjugate shifts of gaze, the purpose of which is to centralize objects of interest on the fovea (the most sensitive area at the back of the eye). The saccades can be produced by instructing a subject to follow a target made to jump instantaneously from one point to another. Saccades are affected by relatively few confounding variables, are reliable, can be repeated regularly and, once initiated, the movement is thought to be without cognitive or conscious input. Saccadic eye movements are highly sensitive to a range of drugs and have been extensively used as pharmacodynamic measures in clinical pharmacology (e.g., Griffiths et al. 1984). One technique has become

widely used in clinical pharmacology, the Cardiff Saccade Generation, and Analysis System (CSGAAS), and shown to be sensitive to a range of treatments and clinical con ditions (e.g., Griffiths et al. 1984; King 2007; Taylor et al. 2008). There are a number of manufacturers of other systems (e.g., www.tobii.com; www.smivision.com).

PROCEDURE

To perform the CSGAAS, the subject has silver/silver chlo ride electrodes placed laterally to the outer canthus of each eye. The electrodes are connected to an IBM compatible PC via a DC amplifier. Since vertical eye movement sig nificantly alters EOG amplitude in a nonlinear way, only lateral saccades are studied. The subject sits at a fixed distance from the monitor (between 50 and 80 cm), and eye movements in response to a light spot moving across a screen are recorded. A number of saccade trials are recorded (usually 45), with a constant interstimulus inter val of 1.5 s, at target displacements of 10 40°. The whole testing session can last as little as 2 3 min. For more details about the procedure, see (Wilson et al. 1993).

EVALUATION

A variety of measures can be derived; the most common are peak saccade velocity, saccade latency, peak accelera tion, peak deceleration, acceleration/deceleration ratio, and saccade error.

CRITICAL ASSESSMENT OF THE METHOD

The aim during development of CSGAAS was to produce a system that was simple enough for use as a routine screen in drug studies but with the flexibility to be a useful research tool. There are standard protocols used in drug studies, whereas more flexibility is used in certain novel research situations. The system is sensitive to compounds that can increase saccade latency and peak velocity, like stimulants, as well as a range of compounds that decrease these param eters (e.g., benzodiazepines). It is a very useful biomarker of drug effects, but like CFF, it is not a direct measure of cognitive function, though many drugs that impair cogni tive function do slow saccade velocity and latency.

MODIFICATIONS OF THE METHOD

The methodology has evolved with technological advances, previous versions using a row of light emitting diodes, but the overall basis of the measurement remains constant. Different systems may use different techniques and report different parameters, but the difficulty in com paring effects between studies using different techniques is unlikely to be as troublesome as many other measures mentioned in this chapter.

C.6.12 Measures of Motor Control and Eye–Hand Coordination

C.6.12.1 Finger Tapping

PURPOSE AND RATIONALE

Finger tapping measures are included in neuropsycholog ical examinations in order to assess subtle motor and other cognitive impairment. The finger tapping task was part of the Halstead Reitan Neuropsychological Test Bat tery developed in the 1960s. Sometimes called Morse tapping, the test involves tapping a button as quickly as possible for predetermined periods of time. The task has long been known to be drug sensitive, an early study showing nicotine had favorable effects (Frith 1967), and these results have been replicated in schizophrenia (Silver et al. 2002). A variety of other compounds impair tapping rates (Bishop et al. 1996).

PROCEDURE

The subject is instructed to press a button as quickly as possible for a predetermined period of time (1 s to 3 min).

EVALUATION

The major measure is the number of taps; some tech niques also measure the inter tap intervals as larger inter vals can indicate drops in attention.

CRITICAL ASSESSMENT OF THE METHOD

Easy to administer, short to perform, is useful as part of a broader assessment battery. Slowed tapping rates may indicate motor failures, but could also be due to attention or motivation failures, and thus the pattern of effects over the battery is important to aid interpretation. There is little standardization of the procedure between studies.

MODIFICATIONS OF THE METHOD

As mentioned already, differing durations of testing are used, and some tests require both dominant and nondominant hands to be used in successive tests.

C.6.12.2 Pegboard Tests

PURPOSE AND RATIONALE

The purpose of this type of test is to measure dexterity and fine motor control. The most common tests are the Lafa yette Grooved Pegboard test and the Purdue Pegboard test, which are both commercially available, extensively

validated, and with comprehensive user manuals (www.lafayetteevaluation.com). Both have been widely used in clinical pharmacology and are sensitive to both disease as well as the effects of drugs (e.g., Duka et al. 1996; Silbert et al. 2004; Stonier et al. 1982).

PROCEDURE

The Grooved Pegboard is a manipulative dexterity test consisting of 25 holes with randomly positioned slots. Pegs with a key along one side must be rotated to match the hole before they can be inserted. The test is explained to the subject, who then has to put all the pegs in the holes, first with the dominant hand, then with the nondominant hand. Each stage is timed with a stopwatch. The test takes around 5 min to administer.

The Purdue Pegboard test measures gross movements of hands, fingers and arms, and fingertip dexterity as necessary in assembly tasks. It consists of a pegboard and a collection of pins, washers, and collars. The subject manipulates the pins and collars and inserts them into the holes on the board according to the test routine. The standard routine is to give the subject 30 s first to insert pins with the right hand, then with the left hand, and finally with both hands. After this, the subject is given 60 s for the assembly stage, which involves placing a pin in a hole with the right hand, then immediately placing a washer over the pin with the left hand, and then a collar with the right hand, and then inserting the next pin, and so on. The whole procedure takes around 10 min.

EVALUATION

The score on the Grooved Pegboard test is simply the total time taken in seconds to perform both stages.

The scores on the Purdue Pegboard test are the number of pins inserted at each of the first three stages, plus a total score. For the assembly stage, it is the number of completed assemblies in the 60 s period.

CRITICAL ASSESSMENT OF THE METHOD

Both are standardized and validated, and the scores can easily be compared between different studies. There are clear training effects, which can be a problem in trials where repeated testing is required, and for these pre study training is recommended. They need careful administration, but do not require specialists.

MODIFICATIONS OF THE METHOD

There are a number of versions of pegboard tests, but the user is recommended to use one of the two described.

C.6.13 Visual Tracking Tasks

PURPOSE AND RATIONALE

The purpose of these automated tests is to assess eye hand coordination and sustained attention. An early electromechanical task, the Pursuit Rotor Test, was used extensively in clinical pharmacology trials (see Hindmarch 1980; Wesnes 1977), but has been superseded by computerized tracking tests in recent years. An adaptive tracking task (Borland and Nicholson 1974) has been widely used (e.g., Mercer et al. 1998; Patat et al. 1995; van der Post et al. 2005), as have various visual tracking tasks involving the use of a joystick.

PROCEDURE

The adaptive tracking test is a pursuit tracking task. A circle moves randomly about a screen, and the subject is instructed to try to keep a dot inside the moving circle by operating a joystick. If this effort is successful, the speed of the moving circle increases. Conversely, the velocity is reduced if the test subject cannot maintain the dot inside the circle. The test is performed for 10 min.

A visual tracking task that has been used in a number of studies involves the subject using a joystick to track a randomly moving target cross on the screen (Beuzen et al. 1999; Williams et al. 1996). The joystick moves a box on the screen, which must be kept as close as possible to the target for 1 min. The task does not vary in difficultly with successful performance as does the adaptive task.

EVALUATION

In the adaptive tracking task, the average performance and the standard deviation of scores over a 10 min period are recorded.

In the visual tracking task, the distance from target each second is recorded, and the scores are the average distance in millimeter (tracking error), and the standard deviation of the error.

CRITICAL ASSESSMENT OF THE METHOD

Like the pursuit rotor, tracking tasks are subject to large practice effects and require pre study training. Comparisons between studies are possible with the adaptive tracking task, and also other standardized visual tracking tasks such as the one described above.

MODIFICATIONS OF THE METHOD

Numerous versions are available, and the next development will be three dimensional (3 D) tracking tasks.

C.6.14 Self-Ratings of Mood, Alertness, Sleepiness, and Sleep Quality Mood and Alertness

C.6.14.1 Bond-Lader Visual Analogue Scales (VAS)

PURPOSE AND RATIONALE

A Visual Analogue Scale (VAS) is a measurement instru ment that tries to measure a characteristic or an attitude that is believed to range across a continuum of values and cannot easily be directly measured (Crichton 2001). For example, how alert a patient feels ranges across a contin uum from "not at all" to "extremely." Operationally a VAS is usually a horizontal line, 100 mm in length, anchored by word descriptors at each end. The patients mark on the line at the point that they feel represents their perception of their current state. The VAS score is determined by measuring in millimeters from the left hand end of the line to the point that the patient marks. As such an assess ment is clearly entirely subjective, these scales are of most value when looking at the change within individuals, and are of less value for comparing between groups of individ uals at single time points. Bond and Lader (1974) created 16 item VAS to assess mood and alertness. The question naire derives three factors that assess change in self rated alertness, self rated calmness, and self rated contentment from 16 analogue scales. It has been used in hundreds if not thousands of trials worldwide, and has proven sensi tive to detecting changes in self rated mood and alertness with a very wide range of compounds (e.g., Hanks et al. 1995; Wesnes et al. 2000).

PROCEDURE

This task examines the mood of the volunteer/patient at the time of testing. For this questionnaire, the individual is required to rate how they feel "at this moment" on 16 analogue scales. Each scale has an adjective describing a mood at one end of each 100 mm line, and an adjective describing the opposite mood at the other end. These are the adjectives used, the left one being at the left hand and the other on the right side of the 10 cm line:

ALERT DROWSY
CALM EXCITED
STRONG FEEBLE
MUZZY CLEAR HEADED
WELL COORDINATED CLUMSY
LETHARGIC ENERGETIC
CONTENTED DISCONTENTED
TROUBLED TRANQUIL
MENTALLY SLOW QUICK WITTED
TENSE RELAXED
ATTENTIVE DREAMY
INCOMPETENT PROFICIENT
HAPPY SAD
ANTAGONISTIC FRIENDLY
INTERESTED BORED
WITHDRAWN SOCIABLE

In the paper and pencil version, participants rate how they feel by crossing the line running between one adjective and the other at the place they feel appro priate to their mood at that time. The procedure lasts approximately 120 s.

EVALUATION

The results are combined to form three factor scores, alertness (nine scales contribute), calmness (two scales contribute), and contentment (five scales contribute). The scoring is arranged such that a larger score reflects a higher degree of the factor, that is, more alertness.

CRITICAL ASSESSMENT OF THE METHOD

The scale is highly sensitive and widely used. Calmness is the weakest factor, having only two scale items, and for this aspect of mood the Spielberger STAI may be more appropriate. When assessing the factor scores, some researchers use the factor weightings for the individual items from the original paper, though this practice is questionable, and the author of the test has confirmed to the writer that this was not the intention in publishing the weightings, and is not their preferred method.

MODIFICATIONS OF THE METHOD

Numerous language versions have been developed and used. A computer version has been developed for the CDR System in which each scale is presented on the screen and the participants move the cursor along the line using the mouse and clicks once they feel the cursor is in the correct place to represent their assessment.

C.6.14.2 Profile of Mood States (POMS)

PURPOSE AND RATIONALE

The Profile of Mood States (POMS) is a 65 item self report questionnaire designed to assess mood state over the previous week or for shorter periods such as "right

now." The original POMS manual was published in 1971, followed in 1992 by a revised edition, that provided addi tional validity and normative data, as well as introducing a smaller version of the POMS. The POMS is sensitive to mood changes produced by a variety of compounds in clinical pharmacology trials (e.g., Parrott and Winder 1989; Silber et al. 2006).

PROCEDURE

Each item consists of an adjective, some of which reflect positive mood states (e.g., lively, clearheaded, and cheerful), whereas others reflect negative mood states (e.g., sad, hopeless, and unhappy). The participants select the number under the answer that best describes how they have been feeling during the period of interest (e.g., "past week including today," "last few hours," or "right now"). For example, patients are shown the fol lowing instructions that are varied to reflect the period of interest:

- "Below is a list of words that describe feelings people have. Please read each one carefully. Then select ONE answer to the right which best describes your mood in past week including today."

The ratings for each item are:

0 = not at all
1 = a little
2 = moderately
3 = quite a bit
4 = extremely

EVALUATION

A scoring template is provided to derive the mood factors. Both the POMS Standard and POMS Brief assessments measure six identified mood factors (Lorr et al. 2003), plus a Total Mood Disturbance score, which is a composite of all six:

1. Tension
2. Depression
3. Anger
4. Vigor
5. Fatigue
6. Confusion

CRITICAL ASSESSMENT OF THE METHOD

This is a widely used and well validated assessment method, which provides a useful evaluation of arrange of mood states.

MODIFICATIONS OF THE METHOD

Numerous language versions have been developed and used. A computer version has been developed under license for the CDR System, in which the scale is presented on the screen and the participant uses the mouse to click on the ratings. This does avoid the prob lem of volunteers missing items, and also facilitates data analysis and storage.

C.6.14.3 Spielberger State-Trait Anxiety Inventory (STAI)

PURPOSE AND RATIONALE

The STAI Form Y is one of the more widely used self report scales for evaluation of anxiety in volunteers and patients (Form Y is a revised version of the original Form X). The instrument includes separate measures of state and trait anxiety. Respondents are asked to indicate on two 20 item scales how they are feeling "right now, at this moment" (state version) and how they "generally" feel (trait version). The STAI shows good correlations with other measures of anxiety such as the Beck Anxiety Inven tory and the Fear Questionnaire. Due to its longevity and ease of acquisition and use, the STAI has been widely used in a variety of research studies (e.g., Girdler et al. 2002) and clinical settings (e.g., Bailie et al. 1987).

PROCEDURE

Participants are shown two 20 item scales. One assesses trait anxiety, the other state anxiety. Each statement is followed by a scale and participants must circle the appro priate number to indicate how they feel, either generally (trait) or right now, at this moment (state). Statements include:

- "I feel calm"
  "I feel comfortable"

The following ratings are used for each statement:

1 = Almost never
2 = Sometimes
3 = Often
4 = Almost always

EVALUATION

A template is used to derive the score.

CRITICAL ASSESSMENT OF THE METHOD

The current gold standard of its type.

MODIFICATIONS OF THE METHOD
A six item short form is also available. It has been computerized.

C.6.15 Self-Ratings of Sleepiness

C.6.15.1 Epworth Sleepiness Scale

PURPOSE AND RATIONALE
The Epworth Sleepiness Scale (ESS) is a simple, self administered scale developed by Johns (1991) for assessing the daytime sleepiness of patients at the Epworth Sleep Centre in Melbourne (www.epworthsleepinessscale.com).

PROCEDURE
Participants are shown a list of eight situations and must decide how likely they would be to "doze" during each one by responding with a score between 0 (no chance of dozing) and 3 (high chance of dozing). The instructions are:

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired? Even if you have not done some of these things recently, try to work out how they would have affected you. Use the following scales to choose the most appropriate number for each situation:

0 = no chance of dozing
1 = slight chance of dozing
2 = moderate chance of dozing
3 = high chance of dozing

Situation	Chance of dozing
Sitting and reading	
Watching TV	
Sitting inactive in a public place (e.g., a theater or a meeting)	
As a passenger in a car for an hour without a break	
Lying down to rest in the afternoon when circumstances permit	
Sitting and talking to someone	
Sitting quietly after a lunch without alcohol	
In a car, while stopped for a few minutes in traffic	

EVALUATION
The total of the eight numbers to the items is totaled to give a score in the range 0 24.

CRITICAL ASSESSMENT OF THE METHOD
The wider range of scores in the ESS compared to the SSS and Karolinska Sleepiness Scale (KSS) increases the sensi tivity of the scale to change, and its everyday relevance is another feature which differentiates it from the other sleepiness assessments. The scale is widely used in clinical practice and research protocols as a simple rapid assess ment of subjective sleepiness. In addition, the ESS is fre quently used in research studies as a means of quantifying changes in habitual self rated sleep propensity after a pharmacological intervention (e.g., Harsh et al. 2006; Ondo et al. 2005).

MODIFICATIONS OF THE METHOD
The ESS has been computerized, which facilitates admin istration by preventing participants missing items or entering numbers outside of the range. This also prevents errors in scoring and entering the total score into a database.

C.6.15.2 Karolinska Sleepiness Scale

PURPOSE AND RATIONALE
The KSS developed by Åkerstedt and Gillberg (1990) to assess self ratings of sleepiness in volunteers and patients.

PROCEDURE
Participants are given the nine point scale and are instructed to indicate their level of sleepiness felt during the 10 min prior to completing the scale by selecting a number from 1 to 9:

1: Very Alert
2
3: Alert Normal Level
4
5: Neither alert nor sleepy
6
7: Sleepy but no effort to keep awake
8
9: Very sleepy, great effort to keep awake, fighting sleep

EVALUATION
The score is simply the digit checked.

CRITICAL ASSESSMENT OF THE METHOD
The simplicity of the scale is an attractive feature of the KSS. It has been used widely, is sensitive to sleep loss

(e.g., Sallinen et al. 2004), and has shown correlations both to performance and physiological indicators of sleepiness (e.g., Kaida et al. 2006). The KSS has been used in a diverse range of clinical trials of new medicines (e.g., Czeisler et al. 2009).

MODIFICATIONS OF THE METHOD

It can be easily computerized, though with its simplicity this just aids data storage.

C.6.15.3 Stanford Sleepiness Scale

PURPOSE AND RATIONALE

The Stanford Sleepiness Scale (SSS) is a momentary self rated assessment scale to detect feelings of sleepiness which was developed by Hoddes et al. (1972). Like the KSS, the SSS is succinct and straightforward to use in clinical pharmacology settings, and can be administered repeatedly at closely timed intervals (e.g., hourly).

PROCEDURE

The SSS consists of seven descriptive phases that describe individual mood states, the participants being instructed to choose the one that best describes how they feel at the time.

1. Feeling active and vital, alert, or wide awake
2. Functioning at high levels, but not at peak; able to concentrate
3. Awake, but relaxed; responsive but not fully alert
4. Somewhat foggy, let down
5. Foggy; losing interest in remaining awake; slowed down
6. Sleepy, woozy, fighting sleep; prefer to lie down
7. No longer fighting sleep, sleep onset soon; having dreamlike thoughts

EVALUATION

It is simply the number of the evaluation selected.

CRITICAL ASSESSMENT OF THE METHOD

As with the KSS, the simplicity of the scale is an attractive feature of the SSS, and being oldest of the three scales, it has been used extensively in a wide variety of situations and for a wide variety of purposes. It has been shown to relate to performance measures (e.g., Glenville and Broughton 1979). It is probably the least sensitive to change due to the small number of possible responses. Nonetheless, it is a useful tool for clinical pharmacology studies and has shown sensitivity to treatment effects (e.g., MacDonald et al. 2002).

MODIFICATIONS OF THE METHOD

It can be easily computerized, though with its simplicity this just aids data storage.

C.6.16 Ratings of Sleep and Sleep Quality

There are a number of instruments for assessing self ratings of sleep quality, which have been used widely in clinical pharmacology research. The two oldest and most widely used will be described as they reflect the two different techniques in this area. The reader is also advised to consider the Pittsburgh Sleep Quality Index (Buysse et al. 1989). Two further questionnaires have been developed based on the ICD 10 Diagnostic Criteria for Insomnia, and will prove useful tools in clinical pharmacology (Athens Insomnia Scale Soldatos et al. 2000; Bergen Insomnia Scale Pallesen et al. 2008).

C.6.16.1 Leeds Sleep Evaluation Questionnaire

PURPOSE AND RATIONALE

The Leeds Sleep Evaluation Questionnaire (LSEQ) developed by Hindmarch and Parrott (1978) is a set of visual analogue scales, which address four aspects of sleep: getting to sleep (GTS), quality of sleep (QOS), awakening from sleep (AFS), and behavior following wakefulness (BFW).

PROCEDURE

The LESQ requires participants to compare their current subjective appreciation of sleep with that of a previous sleep experience. Participants are shown statements relating to sleep, and must make ratings by placing a mark on the anchored scale(s) below the statements. This can be done using a mouse and curser in the computer version or drawing a vertical line in the pen and paper version. The questions and the scale items are listed below:

Question: How would you compare getting to sleep using the medication with getting to sleep normally, that is, without medication?

HARDER THAN USUAL EASIER THAN USUAL
LOWER THAN USUAL QUICKER THAN USUAL

FELT LESS DROWSY THAN USUAL FELT MORE DROWSY THAN USUAL

Question: How would you compare the quality of sleep using the medication with non medicated (your usual) sleep?

MORE RESTLESS THAN USUAL MORE RESTFUL THAN USUAL
MORE PERIODS OF WAKEFULNESS THAN USUAL
FEWER PERIODS OF WAKEFULNESS THAN USUAL

Question: How did your awakening after medication com pare with your usual pattern of awakening?

MORE DIFFICULT THAN USUAL EASIER THAN USUAL
TOOK LONGER THAN USUAL TOOK SHORTER THAN USUAL

Question: How did you feel on waking?

TIRED ALERT

Question: How do you feel now?

TIRED ALERT

Question: How was your sense of balance and coordina tion upon getting up?

MORE CLUMSY THAN USUAL LESS CLUMSY THAN USUAL

EVALUATION

The marked position on the scale is measured, the distance away from the center representing change from ordinary sleep experience. The individual scales are measured from end containing the word that reflects the less positive aspect. These scores are then combined to produce factor scores for GTS, QOS, AFS, and BFW.

CRITICAL ASSESSMENT OF THE METHOD

The LSEQ has been used to assess subjectively perceived changes in sleep in clinical trials involving a variety of compounds including sedatives, antidepressants, anxio lytics, CNS stimulants, and antihistamines, showing good sensitivity (e.g., Parrott and Hindmarch 1980; Wesnes and Warburton 1984b, c).

MODIFICATIONS OF THE METHOD

Computerization of this questionnaire has reduced the respondent time and increased ease of data capture and processing in clinical trials.

C.6.16.2 St Mary's Hospital Sleep Questionnaire (SMHSQ)

PURPOSE AND RATIONALE

As with the LSEQ, the intention of St Mary's Hospital Sleep Questionnaire is to assess self rated sleep quality (Ellis et al. 1981).

PROCEDURE

The SMHSQ is a nine item questionnaire. The instruc tions to the patient/volunteer are:

This refers to your sleep over the last 24 h. Please ensure that you answer every question and circle the answer that best applies to you.

1. Was your sleep?

Very light	1
Light	2
Fairly light	3
Light average	4
Deep average	5
Fairly deep	6
Deep	7
Very deep	8

2. How many times did you wake last night?

Not at all	1
Once	2
Twice	3
Three times	4
Four times	5
Five times	6
Six times	7
More than six times	8

3. How much sleep did you have last night?

	h		min

4. How much sleep did you have during the day yesterday?

	h		min

5. How well did you sleep last night?

Very badly	1
Badly	2
Fairly badly	3
Fairly well	4
Well	5
Very well	6

6. How clearheaded did you feel this morning after get ting up?

Still very drowsy indeed	1
Still moderately drowsy	2
Still slightly drowsy	3
Fairly clearheaded	4
Alert	5
Very alert	6

7. How satisfied were you with last nights sleep?

Very unsatisfied	1
Moderately unsatisfied	2
Slightly unsatisfied	3
Fairly satisfied	4
Completely satisfied	5

8. Were you troubled by waking early and being unable to get off to sleep again?

Yes	No

9. How much difficulty did you have in getting off to sleep last night?

None or very little	1
Some	2
A lot	3
Extreme difficulty	4

EVALUATION

There is no clear factor structure to the SMHSQ (Leigh et al. 1988), and the nine items are generally analyzed separately.

CRITICAL ASSESSMENT OF THE METHOD

This questionnaire addresses important aspects of sleep quality and has been widely used. Several of the individual items have limited responses, which may limit their sen sitivity to change, and also require nonparametric analysis methods. Nonetheless, the SMHSQ has shown sensitivity to changes in sleep quality due to pharmacological inter vention in a wide range of studies (e.g., Hicks et al. 2002; Wesnes and Warburton 1984b).

MODIFICATIONS OF THE METHOD

It is easy to computerize, and computerized versions ensure all items are completed and facilitate scoring and entry into databases.

C.6.17 Measures of Brain Activity

C.6.17.1 Electroencephalography

PURPOSE AND RATIONALE

EEG involves the measurement of the electrical activity of brain neurones, using electrodes placed on the scalp. It has been widely used in clinical pharmacology for over 60 years and is worthy of its own chapter in this volume. The International Pharmaco EEG Society founded in 1980 is a good source of general information, and the reader is recommended to the Special Issue of the Journal of Clinical EEG and Neuroscience, April 2006, entitled "Pharmaco EEG at a Crossroads", for some excellent reviews of the field. There is good standardization of measurement and electrode placement in the field, though there are dozens of manufacturers of EEG equipment and software. As technology has advanced, so have the capa bilities of EEG, for example, source localization and topo graphical mapping.

The uses of EEG in clinical pharmacology are diverse, including characterizing the signature of different classes of drugs or types of compounds, identifying "sedative" or "stimulant" actions of drugs, clinical conditions, etc. EEG also plays a major role in sleep research (see Achermann 2009). The event related potential (ERP) is another valu able assessment, this being tied to a particular stimulus, and the different components of the wave form, for exam ple, P300, can yield valuable information about central processing in attention. The linking of such techniques with the stimuli presented in cognitive tasks is another important development, allowing direct performance based measures of cognitive function to be coupled with

insight into the neural mechanisms involved and their location or source.

EEG techniques can also be used to test sleep latency in tests of wake promoting agents. There are two well established techniques, which involve EEG assessment: the Maintenance of Wakefulness Test (MWT) and the Multiple Sleep Latency Test (MSLT; see Sullivan and Kushida 2008). Both are useful and widely used tools for clinical pharmacology studies.

PROCEDURE

The equipment involved is highly specialized and needs much training and expertise to use. Electrodes (mostly from 19 to 256) are placed on the scalp, generally using international conventions for their placement (e.g., the 10 20 system first described by Jasper in 1956). High power digital amplifiers are used for each pair of electrodes and computers perform the huge processing requirements of the techniques and the storage of the massive amounts of information obtained.

In ERP assessments, a stimulus is presented to the subject and the EEG activity epoch is recorded from the onset of the stimulus until around a second. The stimulus is repeated a number of times (say 30 50), and an averag ing technique is applied to the EEG epochs to cancel out the noise and identify the wave pattern unique to the stimulus. With multiple electrode sites, the ERPs can be compared from numerous locations.

EVALUATION

Often EEG analysis employs fast Fourier methods to iden tify the relative amounts of power in different frequency bands in the range (1 20 Hz), for example, alpha (8 13 Hz). An alternative is to identify the dominant frequency in one of these bands.

ERPs are evaluated for various waveforms, for exam ple, P300, either in terms of the latency at which they appear after the stimulus and/or their amplitude.

In sleep research, different sleep stages are identified by their characteristic EEG profiles, for example, REM sleep and slow wave sleep, and changes to the onset or duration of these stages can be used to infer disturbances in sleep patterns produced by drugs.

CRITICAL ASSESSMENT OF THE METHOD

An invaluable tool for clinical pharmacology and neces sary to profile and classify any novel compound. The high complexity of the various methodologies both in terms of the expertise required in the administration of various EEG techniques as well as the analysis and interpretation, sometimes limits the availability of such techniques in early safety trials of novel medicines, but such techniques can be used even in first to man trials. EEG is valuable often for simply determining if a drug enters the CNS in early stages of development. It should be noted that EEG techniques do not directly measure cognitive function, only tests do this, but when cognitive tests are linked with EEG, the resulting information far surpasses what can be obtained with either technique alone. Sleep labo ratories are expensive to equip and run, but nonetheless the number of centers that can run clinical pharmacology sleep studies is increasing worldwide.

MODIFICATIONS OF THE METHOD

Magnetoencephalography (MEG) techniques that mea sure the magnetic fields produced by brain electrical activ ity are now entering clinical pharmacology units, and confer a number of benefits over EEG including better spatial resolution.

C.6.18 Imaging Techniques MRI, fMRI, PET, and SPECT

PURPOSE AND RATIONALE

These four techniques have made huge inroads into clin ical research and, more recently, clinical pharmacology. As with EEG, a full coverage is beyond the scope of this chapter, but an overview of their utility in early drug development will be worthwhile.

PROCEDURE AND EVALUATION

Magnetic Resonance Imaging (MRI) uses magnetic fields and radio waves to produce high quality 2 D or 3 D images of brain structures without injecting radioactive tracers. Functional MRI (fMRI) measures changes in blood oxygen levels in the brain to produce real time images of blood flow in the brain. This identifies which areas are active during a task, or by a compound, and can be done over short time intervals. PET involves introduc ing a low activity, short lasting radioactive label to com pounds like glucose or oxygen in the brain. The radioactive labels decay in a characteristic way, emitting positrons. The technique creates images of the brain show ing different levels of radioactivity, which reflect activity in differing regions. Using different compounds, PET can show blood flow, oxygen, and glucose metabolism, as well as drug concentrations in the brain. Single photon emission computed tomography (SPECT) is similar to PET, but SPECT tracers are more limited than PET tracers

in the kinds of brain activity they can monitor, and deteriorate more slowly than many PET tracers, which is disadvantageous procedurally as it requires longer test and retest periods, but advantageous because the longer lasting tracers do not require an onsite cyclotron to produce them, and also the procedure requires less technical and medical support, both making it less expensive.

CRITICAL ASSESSMENT OF THE METHOD

Overall, fMRI provides superior image clarity along with the ability to assess blood flow and thus localized brain function in seconds. PET is more versatile than SPECT and produces more detailed images with a higher degree of resolution. For clinical pharmacology, PET has the important advantage of being able to identify which brain receptors are being activated by neurotransmitters, drugs, and new pharmacological therapies. Further, the degree of receptor occupancy with a pharmacological compound can be determined, a hugely important method for CNS drugs and a major advance over traditional pharmacokinetic methods.

The procedures have clearly brought numerous benefits to clinical pharmacology and will continue to do so. However, there is an issue of stress for participants, independently of possible claustrophobia, the noise in MRI scanners can also be disturbing, and up to 25% of patients experience moderate to severe anxiety in first time MRI procedures (Mcisaac et al. 1998). These scanning procedures are also hugely expensive and often time consuming. Nonetheless, some clinical research units in Europe and the USA have fMRI and SPECT capabilities, either on site, or nearby, and these are currently being used in early stage drug development.

MODIFICATIONS OF THE METHOD

Cognitive testing can be incorporated during fMRI to enable the brain areas involved in various tasks and cognitive processes to be delineated. As with EEG, these techniques do not directly measure cognitive function, but when linked with tests, provide hugely valuable information.

C.6.19 Models

A number of models have been used in early clinical pharmacology studies to identify potential future efficacy of new compounds. These proof of concept/principle studies are becoming increasingly popular in drug development, and some have proved to be valuable aids to drug development. Two widely used models in translational medicine clinical pharmacology trials will be described. The reader is also referred to the hypoxia model, which has been proven a sensitive and valuable procedure in drug studies (see Schaffler and Wauschkuhn 1992). Other models have been employed in clinical pharmacology, for example, the ketamine model of schizophrenia (e.g., Abi Saab et al. 1998), social anxiety models for screening novel anxiolytics (e.g., Graeff et al. 2002), and various depletion models (e.g., the tryptophan depletion test; Neumeister et al. 1997). A number of other innovative clinical models are also available or are soon becoming available for a range of conditions, including anxiety, schizophrenia, obesity, ischemia, depression, and cognition (see e.g., www.p1vital.com, www.forenap.com).

C.6.19.1 The Scopolamine Model of Dementia

PURPOSE AND RATIONALE

Scopolamine blocks brain cholinergic function and produces temporary cognitive deficits in volunteers (e.g., Drachman and Leavitt 1974), which resemble closely the core cognitive deficits seen in Alzheimer's disease and other cholinergically based dementias (e.g., Huff et al. 1988; Wesnes and Simpson 1988; Wesnes 2001). This provides the opportunity to determine whether these deficits can be reversed by potential anti dementia compounds early in drug development. The scopolamine model has been the most widely used model in early phase drug development over the last 30 years. A wide variety of compounds have been found to reduce the cognitive deficits of scopolamine in young and elderly volunteers (e.g., Baker et al. 2009; Sitaram et al. 1978; Wesnes and Revell 1984; Wesnes et al. 1991), even compounds without a direct cholinergic mechanism (e.g., Anand and Wesnes 1990; Wesnes et al. 1987) and some of these compounds have shown efficacy when administered to patients (e.g., Coelho and Birks 2001;Siegfried 1993; Sourander et al. 1987).

PROCEDURE

This procedure is easily applied in any clinical pharmacology unit. Although orally administered scopolamine hydrobromide has been used, for pharmacokinetic reasons subcutaneous injection is the preferred technique. Doses vary, but the ideal range is 0.4 0.7 mg in healthy young volunteers, with 0.5 mg giving the optimum magnitude and duration of impairment while minimizing the side effects. The elderly are more sensitive, and doses in the

range 0.2 0.3 mg have been used successfully. The effects of scopolamine administered in this fashion appear within 30 min and last up to 5 h. There are three main paradigms employed. The first is to administer scopolamine, estab lish the cognitive deficit 45 60 min later, and then admin ister the study compound and repeat testing at regular intervals to determine the rate of recovery. The second is to pre dose volunteers with the study compound, for hours, days, or weeks, and then to administer scopolamine and evaluate the profile of impairment. The third is to administer scopolamine and the study compound at the same time. The choice between paradigms is generally based either on the mechanism of action of the compound or pharmacokinetics.

Cognitive function should be measured throughout any study, with baselines, and as many test sessions as feasible following scopolamine, at least one per hour. Crossover designs are the most sensitive, and reference compounds like physostigmine or Aricept can be added to the design to ensure the sensitivity of the model and also to help evaluate the clinical relevance of the effects of the study compound. At least three active doses of the study compound should be administered to establish the dose response profile.

EVALUATION

The method depends on the paradigm, but time point comparisons of the post scopolamine deficits are the most appropriate, made between the placebo and differing doses of the study compound, as well as the reference compound if used.

CRITICAL ASSESSMENT OF THE METHOD

Like any model, it is not without its critics, but the model has stood the test of time, is widely used and has proven sensitivity for predicting effects in patients, as well as providing valuable information on the nature of the dose relationship and the time profile of the effects of the study compound. Physostigmine, for example, works well in the model, but requires subcutaneous administration, and even then the effects fade within an hour (e.g., Ebert et al. 1998), reflecting the clinical experience with the compound (Coelho and Birks 2001). No go decisions have been made, and negative data from the model can predict the lack of efficacy in patients (e.g., Herting 1991). The method is also suitable for aiding decisions on which of various potential candidates should be selected for future development. Sample sizes of as low as 10 have been used successfully, and if the study drug is adminis tered once as opposed to multiply, an entire study can be completed in a matter of weeks.

MODIFICATIONS OF THE METHOD

The model is generally run as an early pharmacodynamic study after initial safety testing has been conducted. How ever, the model was used in one Phase I program, the volunteers first receiving the first to man dose of the study compound to check tolerance and safety, and then a week later the same dose was readministered to the same volunteers in the scopolamine model. Other trials have added a scopolamine administration to the last day(s) of multiple dosing safety and tolerability trials. Elderly vol unteers have been successfully used, and the effects predicting to patients in a trial with a Huperzine derivative and donepezil administered as an internal control at the clinically effective dose (Zangara 2003). Other assessments described in this chapter can also be employed, for exam ple, EEG (e.g., Ebert et al. 1998).

C.6.19.2 Sleep Deprivation Models

PURPOSE AND RATIONALE

Sleep deprivation is a relatively noninvasive technique, which has been employed in clinical pharmacology stud ies. In one study, the ability of a hypnotic to improve daytime sleep quality and duration following a night of sleep deprivation was established in healthy volunteers (Wesnes and Warburton 1986). In other studies, healthy volunteers have been kept awake for periods ranging from 24 to 60 h, and the ability of wake promoting agents to reduce the sleep induced cognitive deficits has been established (e.g., Stivalet et al. 2009; Wesnes and Macher 2004). Another approach for clinical pharmacology is to study people who are sleep deprived due to their occupa tion; in one trial, nurses working 3 day night shifts were tested before and immediately shifts over a 3 month period and the study treatment was found to reduce the negative effects of shift work on mood, fatigue, and cog nitive function (Wesnes et al. 2003).

PROCEDURE

Such trials can be conducted in any clinical pharmacology unit, in volunteer trials the subjects are kept in the unit for the study periods under controlled conditions, in shift work trials; the workers visit the units for assessments before and after their shifts. Any number of the assess ments described in this chapter can be employed in such work.

EVALUATION

Varies according to the design and the outcome measures.

CRITICAL ASSESSMENT OF THE METHOD

Such trials can be highly useful for screening compounds for subsequent efficacy. For example, a wake promoting agent was studied in the laboratory in volunteers (Wesnes and Macher 2004), and the results replicated in registration trials in patients with sleep apnea (Roth et al. 2006), narcolepsy (Harsh et al. 2006) and shift work sleep disorder (Czeisler et al. 2009). In the latter study that involved several measures described in this chapter, the study compound significantly reduced the attentional declines in the patients on the CDR System, reduced sleepiness as reported using the KSS, and increased sleep latency as assessed using the MSLT. This converging evidence provides a convincing profile of improved attentional ability, reduced sleepiness, and a greater ability to stay awake. Further the Clinical Global Impression of Severity of Illness (CGI S) was significantly reduced by treatment, and significant improvement was identified in most items assessed in the electronic diaries, including maximum level of sleepiness during the night shift and commute home, as well as the mean number of mistakes, accidents, or near misses compared with placebo (Czeisler et al. 2009). This study is an excellent illustration of how the various techniques described in this chapter can be used together in clinical pharmacology to identify independent but mutually supportive effects, which also relate to CGI S and self reported behavior by the patients.

MODIFICATIONS OF THE METHOD

The opportunities for developing this methodology are numerous.

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C.7 Pharmacodynamic Evaluation: Diabetes Methodologies

Methods in Clinical Pharmacology

Reinhard H. A. Becker

C.7.1 Introduction

C.7.1.1 Endogenous and Exogenous Insulin

Endogenous insulin is the chief regulator of glucose homeostasis. It is formed in pancreatic beta cells and released into the portal vein in a pulsatile manner to act on the liver, muscle, other body organs, and on peripheral tissues. Insulin reduces hepatic glycogenolysis and gluco neogenesis, promotes glucose uptake and glycogenesis in liver and muscle, and triggers conversion of excess glucose to fatty acids for storage as triglycerides in adipose tissue (Hellman 2009; Zierler 1999).

While endogenous insulin is secreted in response to a given glucose load, the distinct glucodynamic response profiles of different pharmaceutical insulin products result from the given exogenous insulin load (Gaohua and Kimura 2009; Owens et al. 2001; Zinman 1989).

C.7.1.2 Insulin Products

Subcutaneously given insulin products are the mainstay in treatment of diabetes mellitus since the independent elab orate discovery and characterization of a hypoglycemic extract of pancreas in dogs by *Banting* and his assistant *Best*, and purification of the extract, advanced by *Collip* for first successful clinical use (Bliss 2005; Rosenfeld 2002), after which *Banting* and *Macleod* were awarded the Nobel prize (Lindsten 2001) although the first description and use in dogs was apparently made by Paulescu (1921).

While early insulin products were based on animal insulin extracted from pancreata following a licensed recipe from the University of Toronto (Rosenfeld 2002), advancement in biotechnology based on recombinant DNA technology allowed mass production of human insulin by bacteria (*E. coli*) and yeast (*S. cerevisiae*) to yield human insulin products (regular human insulin, NPH insulin) (Goeddel et al. 1979; Miller 1979), and more recently human insulin analogue products (Brange et al. 1988). Analogues of human insulin differ from human insulin by the substitution of amino acids or other chemical changes, for example, an additional fatty acid chain (U.S. Department of Health and Human Services, Food and Drug Administration 1995; U.S. Department of Health and Human Services, Food and Drug Administration 2000a; U.S. Department of Health and Human Services, Food and Drug Administration 2000b; U.S. Department of Health and Human Services, Food and Drug Administration 2005; Brange et al. 1988; Havelund et al. 2004; Heise et al. 2007; Robinson and Wellington 2006).

Regardless of source, subcutaneous insulin prepara tions differ mainly by their pharmacokinetic and, conse quently, their pharmacodynamic time effect profiles (Binder et al. 1984; Holmes et al. 2005). As of today, they are divided into short , intermediate , and long acting products, of which a subgroup of short acting products is defined rapid acting, comprising the group of rapidly absorbed human insulin analogue products (American Diabetic Association 1998).

While insulin products addressing insulin need in response to a meal are termed prandial insulins, the term basal insulin is used for products addressing interprandial or fasting glucose control.

Subcutaneous insulin products are used alone or as combination medications of free mixtures or premixed preparations of short or rapid acting insulin and inter mediate (long) acting insulin preparations in various proportions.

Inhaled insulin products provide for a different route of administration; they are rapidly absorbed and there fore classified as short acting (U.S. Department of Health and Human Services, Food and Drug Administration 2006).

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C.7.1.3 Guidelines on Pharmacodynamic and Pharmacokinetic Evaluation

Following the Note for Guidance on Clinical Investigation of Medicinal Products in the Treatment of Diabetes Mellitus (EMA guideline 2010), due to the wide intra and intersubject variability in the response to insulin in type 1 diabetes, pharmacodynamic data are of primary importance to demonstrate therapeutic equivalence or differences between insulin preparations, including their use in mixtures. Similar guidance is given by the Center for Drug Evaluation and Research (CDER) at FDA (FDA guideline 2008). More recently, the scope of the guidance has been broadened to cover clinical investigation of cardiovascular risk (U.S. Department of Health and Human Services, Food and Drug Administration 2008) and to similar medicinal products containing recombinant human soluble insulin (EMEA/CHMP/BMWP/32775/2005).

For the evaluation of a new insulin preparation, the guideline requests the comparator drug to be an insulin product with a pharmacological profile similar to the product under investigation.

It is commonly considered mandatory to demonstrate equipotency of insulin analogues to human insulin (Becker et al. 2003; Scholtz et al. 2003) or to define potency ratios reflected in insulin units specific to the product (EMEA/CHMP/BWP/124446/2005; U.S. Department of Health and Human Services, Food and Drug Administration 2000b). Historically, insulin preparations were characterized by the potency relative to the activity contained in 1/22 mg of an international standard preparation (One mole of the most recent WHO *human* insulin standard, which has a potency of 26,000 $IU.g^{-1}$, corresponds to 166.8×10^6 $IU.mol^{-1}$, or 1 IU equals 6.00 nmol (Vølund 1993 Nov). One International Unit (IU) was equivalent to 0.04167 mg of the 4th International Standard of 1959 (a mixture containing 52% beef insulin and 48% pork insulin plus some water and salts). The 3rd International Standard of 1952 comprised of bovine insulin had a potency of 24,500 $IU.g^{-1}$ which defined an IU as equivalent to 0.04082 mg. The 2nd International Standard of 1935 had a potency of 22,000 $IU.g^{-1}$ which defined an IU as equivalent to 1/22 mg or 0.0455 mg. The 1st International Standard of 1925 defined one Unit equals 0.125 mg (Lacey 1967; Sinding 2002)). Today, insulin preparations are defined by actual human insulin content, that is, 1 International Unit *is* 0.0347 mg or 6.00 nmol of pure human insulin (Bristow et al. 2006; Vølund 1993). Accordingly, all human insulin and insulin analogue preparations contain 6 $nmol \cdot U^{-1}$ of the respective insulin molecule in U100 preparations, apart from Levemir (insulin detemir), which contains 24 $nmol \cdot U^{-1}$ to aim for equipotency (U.S. Department of Health and Human Services, Food and Drug Administration 2005).

Comprehensive data should be provided on the pharmacodynamic effect, based on the area under the glucose infusion rate (GIR) time curves, and on the insulin bioavailability, based on peak insulin concentration and on the area under the insulin concentration time curves. For long acting insulin preparations endowed with little to no fluctuation in serum concentration and activity, times to percentages of total exposure or effect may be more appropriate [Sect. C.7.3.2.2, Long acting Insulin Preparations; Becker et al. 2008].

For pharmacokinetics, the guideline (EMA guideline 2010) states that apart from studies in healthy subjects, studies should be performed in type 1 and in type 2 diabetic patients and in children (Danne et al. 2005) and in various conditions with respect to factors associated with variability in pharmacokinetics (PK): insulin dose (Becker et al. 2007), site of injection (Becker et al. 2003; Becker and Frick 2008), and thickness in fat layer contribute (Becker et al. 2005b) to the rather considerable variation in PK parameters seen with insulin even in the same individual over time. Moreover, these issues should be addressed in clinical trials. Age, ethnicity (Picchini et al. 2006), and conditions such as impaired renal (Becker and Frick 2008; Holmes et al. 2005) or liver (Holmes et al. 2005) function may also contribute to PK variability, particularly with long acting preparations (U.S. Department of Health and Human Services, Food and Drug Administration 2000b), that is, with increased systemic residence time.

It is desirable to have steady state PK data (multiple dose concentration time profiles), particularly with long acting insulin preparations (Heise et al. 2002; Heise and Pieber 2007).

Particular interest should be evinced in showing that pharmacokinetic characteristics remain unchanged if an insulin product is used in mixtures. Also, considering actual use, fresh mixtures should be tested versus mixtures made several hours prior to administration (Fogt et al. 1978).

Given that insulin compounds can be analyzed specifically in the presence of endogenous human insulin, pharmacokinetic studies can be performed without glucodynamic assessments, provided development of hypoglycemia is blunted, for example, by meals. If an assay specific to the insulin compound is not available, studies in subjects with type 1 diabetes are the choice to account for endogenous human insulin contributions (Rave et al. 2006). This finding is particularly relevant to long acting preparations with sustained low rate absorption (Lepore et al. 2000).

In order to generate a time concentration profile for an insulin product, a sufficient number of samples need to be taken and adequate methods for determination to be applied, of which immunoassays (radioimmunoassay (RIA), enzyme linked immunosorbent assay (ELISA), immunoenzymometric assay (IEMA), immunochemilu minescent assay (ICMA), immunoelectrochemilumi nometric assay (IECMA)), specific for the analyte, human insulin, or insulin analogue, and high pressure liquid chromatography with tandem mass spectrometry (HPLC MS MS) are the most appropriate (Sapin 2003). Combined use of cross reactive immunoassays with assays specific for human insulin may help to analyze insulin analogue concentrations in the presence of human insulin (Anderson et al. 2000).

C.7.2 The Hyperinsulinemic Euglycemic Clamp

Created to assess insulin sensitivity (Ferrannini and Mari 1998; Vølund 1993), the hyperinsulinemic euglycemic clamp technique measures the amount of glucose neces sary to maintain euglycemia upon an exogenous increase in insulin. It is the method of choice to characterize novel insulin preparations by determining their time action profiles based on the GIR (pharmacodynamics) and their exogenous insulin serum concentration profiles (pharma cokinetics) in both nondiabetic and diseased subjects (DeFronzo et al. 1979; Heinemann and Anderson 2004; Heinemann 2004; Roden 2007).

At basal insulin secretion (about 1.4 $pmol \cdot kg^{-1} \cdot min^{-1}$ (Polonsky et al. 1988a; Polonsky et al. 1988b)) and normoinsulinemia (about 30 60 $pmol \cdot L^{-1}$ or 5 10 $\mu \cdot mL^{-1}$), baseline endogenous glucose production and disposition are in equilibrium (about 10 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ (Horvath et al. 2008; Zierler 1999)). Exogenous hyperinsulinemia in the fasting state causes the body to dispatch more glucose than delivered by endoge nous glucose production and hence requires compensatory infusion of glucose to keep blood glucose concentration constant. If blood glucose concentrations close to fasting levels of nondiabetic subjects (about 4.0 5.5 $mmol \cdot L^{-1}$) are maintained, that is, clamped, the term hyperinsulinemic euglycemic clamp is used.

The ability of insulin to induce disposition of blood glucose is quite large, and while large doses of exogenous insulin can be administered, the actual disposable amount of fasting blood glucose is small, about 22 28 mmol (4 5 g) at 4.4 $mmol \cdot L^{-1}$ (80 $mg \cdot dL^{-1}$) concentration and a blood volume of 5 6 L. At steady state in nondiabetic subjects, about 40 $\mu mol \cdot min^{-1} \cdot kg^{-1}$ glucose is disposed at insulin concentrations of 360 $pmol \cdot L^{-1}$ (60 $\mu U \cdot mL^{-1}$), represen tative of postprandial elevations (Becker et al. 2003). Thus, an 80 kg person disposes 3.2 $mmol \cdot min^{-1}$ or the entire blood glucose within less than 10 min.

Well balanced blood glucose equilibrium is manda tory for life, in particular for cerebral functions (Gaohua and Kimura 2009). Therefore, changing the glucose equi librium with an exogenous insulin load causes immediate counterregulatory responses to reestablish the equilib rium. Rapid disposition of glucose with resulting hypo glycemia provokes glucagon release, triggering hepatic glycogenolysis and glucose release. This process increases the amount of glucose disposed, leading to an underesti mation of the genuine glucose disposing effect. Keeping blood glucose concentration constant at euglycemic concentrations avoids this effect.

Thus, by identifying the amount and kinetics of glucose required to maintain euglycemia upon an exoge nous insulin challenge, the hyperinsulinemic euglycemic clamp is a highly valuable technique to characterize the metabolic activity profiles of insulin products.

The hyperinsulinemic euglycemic glucose clamp may be combined with tracer technologies to assess effects on hepatic gluconeogenesis (Horvath et al. 2008) and is also subject to mathematical modeling (Picchini et al. 2006).

C.7.2.1 Characterization of Prandial Versus Basal Insulin Products

Effect profiles of prandial insulin products, that is, short or rapid acting insulin products, aim to mimic glucose dispo sition after a meal. Using the hyperinsulinemic euglycemic clamp technique, the net glucodynamic effect profile is characterized by the exogenous glucose required in time for each unit of injected insulin to keep the blood glucose concentration constant, or clamped, at a predefined level.

Effect profiles of basal insulin products, that is, inter mediate and long acting insulin products, aim to mimic the glucose disposition between meals. Basal insulin prod ucts dosed to produce hyperinsulinemia also require glucose infusion and can thus be characterized in the same manner as short or rapid acting insulin products.

However, in subjects without endogenous insulin, that is, subjects with type 1 diabetes, basal insulins are dosed to match endogenous glucose formation and thus to provide for balanced insulinemia without the need for glucose infusion. Characterization of basal insulin products should therefore extend beyond euglycemic clamp settings and assess the duration of blood glucose control within

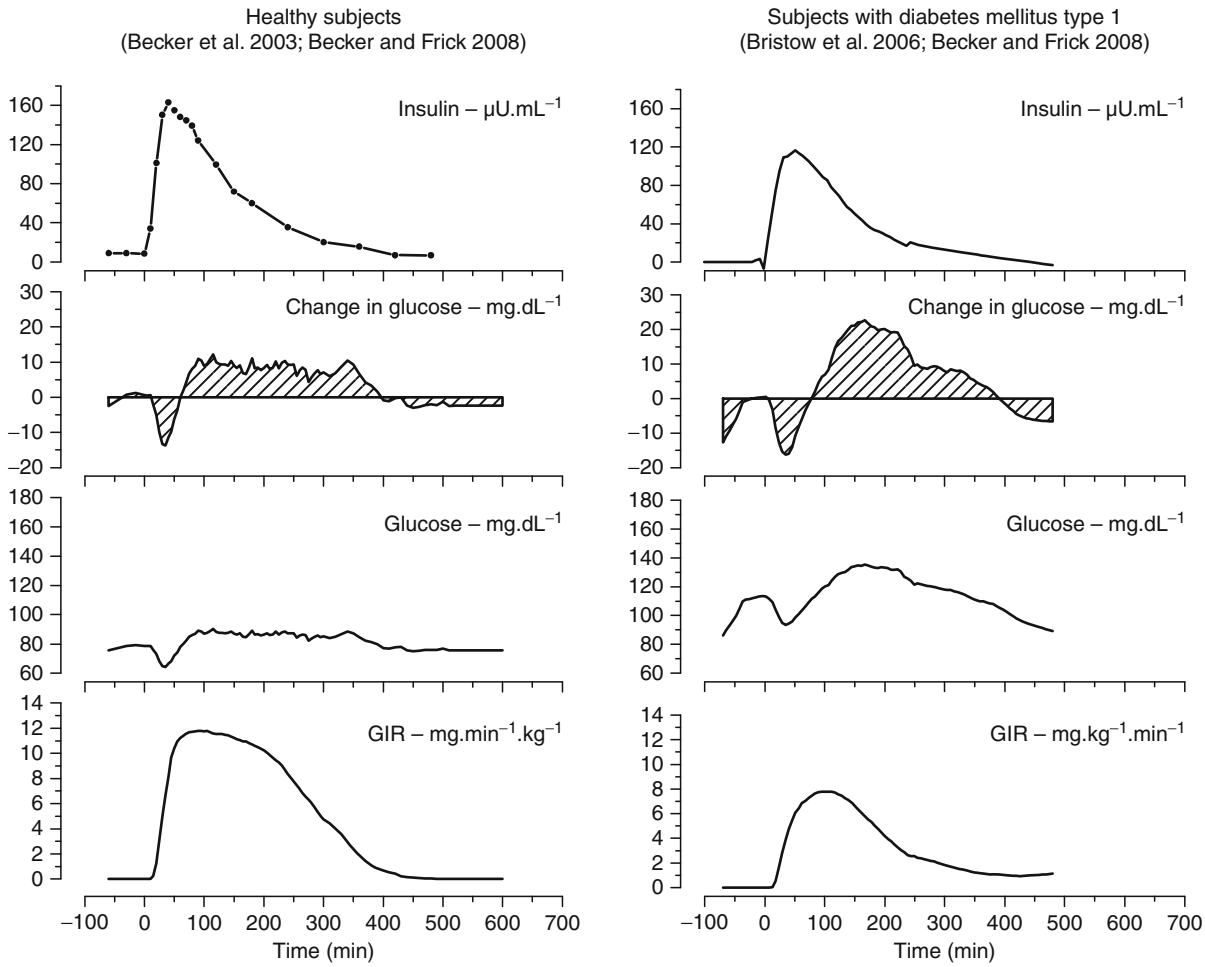
predefined acceptance limits, for example, below 7.5 mmol·L^{-1} without provoking hypoglycemia.

Thus, dose response relationships established with hyperinsulinemic euglycemic clamps provide useful information for prandial insulin products but are of limited use for basal insulin products (Becker 2008; Heise and Pieber 2007; Swinnen et al. 2008).

For any insulin product, the corresponding insulin concentrations are described as observed, as requested by regulatory bodies, although predicted or modeled values are also appropriate.

C.7.2.2 Automated Versus Manual Techniques

While some hyperinsulinemic euglycemic clamp protocols rely on manual infusion with 3 5 min interval blood glucose readings and adjustments, state of the art techniques employ an automated reading and adjusting system with more frequent (minute by minute) feedback to keep the blood glucose concentration constant (Biostator® instrument, Life Sciences instruments, Elkhart, IN, USA; Glucose Controlled Insulin Infusion System GCIIS,



Insulin concentration, change in glucose concentration, glucose concentration, and glucose infusion rate in healthy subjects following sc administration of 0.3 U/kg insulin glulisine

Insulin concentration, change in glucose concentration, glucose concentration, and glucose infusion rate in subjects with diabetes mellitus type 1 following sc administration of 0.2 U/kg insulin glulisine

Figure C.7-1
Short-acting Manual

MTB Medizintechnik, Ulm, Germany; more appropri ately also named Glucose Controlled Dextrose Infusion System) (Fogt et al. 1978; Heinemann 2004).

Compared to automated glucose clamping using the Biostator, the manual method is thus associated with greater fluctuation in blood glucose concentrations and greater lag time between onset of effect, drop in blood glucose, and adjustment of the GIR (› *Fig. C.7 1*). Con sequently, manual adjustments are less sensitive to changes in insulin concentrations and result in fewer changes in GIR at the expense of tightness of blood glucose control, while the tight control achieved with the Biostator minute by minute reading occurs at the expense of stability of the GIR. This difference is espe cially relevant when low doses of short acting products or long acting products with subtle onset of effect are tested.

Therefore, visualization of the glucodynamic effect profile obtained with the Biostator affords more robust smoothing of the recorded GIR; however, it does not affect quantification of total glucose disposition (› Sect. C.7.3.4, Statistical Analysis; › *Fig. C.7 2*).

C.7.2.3 Examples of Glucose Clamp Profiles

To illustrate the use of the hyperinsulinemic euglycemic clamp technique in the course of drug development and the subtle differences between characterization in healthy subjects versus subjects with diabetes mellitus type 1 and in employing the manual (› *Figs. C.7 1* and › *C.7 3*; › *Tables C.7 1* and › *C.7 2*) versus automated technique (› *Figs. C.7 4* and › *C.7 5*; › *Tables C.7 3* and › *C.7 4*), examples are given below for all variations. As available, a rapid , short acting human insulin analogue product is compared to a reference short acting human insulin prod uct and a long acting human insulin product is compared to the intermediate acting NPH insulin.

C.7.3 Study Set-Up

Any clinical study design has to follow the principles of the Good Clinical Practice guidelines of the European Union and the Declaration of Helsinki, and has to be reviewed and approved by the local Ethics Committee. Subjects are

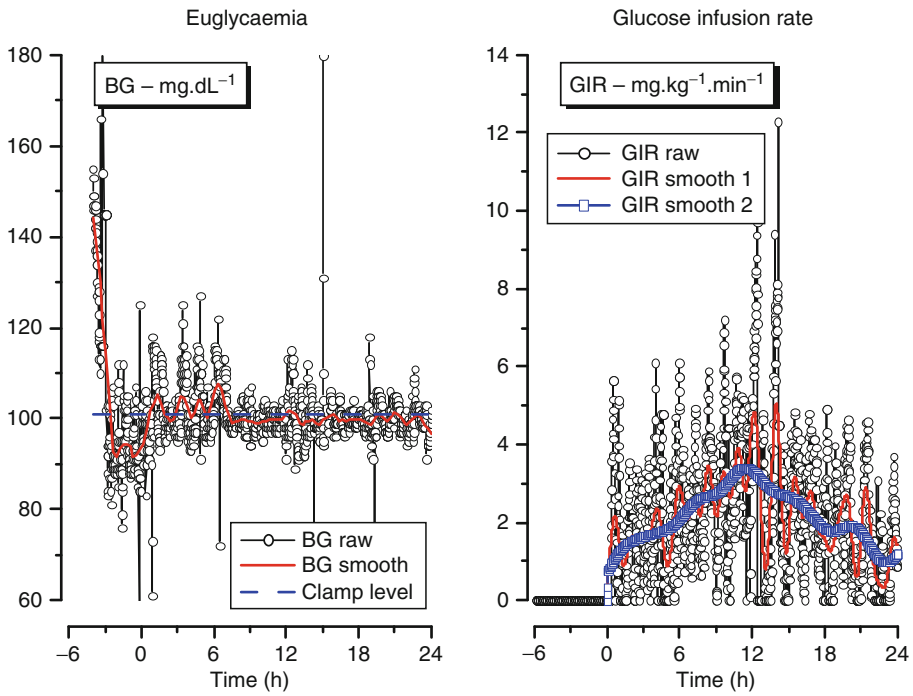
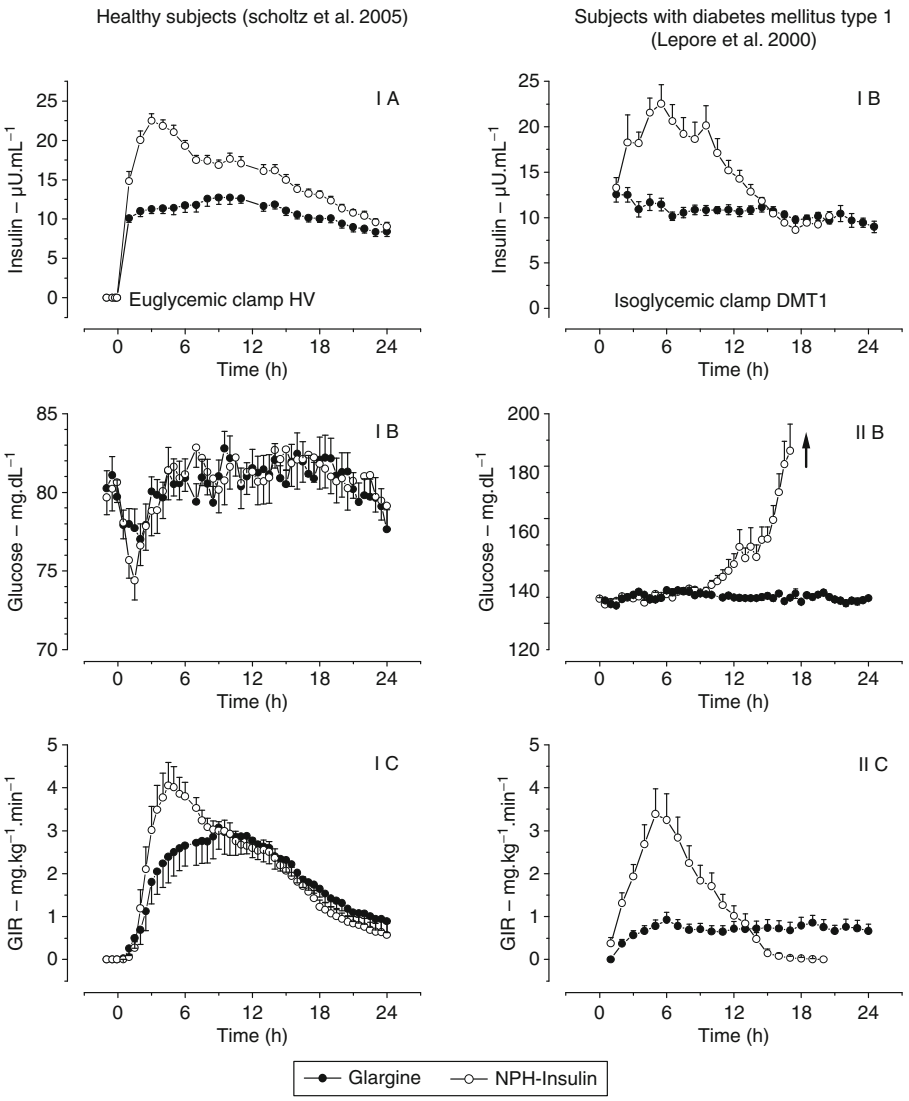


Figure C.7-2

Long-acting insulin product Biostator®. Blood glucose (BG), BG raw (blood glucose raw data), BG smooth (blood glucose smoothed data) Glucose infuions rate (GIR), GIR raw (GIR raw data), GIR smooth 1 (GIR week smooth factor), GIR smooth 2 (GIR strong smooth factor)



Insulin concentration [I A], glucose concentration [I B], and glucose infusion rate [I C] in healthy subjects following sc administration of 0.4 U/kg insulin glargine

Insulin concentration [II A], glucose concentration [II B], and glucose infusion rate [II C] in subjects with diabetes mellitus type 1 following sc administration of 0.3 U/kg insulin glargine

Figure C.7-3
Basal/Long-acting Manual

to be provided with written information about the study and a consent form with which to give written informed consent prior to the start of the study.

A standard euglycemic clamp trial follows a single center, randomized, two way crossover design. Trial periods comprise a screening visit, two glucose clamp visits with reference and test insulin, and a follow up visit.

While two way crossover studies are sufficient to compare two products for their pharmacodynamic and pharmacokinetic profiles, replicate designs serve to add information on intra subject variability and enhance the test power (U.S. Department of Health and Human Services, Food and Drug Administration 2001; Chow and Liu 2000; Chow and Shao 2002).

Table C.7-1

Pharmacokinetic and pharmacodynamic data short-acting insulin Manual subjects with diabetes mellitus type 1

Dose 0.2 U.kg^{-1}	GLU[a]	RHI[a]	GLU/RHI[b]
INS AUC_{0-2h} (μU·min·mL^{-1})	10,625	5,412	178 (136.7; 232.4)
INS AUC_{total} (μU·min·mL^{-1})	16,120	16,610	97 (80.3; 118.1)
INS C_{max} (μU·mL^{-1})	120	57	192 (145; 254)[c]
INS t_{max} (min)	51[d]	82[d]	−33 (−49, −18)[d]
MRT (min)	96	185	55 (45; 68)[c]
GIR AUC_{0-2h} (mg·kg^{-1})	625	348	145 (104.6; 186.0)
GIR AUC_{total} (mg·kg^{-1})	1,547	1,473	96 (73; 119)[e]
GIR_{max} (mg.min^{-1}·kg^{-1})	8.2	5.9	125 (98; 151)[e]
GIR $t_{20\%}$ (min)	81[f]	112[f]	−30 (−45; −18)[d]
GIR $t_{80\%}$ (min)	218[f]	306[f]	−88 (−123; −48)[d]
GIR t_{max} (min)	98[f]	161[f]	−63 (−120; −17)[d]
GIR t_{early} (min)	34[f]	43[f]	−10 (−18, −3)[d]

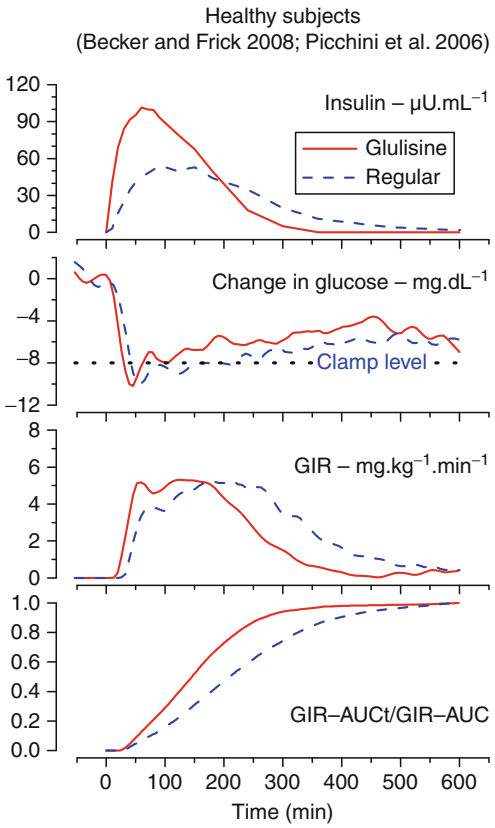
[a]Values are expressed as geometric means unless specified otherwise
[b]Point estimate% (95% CI)
[c]Based on log transformed data
[d]Median differences from nonparametric data analysis
[e]Based on untransformed data
[f]Median
AUC area under the blood/serum concentration−time curve, *GIR* glucose infusion rate, *GIR AUC0 2h* glucose disposal at 2 h, *GIR* AUC_{total} total glucose disposal, GIR_{max} maximum glucose infusion rate, *GIR* t_{early} time to early half maximum GIR, *GIR* t_x% time to x% of total glucose disposal, *GIR* t_{max} time to GIR_{max}, *INS AUC0 2h* insulin exposure at 2 h, *INS* AUC_{total} total insulin exposure, *INS* C_{max} maximum insulin concentration, *INS* t_{max} time to INS C_{max}

Table C.7-2

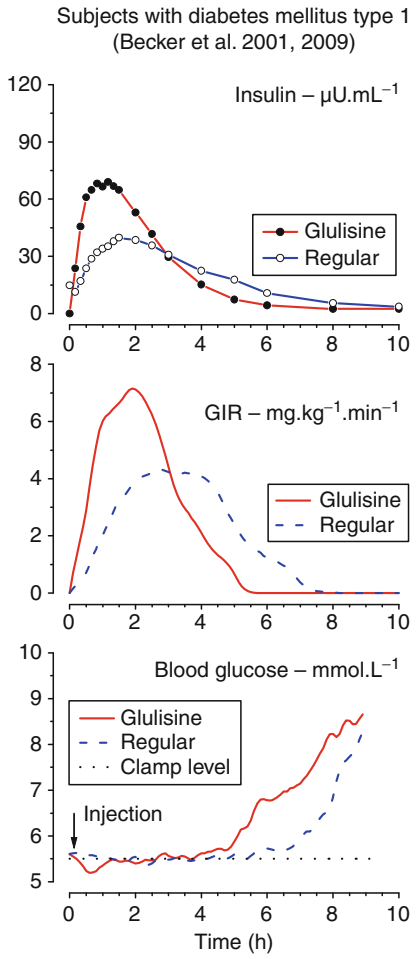
Pharmacokinetic and pharmacodynamic data short-acting insulin Manual subjects with diabetes mellitus type 2

Dose 0.2 U.kg^{-1}	GLU[a]	RHI[a]	GLU/RHI[b]
INS C_{max} (μU·mL^{-1})	92	46	216 (177; 264)[d]
INS t_{max} (min)[c]	83	92	−5 (−28; 20)
INS AUC_{0-2h} (μU·min·mL^{-1})	7,661	4,221	202 (160; 256)[d]
INS AUC_{total} (μU·min·mL^{-1})	18,408	19,731	101 (77; 131)[d]
GIR $t_{20\%}$ (min)[c]	121	194	−64 (−103; −37)[d]
GIR $t_{80\%}$ (min)[c]	350	435	−72 (−133; −26)[d]
GIR AUC_{total} (mg·kg^{-1})[e]	906	943	120 (−370; 609)

[a]Values are expressed as geometric means unless specified otherwise
[b]Point estimate% (95% CI)
[c]Median
[d]$p < 0.05$ vs RHI
[e]Arithmetic mean
*AUC*area under the blood/serum concentration−time curve, *GIR* glucose infusion rate, *GIR* AUC_{total}total glucose disposal, *GIR* $t_{x\%}$time to x% of total glucose disposal, *INS* $AUC_{0\ 2h}$insulin exposure at 2 h, *INS* AUC_{total}total insulin exposure, *INS* C_{max}maximum insulin concentration, *INS* t_{max} time to INS C_{max}



Insulin concentration, change in glucose concentration, glucose concentration, and glucose infusion rate in healthy subjects following sc administration of 0.15 U/kg insulin glulisine and regular human insulin



Insulin concentration, glucose concentration, and glucose infusion rate in subjects with diabetes mellitus type 1 following sc administration of 0.15 U/kg insulin glulisine and regular human insulin

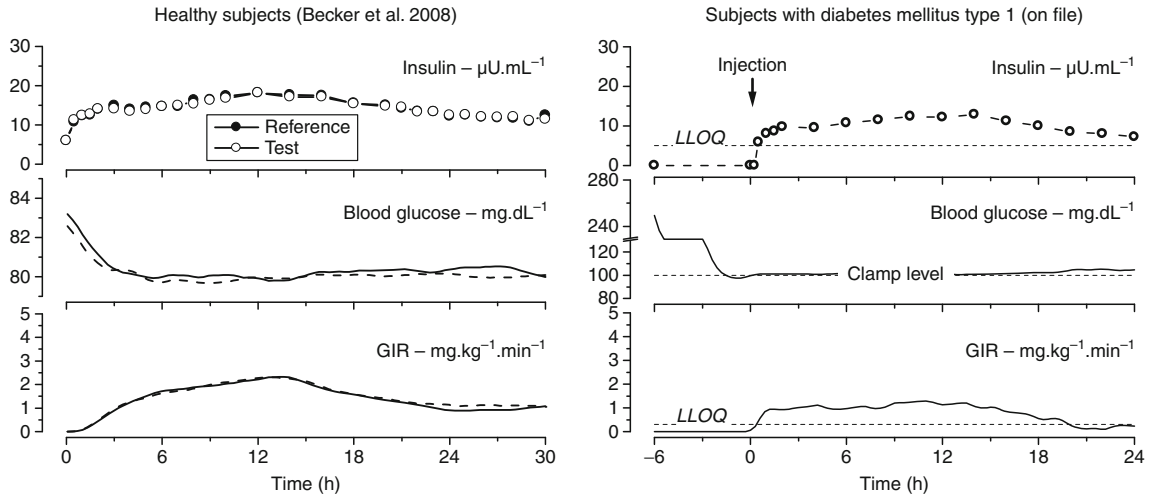
Figure C.7-4
Short-acting Biostator®

C.7.3.1 Study Population and Baseline Characteristics

To cover varieties in insulin sensitivity, participants may be subjects with diabetes mellitus type 1, subjects with diabetes mellitus type 2, or healthy subjects with and without obesity (American Diabetic Association (ADA) 1998; Becker et al. 2003; Becker et al. 2005b; Becker et al. 2007; Becker et al. 2009; Kapitza et al. 2004; Picchini et al. 2006).

No strenuous physical activity is to be allowed within 2 days before each study medication administration. Subjects who smoke less than five cigarettes a day may be included in the study and subjects may smoke during the study, except on trial days.

The subjects are not to take any non trial medication, which will interfere with the metabolic control or the insulin sensitivity of subjects throughout the study and in the 2 weeks before the study. Other non study medications (e.g., β blocker or ACE inhibitor) necessary to treat concurrent diseases are permitted. Consumption of alcoholic beverages, grapefruit juice, and stimulating beverages containing xanthine derivatives (tea, coffee, Coca



Insulin concentration, glucose concentration, and glucose infusion rate in healthy subjects following sc administration of 0.4 U/kg insulin glargine

Insulin concentration, glucose concentration, and glucose infusion rate in subjects with diabetes mellitus type 1 following sc administration of 0.4 U/kg insulin glargine

Figure C.7-5
Long-acting Biostator®

Cola like drinks, chocolate) are not permitted from 24 h before administration of each study medication until the end of the clamp. Water supply should be at least 1,500 mL for each 24 h period.

C.7.3.1.1 Subjects with Diabetes Mellitus Type 1

Subjects with non progressed type 1 diabetes (e.g., with out progressive diabetic retinopathy) who meet the inclu sion criteria are eligible for the study (Becker et al. 2007; Becker et al. 2009). Basically, participants are to be of age between 18 and 50 years with a body mass index (BMI) below 30 $kg \cdot m^{-2}$, and with glycosylated hemoglobin HbA_{1c} levels at screening of below 9.0% at a stable insulin regimen with less than 1 $U \cdot kg^{-1} \cdot day^{-1}$ for at least 2 months.

Subjects have their last usual insulin treatment with long acting insulin at the latest at 12:00 noon on Day 2 of each clamp setting. The last sc injection of NPH or any other intermediate insulin should be no later than 12:00 noon on Day 1. Thereafter, the blood glucose levels are controlled solely by multiple subcutaneous (sc) injections of the usual short acting insulin prescribed by the subject's treating physician (e.g., family practitioner).

The last subcutaneous injection of short acting insulin should occur no later than 03:00 the night before study medication administration on the first study day.

Subjects on pump therapy may remain on their basal infusion rate without bolus injections from 03:00 the night before study medication administration.

C.7.3.1.2 Subjects with Diabetes Mellitus Type 2

Studies on short acting insulin products in subjects with diabetes mellitus type 2 (Kapitza et al. 2004) are feasible and should follow the same scheme as for subjects with diabetes mellitus type 1. However, the results show a broad range of patterns given that obesity results in high vari ability in insulin sensitivity and bioavailability ((Becker and Frick 2008; Rave et al. 2004b); *Table C.7 3*).

C.7.3.1.3 Healthy Subjects

Nondiabetic subjects who meet the inclusion criteria are suitable to participate in the study (Becker et al. 2003; Picchini et al. 2006; Rave et al. 2005a; Scholtz et al. 2003; Scholtz et al. 2005). In general, male subjects aged between 18 and 50 years with a BMI between 18 and 27 $kg \cdot m^{-2}$ and with glycosylated hemoglobin HbA_{1c} levels at screening of below 6.0%, normal glucose tolerance test, and no clini cally relevant findings in their medical history and phys ical examination are eligible for hyperinsulinemic euglycemic clamp studies.

Table C.7-3

Pharmacokinetic and pharmacodynamic data short-acting insulin Biostator subjects with diabetes mellitus type 1 (Becker et al. 2009)

Dose 0.15 U.kg^{-1}	Insulin glulisine	Regular human insulin	Insulin glulisine/regular human insulin PE (95% CI)
Concentration parameters			
INS C_{max} (μU·mL^{-1})[a]	73	40	1.9 (1.7; 2.1)[b]
INS AUC_{0-2h} (μU·min·mL^{-1})[a]	6,832	3,630	1.9 (1.7; 2.2)[b]
INS AUC_{total} (μU·min·mL^{-1})[a]	11,284	10,932	1.1 (0.9; 1.2)
INS T_{max} (min)[c]	57	104	−48 (−64; −36)
INS $T_{10\%}$ (min)[c]	31	53	−20 (−27; −15)
INS $T_{90\%}$ (min)[c]	205	348	−141 (−186; −101)
Effect parameters			
GIR_{max} (mg·kg^{-1}·min^{-1})[a]	6.4	4.6	1.4 (1.2; 1.6)
GIR AUC_{0-2h} (mg·kg^{-1})[a]	491	219	2.2 (1.7; 3.1)[b]
GIR AUC_{total} (mg·kg^{-1})[a]	1,090	1,076	1.0 (0.9; 1.2)
GIR T_{max} (min)[c]	114	169	−86 (−118; −51)
GIR $T_{10\%}$ (min)[c]	45	88	−39 (−63; −25)
GIR $T_{90\%}$ (min)[c]	238	330	−115 (−157; −70)
Time parameters			
BG T_{EU} (min)	316[d]	410[d]	100 (149; 50)[e]
BG T_{130} (min)	356[d]	465[d]	103 (148; 58)[e]
BG T_{150} (min)	415[d]	505[d]	91 (138; 44)[e]
BG T_{180} (min)	510[d]	573[d]	77 (145; 10)[e]

[a]Data are arithmetic means or ratios of least square means
[b]Significantly different to regular human insulin ($p < 0.05$)
[c]Data are medians or differences of medians
[d]Data are medians: *BG* T_{EU} time of euglycemia/clamp level, BG T_{130}, BG T_{150} and BG T_{180}, time to 7.2, 8.3, and 10.0 mmol.L^{-1} (130, 150, and 180 mg.dL^{-1}). Censored data (i.e., affected by the end of the observation period [maximum 600 min]) are italicized
[e]Data are point estimates for treatment differences with 95% CI estimated by the LIFEREG procedure (SAS)
GIR$_{max}$ maximum glucose infusion rate, *GIR* $AUC_{0\ 2h}$ and *GIR* AUC_{total} area under the glucose infusion rate (GIR) time curve between 0 and 2 h; and to the end of clamp; *GIR* $T_{10\%}$ and *GIR* $T_{90\%}$ time to the maximum, to 10% and to 90% of GIR AUC_{total}, *INS* AUC_{total} and *INS* $AUC_{0\ 2h}$ area under the serum insulin concentration curve at the end of the study and after 2 h, *INS* C_{max} maximum insulin concentration; INS T_{max}, *INS* $T_{10\%}$ and *INS* $T_{90\%}$ time to the maximum; to 10% and 90% of INS AUC_{total}

Nondiabetic subjects with a BMI of above 30 kg·m^{-2} may be invited for studies in more insulin resistant populations (Becker et al. 2005b; Heise et al. 2007).

C.7.3.2 Study Procedures

C.7.3.2.1 Short-Acting Insulin Preparations

Subjects with Diabetes Mellitus Type 1

Automated Adjustments For testing of short acting insulin products, subjects should be admitted to the research unit on the evening prior to the trial days and to receive dinner.

The subjects are randomized and then prepared with three in dwelling venous lines and connected to the auto matic glucose reading device, the Biostator (Becker et al. 2009). To this end, a dorsal hand vein or lateral wrist vein of the left arm is cannulated (preferred in retrograde fashion) in order to continuously draw arterialized venous blood for the determination of blood glucose concentra tion. The left hand is placed into a heated box ("Hot Box"), which provides for an air temperature of about 55°C, allowing arterialization of venous blood. A second venous line is placed into the antecubital vein of the left arm and is used to collect samples for serum insulin and reference blood glucose determination. A third vein is cannulated on the contralateral forearm allowing the

Table C.7-4

Pharmacokinetic and pharmacodynamic data long-acting insulin Biostator healthy subjects point estimates and confidence bound for treatment ratios (Becker et al. 2008)

Variable	Test	Reference	ABE, point estimate (90% CI)	IBE, upper CI[a]	PBE, upper CI[a]
Geometric mean					
INS AUC_{0-24h} ($\mu U \cdot h \cdot mL^{-1}$)	343	355	96.9 (91.0, 102.6)[b]	0.0021	−0.0276[c]
INS AUC_{0-30h} ($\mu U \cdot h \cdot mL^{-1}$)	414	425	97.3 (92.1, 102.9)[b]	−0.0032[c]	−0.0304[c]
INS C_{max} ($\mu U \cdot mL^{-1}$)	20	22	89.6 (83.5, 96.1)[b]	−0.0109[c]	−0.0501[c]
INS T_{max} (h)	14.4[d]	12.5[d]	0.5 (−2.5, 3.5)		
Arithmetic mean					
GIR AUC_{0-24h} ($mg \cdot kg^{-1}$)	2,373	2,367	100.1 (88.1, 113.8)[b]	−0.2414[c]	−0.2036[c]
GIR AUC_{0-30h} ($mg \cdot kg^{-1}$)	2,796	2,743	101.9 (90.6, 114.7)[b]	−0.2241[c]	−0.1743[c]
GIR_{max} ($mg \cdot min^{-1} \cdot kg^{-1}$)	3	3	95.6 (83.3, 109.7)[b]	−0.0749[c]	−0.2124[c]
GIR T_{max} (h)	12.8[d]	12.5[d]	−0.2 (−2.5, 1.4)		

[a]Confidence interval of reference scaled criterion

[b]Within the limits (80 125%) establishing bioequivalence

[c]Negative value, establishing bioequivalence

[d]Median

ABE average bioequivalence, *AUC*, area under the curve; *CI* confidence interval, *GIR* glucose infusion rate, GIR_{max} maximum GIR, *GIR* T_{max} time to GIR_{max}, *IBE* individual bioequivalence, *INS* serum insulin glargine concentration, *INS* C_{max} maximum INS, *PBE* population bioequivalence, T_{max} time to INS C_{max}

infusion of a 0.9% saline and 20% glucose solution with a pump in the Biostator or regular human insulin (or any other appropriate insulin) with an external pump. The subjects will remain in semi recumbent position for the entire duration of the sampling period.

From insertion of the vascular catheters until 60 min before study medication administration, that is, for about 4 h, the blood glucose level is maintained within 4.4 6.6 $mmol \cdot L^{-1}$ (80 120 $mg \cdot dL^{-1}$). Insulin solution has to be infused by means of a high precision infusion pump (e.g., Terumo Spritzenpumpe TE 311); 20% glucose solution is also given by a high precision infusion pump (e.g., Terumo Infusionspumpe TE 171). Depending on the blood glucose level, additional intravenous bolus injec tions of insulin are given to keep the blood glucose within the target range. In the 3 h before study medication administration and until clamp end, no intravenous bolus injections are given. Insulin infusion rates are adjusted individually. While keeping blood glucose at the target level, both insulin and GIR should be minimized during the clamp run in phase.

Arterialized venous blood is to be continuously drawn at a rate of 2 $mL \cdot h^{-1}$ for determination of arterial blood glucose concentration every minute from 4 h prior to medication to end of clamp. Additional arterialized venous blood samples (0.02 mL) for concurrent Biostator calibration, which is a technical requirement, are collected at 30 min intervals after connection to the Biostator until end of clamp, and once 5 min prior to dosing. These samples are checked against a laboratory reference based on the glucose oxidase method.

The clamp level is adjusted 60 min before study medication administration to maintain the blood glucose at about 5.5 $mmol \cdot L^{-1}$ (100 $mg \cdot dL^{-1}$) until the end of the clamp period. Study medication administration may be postponed for up to 2 h in case the target glucose level has not been met after 4 h. If the target glucose level is not established after 6 h, the visit is terminated and the subject may be scheduled for a new dosing visit 1 7 days later.

The insulin infusion is discontinued immediately before study medication administration. The first serum insulin sample is taken immediately thereafter. At about noon, a physician administers the study medication, at a periumbilical site, 5 cm lateral to the umbilicus, within 1 min, according to the randomization plan, using a stan dardized skin fold technique and insulin syringes (Microfine IV syringe, Becton Dickinson, Heidelberg, Germany). Alternatively, pen injectors (e.g., SOLOstar, sanofi aventis) may be used. The study medication should preferably be administered by the same physician at each study day.

The end of the injection defines time zero, the starting time of the subsequent clamp period. Every clamp observation period will last a maximum of 10 h for short acting products or 24 and 30 h for long acting products. The clamp experiment is complete when the blood glucose values registered every minute are equal to or greater than the predefined level, for example, 10 or 11 $mmol \cdot L^{-1}$ (180 or 200 $mg \cdot dL^{-1}$), for 30 min after cessation of glucose infusion and the investigator confirms that any possible errors leading to false blood glucose levels above have been excluded, or after the predefined end of observation period, for example, 10, 24, or 30 h, whichever comes first.

Injection site are to be assessed 15 min as well as 1 h after injection of the study medication.

Samples for serum insulin are to be taken as requested by pharmacokinetics. After the clamp, a meal ad libitum is served and the usual insulin treatment resumed.

Manual Adjustments The preparation of participants follows the same scheme as for automated readings with the exception that, after manual adjustment of the blood glucose concentration to 5.5 $mmol \cdot L^{-1}$ (100 $mg \cdot dL^{-1}$), euglycemia is maintained by an algorithm based manual infusion of 20% glucose solution until the end of clamp. Blood glucose and GIRs are recorded throughout the glucose clamp periods every 3 5 min by the study operator, for example, study nurse or investigator (Becker and Frick 2008; Bristow et al. 2006).

Healthy Subjects and Subjects with Diabetes Mellitus Type 2

Manual and Automated Adjustments The preparation of nondiabetic participants follows the same scheme as for automated or manual readings with early morning admission and preparation of intravenous lines and thus without overnight adjustment of blood glucose concentration. The preparation period prior to study medication administration with 60 min maintenance of fasting blood glucose concentration may be confined to 2 h. Subjects are clamped at 5% or 10% below their fasting blood glucose concentration, which is essential to avoid release of confounding endogenous insulin (Becker et al. 2003; Becker and Frick 2008; Picchini et al. 2006; Rave et al. 2005a; Scholtz et al. 2003; Scholtz et al. 2005).

C.7.3.2.2 Long-Acting Insulin Preparations

Characterization of long acting insulin products requires a somewhat different preparation of subjects as compared to characterization of short acting insulin products (Becker 2007; Becker et al. 2008; Becker 2008; Heise and Pieber 2007; Swinnen et al. 2008). Strong increases in insulin concentration with rapid acting products or other exogenous insulin supply serve to digest a meal and hence can be measured in any subject without or with a little interference from endogenous insulin, the release of which is suppressed by exogenous insulin. Long acting products are to provide for the basal insulin supply sufficient to dispose hepatic (95%) and renal (5%) formation of endogenous glucose (DeVries et al. 2007). Thus, mere doubling, modest elevation or sole reestablishing of physiological basal insulin concentration is the goal of long acting insulin products. Therefore, in order to avoid confounding by endogenous insulin, subjects with diabetes mellitus type 1 are preferred for characterization. The average therapeutic basal insulin product dose is slightly below 0.4 $U \cdot kg^{-1}$, which renders this dose most appropriate for a euglycemic clamp experiment. It creates a mild glucose demand, which can be pursued. For this, subjects with diabetes mellitus type 1 may be clamped at a predefined euglycemic level (e.g., about 5.5 $mmol \cdot L^{-1}$), while nondiabetic subjects are to be clamped at 5% below fasting level (e.g., about 4.4 $mmol \cdot L^{-1}$) to yield a reliable assessment.

For testing of long acting insulin products, subjects are to be admitted early in the morning of the study day in order to avoid two overnight stays.

Studies on long acting insulin preparations in subjects with diabetes mellitus type 2 are feasible; yet, results show an even broader range of patterns than studies with short acting products due to high variability in low insulin sensitivity.

Beyond characterization of pharmacokinetics and pharmacodynamics of long acting insulin products in healthy subjects or subjects with diabetes mellitus type 1, objectives of such a study may be to assess fluctuation and reproducibility of exposure and effect (Heise et al. 2004). Fluctuation is to be calculated from exposure and effect profiles as described in Statistical Analysis (Sect. C.7.3.4; Becker et al. 2008; Fogt et al. 1978).

C.7.3.2.3 Safety Assessments

Assessment of the treatment effect on body systems not targeted by the product under investigation is an essential part of any drug testing. Blood samples for laboratory tests should be taken under fasted conditions (10 h overnight fast).

Routine laboratory tests including hematology, clinical chemistry, and urinalysis are performed in addition to

physical examination, 12 lead electrocardiography (ECG), and monitoring of vital signs.

Subjects return for an end of study (EOS) visit within a week after the last administration of the investigational product. The EOS should include the following investigations:

- Physical examination (including weight, body temperature) with updated medical history
- ECG, vital signs measurements
- Laboratory tests with hematology, biochemistry, urinalysis, and if female a beta human chorionic gonadotropin (β HCG) blood test and follicle stimulating hormone (FSH)
- Any adverse event (AE) occurring or concomitant medication taken since last treatment

Local Tolerability at Injection Site

The evaluation of injection site reactions is standardized. Findings (such as erythema, edema, papules, induration, vesicles, blisters) are graded according to a Global Irritation Score. A local injection site reaction with a score of $\geq$2 according to the rating scale is documented as an AE. Also, the subjects are asked to report sensations at the injection site.

Adverse Events

Adverse events (AEs) are reported by the subject or noted by the investigator.

Detailed Safety Assessments

In detail, investigations include physical examination at screening (past and current smoking status, cardiovascular system, chest and lungs, thyroid, abdomen, nervous system, skin and mucosae, and musculoskeletal system) and relevant medical and surgical history, diabetes history (diagnosis of diabetes, onset of insulin treatment, late complications), and at pre dose and during the study (cardiovascular system, abdomen, and lungs); only findings relevant to the study are to be documented.

C.7.3.3 Pharmacokinetic and Pharmacodynamic Assessments

C.7.3.3.1 Insulin Concentrations

Serum insulin concentrations (INS) of test and reference insulin are to be measured using appropriate immunoassay methods, with or without correction for endogenous insulin, or direct reading by HPLC MS MS (Sapin 2003).

Areas under the curve (AUCs) of serum insulin concentrations (INS) are calculated by the trapezoidal rule. The AUCs of INS time profiles are characterized between 0 and t hours (INS $AUC_{0\text{-}th}$) and to the end of the glucose clamp (INS AUC_{total}). Times to 10% and 90% of INS AUC_{total} (INS $T_{10\%}$ and INS $T_{90\%}$) are derived from the ratios of AUCs per time point (AUC_{0-t}/AUC_{total}). Maximum INS (INS C_{max}) and corresponding time to INS C_{max} (INS T_{max}) values can be derived from predicted data; however, observed data are preferred by regulatory bodies (*Tables C.7 1* and *C.7 2*).

C.7.3.3.2 Glucose Infusion Rates

Areas under the curve of GIRs (GIR $AUC_{0\text{-}th}$ and GIR AUC_{total}) are calculated as the sum of rectangles (i.e., stepwise constant function). Times to 10% and 90% of GIR AUC_{total} (GIR $T_{10\%}$ and GIR $T_{90\%}$) are derived from the ratios of AUCs per time point (AUC_{0t}/AUC_{total}), while maximum GIR (GIR_{max}) and time to GIR_{max} (GIR T_{max}) are obtained from curves smoothed, for example, with a weighted regression technique (e.g., procedure locally weighted scatterplot smoothing (LOESS), factor 0.15, SAS version 8.2, SAS Institute Inc., Cary, NC, USA). Time to end of euglycamic clamp level (BG T_{EU}) and time to first occurrence of spontaneous rise in blood glucose concentrations $\geq$7.2, $\geq$8.3, and 10.0 $mmol \cdot L^{-1}$; BG $T_{7.2}$, BG $T_{8.3}$, and BG $T_{10.0}$ (or $\geq$130, $\geq$150, and $\geq$ 180 $mg \cdot dL^{-1}$; BG T_{130}, BG T_{150}, and BG T_{180}) during the glucose clamps are derived from blood glucose readings confined to the duration of the clamp (e.g., 10, 24, or 30 h) and thus are right censored (*Tables C.7 1* and *C.7 2*).

C.7.3.3.3 Long-Acting Products

Blood samples for determination of insulin concentrations (INS) are drawn immediately prior to insulin administration (minute 0, baseline), at 15 min and at 30 min intervals for the first 2 h thereafter. Samples are then taken every 2 h for the final glucose clamp period.

GIR and blood glucose concentration are registered continuously for 24 h in subjects with diabetes mellitus type 1 after insulin administration, or beyond (e.g., 30 h) in subjects with diabetes mellitus type 2 or healthy subjects.

As INS C_{max} and so INS T_{max} can be ill defined with basal insulin products, time to 50% of INS AUC_{0-24h} or INS AUC_{0-30h} may be more appropriate to define the exposure profile.

Similarly, as GIR_{max} and so GIR T_{max} values are derived from smoothed curves and thus are subject to

the smoothing algorithm, they may be grossly biased for long acting products with flat effect profiles or not to be established at all, which asks for time to 50% of GIR $AUC_{0–24h}$ or GIR $AUC_{0–30h}$ to better define the effect profile.

As given for short acting insulins, time to end of euglycemic clamp level (BG T_{EU}) and to first occurrence of spontaneous rise in blood glucose concentrations, BG are derived from blood glucose readings confined to the duration of the clamp and thus are right censored (*Tables C.7 3* and *C.7 4*).

C.7.3.4 Statistical Analyses

C.7.3.4.1 AUC and C_{max}

Analyses of variance (ANOVA) models, allowing the estimation of least square (LS) means with corresponding 95% confidence limits, are applied on untransformed GIR AUC and GIR_{max} and on natural logarithm transformed INS AUC and INS C_{max} data. For INS data, the 95% confidence intervals (CIs) for the differences between LS means are calculated and retransformed to derive the respective confidence limits for the mean ratios of the pair wise treatment comparisons. For GIR data, the 95% confidence limits for the mean ratios of the pair wise treatment comparisons are calculated according to Fieller's theorem.

C.7.3.4.2 T_{max}, times to $T_{x\%}$

Time parameters based on INS or GIR (INS $T_{10\%}$, INS $T_{90\%}$, GIR $T_{10\%}$, GIR $T_{90\%}$, INS T_{max}, GIR T_{max}) are subjected to nonparametric analysis for pair wise comparisons between treatments. The 95% nonparametric confidence intervals for the respective median difference in treatment are calculated using ranks, based on the method of *Steinijans* and *Diletti* (Steinijans and Diletti 1983).

Estimation of treatment differences in BG derived time parameters (BG T_{EU}, BG T_{130}, BG T_{150}, BG T_{180}, and related differences), based on observations that are right censored by the duration of the clamp (10 h), are analyzed by parametric failure time modeling (procedure LIFEREG) (Becker et al. 2008). Estimations are not given for parameters with right censored data.

C.7.3.4.3 Dose Proportionality

In case of dose proportionality assessments, insulin exposure and metabolic response can be individually investigated for strict monotonic increases with dose. Dose proportionality is assessed by pair wise dose comparisons of the least squares means for: INS $AUC_{0\text{-}xh}$, INS AUC_{total}, INS C_{max}, GIR $AUC_{0\text{-}xh}$, GIR AUC_{total}, and GIR_{max}, applying bioequivalence (BE) criteria for dose normalized results, with half the "investigational" dose as the reference dose (BE; 80 125%). Dose proportionality within the BE criteria is confirmed when the 95% confidence interval for a treatment ratio is within 1.6 2.5 (Becker et al. 2009).

Alternative approaches to assess dose proportionality are discussed by Smith et al. (2000).

C.7.3.4.4 Bioequivalence

Bioequivalence approaches are to be used to compare measures of exposure and effect between formulations, and within subject variability of these measures of either formulation is to be taken for reproducibility within formulation (EMEA/CPMP Note for Guidance 2002). Bioequivalence is to be assessed for the average of observed measures (average bioequivalence, ABE) (Haidar et al. 2008a).

Criteria of population bioequivalence (PBE), which controls both, deviations in averages and in total variability of the measures, are to be assessed to attest *prescribability* (to initiate treatment) of the test formulation. Individual *switchability* (to switch treatments) between formulations is to be verified based on the concept of individual bioequivalence (IBE), which simultaneously demands equivalence between formulations in averages of measures, and in within subject variabilities and of subject by formulation interaction. Derived time parameters are to be compared non parametrically.

Insulin formulations (e.g., reference R and test T) are assessed for ABE in insulin exposure (ABE in INS $AUC_{0–24h}$, INS C_{max}) and effect (ABE in GIR $AUC_{0–24h}$, GIR C_{max}) as well as for IBE and PBE. Analysis of variance (ANOVA) of natural log (ln) transformed INS AUC_{time} and INS C_{max} is used to test for ABE of R and T. Geometric means are calculated for pharmacokinetic parameters. Anti log point estimates and 90% confidence intervals (CIs) for the difference in means (T R) are calculated. To test for ABE in effect, untransformed GIR AUC_{time} and GIR_{max} values are

Table C.7-5

Within-subject variability (coefficient of variation) for pharmacokinetic and pharmacodynamic data long-acting insulin Biostator healthy subjects (Becker et al. 2008)

Variable	Estimate for combined R+T (95% CI)	Test (CV%)	Reference (CV%)	Estimated ratio (90% CI)
INS AUC_{0-24h} ($\mu U \cdot h \cdot mL^{-1}$)	19 (14, 25)[a]	21	14	150 (120, 216)
INS AUC_{0-30h} ($\mu U \cdot h \cdot mL^{-1}$1)	18 (13, 22)[a]	19	13	146 (117, 213)
INS C_{max} ($\mu U \cdot mL^{-1}$)	24 (17, 30)[a]	25	23	109 (101, 130)
INS T_{max} (h)	54 (38, 69)[b]			
GIR AUC_{0-24h} ($mg \cdot kg^{-1}$)	34 (24, 43)[b]	32	36	89 (76, 96)
GIR AUC_{0-30h} ($mg \cdot kg^{-1}$)	32 (23, 41)[b]	30	34	88 (75, 96)
GIR_{max} ($mg \cdot min^{-1} \cdot kg^{-1}$)	36 (26, 46)[b]	42	30	140 (121, 173)
GIR T_{max} (h)	41 (29, 53)[b]			

[a]Natural log transformed data

[b]Raw data

AUC area under the curve, *CI* confidence interval, *CV* coefficient of variation, *GIR* glucose infusion rate, GIR_{max} maximum GIR, *GIR* T_{max} time to GIR_{max}, *INS* serum insulin glargine concentration, *INS* C_{max} maximum INS, *R* = reference, *T* = test, T_{max} time to INS C_{max}

Table C.7-6

Average concentration within clamps, and absolute and relative fluctuation around average concentration within clamps long-acting insulin Biostator healthy subjects (Becker et al. 2008)

	C_{AVG}($\mu U \cdot mL^{-1}$)	*F*($\mu U \cdot mL^{-1}$)	PF (%)
All clamps	14.6 (13.7 15.5)	2.9 (2.6 3.3)	19.9 (18.5 21.4)
Test	14.5 (13.1 16.0,)	2.8 (2.4 3.3)	19.2 (17.3 21.2)
Reference	14.7 (13.5 15.8)	3.1 (2.6 3.5)	20.7 (18.5 22.8)

Data are means with 95% confidence intervals

C_{AVG} average concentration; *F* absolute fluctuation; *PF* percentage of the average concentration

subjected to an ANOVA with subject, treatment, and period as the main effects. Point estimates and 90% CIs for the ratio T/R (means) are calculated. ABE is inferred if the 90% CIs are within the equivalence limits of 80% and 125% of the T/R ratio.

IBE and PBE analyses, scaled to the corresponding reference variability, are calculated using *methods of moments* as discussed by the Food and Drug Administration Individual/Population Bioequivalence Working Group and described in their guidance (U.S. Department of Health and Human Services, Food and Drug Administration 2001). IBE and PBE are inferred if both the geometric means of the parameters are within the limits of 80 125% and the 95% upper confidence bound for the reference scaled criterion does not exceed 0 ((Chow and Liu 2000); *Tables C.7 3* and *C.7 4*).

C.7.3.4.5 Within-Subject Variability

To provide an assessment of within subject variability, intra individual coefficients of variation (CV) are calculated from the mean sum of the error terms as calculated by ANOVA. The statistical F distribution is used to calculate 95% CIs on the ratio of the two within subject variance components (T/R) ((Haidar et al. 2008b; Karalis et al. 2008); *Table C.7 5*).

Table C.7-7

Absolute and relative fluctuation around average glucose infusion rate long-acting insulin Biostator healthy subjects (Becker et al. 2008)

	GIR_{AVG} (mg·kg^{-1}·min^{-1})	*F* (mg·kg^{-1}·min^{-1})		PF (%)	
	Smoothed/Raw	Smoothed	Raw	Smoothed	Raw
All clamps	1.54 (1.40, 1.68)	0.68 (0.61, 0.76)	0.95 (0.88, 1.02)	45.4 (42.2, 47.9)	67.1 (63.5, 70.8)
Test	1.55 (1.34, 1.77)	0.65 (0.59, 0.76)	0.93 (0.83, 1.03)	43.4 (39.1, 47.7)	64.8 (59.5, 70.0)
Reference	1.52 (1.33, 1.72)	0.72 (0.60, 0.83)	0.99 (0.89, 1.09)	46.7 (42.9, 50.5)	69.5 (64.4, 74.6)

Data are given as mean with 95% confidence intervals
F absolute fluctuation; GIR_{AVG} average glucose infusion rate; *PF* percentage of the average concentration

C.7.3.4.6 Fluctuation

To provide an assessment of the nature of the time concentration profile, the fluctuation ($F_{0\text{-th}}$) around the average INS (C_{AVG}) is calculated. $F_{0\text{-th}}$ is defined as the ratio of the cumulative area of the absolute deviation from the average concentration over t hours (Δ_{abs} INS $AUC_{0\text{-th}}$) to the duration of *t* hours, and is also expressed as a percentage of the average concentration ($PF_{0\text{-th}} = 100 \cdot F_{0\text{-th}} \cdot C_{AVG}{}^{-1}$) (Becker et al. 2008; Fogt et al. 1978).

Fluctuations in time action (GIR) profiles may be determined on raw GIR data as outlined for INS, as well as on, for example, LOESS smoothed data, as the shape of the GIR profile and hence the deviation (Δ_{abs} GIR $AUC_{0\text{-th}}$) from the average GIR (GIR_{AVG}) is subject to selection of mathematical smoothing algorithms. Standardized fluctuation ($PF_{0\text{-th}}$) is analyzed by ANOVA on raw data with treatment, subject, and sequence as main effects. Point estimates and 95% CIs are calculated. A stem and leaf plot is applied to analyze the distribution of fluctuations and to classify outliers, defined as greater than 2.7 times the standard deviation (SD) from the mean.

It is understood that GIR fluctuation parameters are useful for sustained euglycemic clamps only; clamps with less tight blood glucose concentrations, which may occur when insulin dose just matches endogenous glucose production or and where no extra glucose is needed, present with zero glucose infusion rate (*Tables C.7 6* and *C.7 7*).

C.7.4 Guidelines on Diabetic Disease Management

Patients with diabetes mellitus type 1 require intensive insulin substitution for life, which serves to control blood glucose under daily life activities where patients have to cope with meals of different content and duration (Danemann 2006; Diabetes Control and Complication Trial/Epidemiology of Diabetes Interventions and Complications Research Group 2002). Basal bolus insulin regimens are today's standard care to achieve near normal glycemia and HbA1c targets for prevention and delaying the onset of micro (retinopathy, nephropathy, neuropathy) and macrovascular (cardiovascular, cerebrovascular, peripheral vascular) complications.

For patients with diabetes mellitus type 2, fasting blood glucose concentrations <110 mg/dL and 2 hr post prandial blood glucose concentrations <135 mg/dL at $\leq$ 6,5% (IDF guideline (International Diabetes Federation (IDF). Clinical Guidelines Task Force. Global guideline for type 2 diabetes. Brussels: International Diabetes Federation 2005); best) or ≤ 7.0 (ADA guideline (Nathan et al. 2006); sufficient) glycosylated hemoglobin (HbA1c) are ambitious goals defined by diabetes associations in order to prevent micro and macrovascular complications, severe hypo and hyperglycemic events and to maintain quality of life (American Diabetes Association (ADA) 2008; Diabetes Control and Complication Trial/Epidemiology of Diabetes Interventions and Complications Research Group 2002; Holman et al. 2008). If not well controlled under metformine and lifestyle modifications, initiation of a preferred basal insulin treatment (basal supported oral therapy) followed by intensification (basal plus and eventually basal bolus) is a recommended option (Nathan et al. 2006).

C.7.5 Epilog

The beginning of the clinical characterization of insulin preparations was the collection of reports assessing the

potency of pancreatic extracts of animal origin in humans. The creation of an international standard allowed better comparison of insulin products for decades despite the unknown nature of the active principle.

Today, after elaborate investigations into the molecular structure of insulin and the advent of bioengineering, a variety of rapid , short , intermediate , or long lasting and premixed insulin products containing human insulin or human insulin analogues are available to choose from for an individualized insulin therapy regimen. Insulin products are now well defined by content and are pharmacologically characterized with the hyperinsulinemic euglycemic clamp technique, which has become the standard assessment method recommended by regulatory bodies.

C.7.6 Alternative Routes of Insulin Delivery

Despite the development of patient friendly pen injectors that allow easy and painless subcutaneous injections, there is the aspiration by many patients and prescribers for alternative routes of administrations. Apart from continuous subcutaneous infusion and inhaled insulin, which failed on acceptance, no further viable approach has been introduced into general medical practice (Owens et al. 2003).

There are comprehensive reviews on alternative deliveries for insulin (Heinemann and Jacques 2009; Henkin 2010; Khafagyel et al. 2007; Peppas and Kavimandan 2006).

C.7.6.1 History

A historical review on the attempts to treat diabetes with pancreas extracts reveals quite a number of experiments prior to report of the Toronto group (Leickert 1975). After the publication about pancreatic islet cells by Langerhans in 1869 and the fluke finding by Minkowski, working in 1889 with von Mehring, that pancreatectomy produces diabetes mellitus in dogs, and the hypothesis that a key normal function of the pancreas is to mediate glucose use of the body, and the failure of this function is the cause of diabetes, many interested in research set out to discover this pancreatic principle (Von Mering and Minkowski 1889; Von Mering and Minkowski 1890). Intravenous injections of pancreatic extracts of various animal origins into dogs and some experiments in humans testified the general possibility to reduce hyperglycemia and glucosuria (Ionescu Tirgoviste 1996; Leickert 1975).

In 1922, Banting assisted by Best, independently confirmed these findings in dogs and upon use of bovine pancreatic extracts by intravenous route in rabbits showed consequences of immediate action, namely brisk reduction in glucose concentration up to hypoglycemic seizures (Best 1972; Bliss 1993; Bliss 2005; Rosenfeld 2002). Rectal administration in rabbits was tried without success and hence they switched to subcutaneous injections of more purified pancreatic extracts allowing the hypoglycemic activity to develop. It was the result of determined, in the end directed interactions in the Toronto unit, which eventually established purified bovine pancreatic preparations for subcutaneous use (Jurdjevic and Tillman 2004; Liebl et al. 2009; Staub 1924).

However, not knowing the nature of the pancreatic principle, oral administration to human was also tried to alleviate the discomfort of many injections, and as is known without success (Joslin et al. 1922).

C.7.6.2 Inhaled Insulin

C.7.6.2.1 Inhalation

The availability of crystalline pancreas extract enabled investigators to seek alternative routes for administration. The very first mentioning of inhalation of insulin addresses compliance as issue with several subcutaneous or even intravenous injections a day (Laqueur and Grevenstuk 1924). The authors also mention that they have tried oral, rectal, vaginal, cutaneous, perlingual routes with little to no success in rabbits. Nevertheless, in a later publication they did not completely disregard percutaneous and nasal administrations and mention perlingual administrations to be quite common (de Jongh et al. 1925).

Tracheal installation in rabbits was tried for future inhalation of insulin solutions and investigators did not find a general difference between subcutaneous and tracheal administration and emphasized a more rapid though weaker effect as compared to sc injection, which is why they increased the insulin load. Eventually, they confirmed the hypothesis that inhalation as delivery route is feasible. These studies were jointly done by Grevenbrueck and Lacquer in their laboratory in Amsterdam and by Heubner in Göttingen (Heubner et al. 1924).

Thus, these early experiments already uncovered the pharmacokinetic and pharmacodynamic issues to overcome in the following years, inhaled insulin takes effect more rapidly while dosing needed to be much greater. Later this year, first experiments in patients confirmed these findings (de Jongh et al. 1925; Grevenstuk et al. 1925;

Heubner 1925; Laqueur and Grevenstuk 1924; Robitschek 1925). The authors concluded, the 30 fold greater amount of insulin needed compared to subcutaneous insulin administration precludes active inhalation treatment. Independent from the Amsterdam Göttingen group and apparently at the same time, Gännslein described the effects of nebulized insulin in diabetic patients (Gännslein 1925). He reports the use of many inhalations over the day to be more physiologic and also achieved higher availabil ity as compared to the Amsterdam Göttingen group.

The following years have seen many attempts to improve dosing and to reduce the amount to deliver. Basically, many dispersion devices were constructed and applied. Although, all authors considered inhalation as a feasible approach they denied becoming this useful as therapeutic alternative to sc injection. Most of this information was published in German, Italian, or French journals and needed to be rediscovered (Patton and Byron 2007).

A most enlightening description of these very facts was given by O. Oe (Kyoto 1938), who considered tenfold amounts needed, rapid onset, recovery within 5 6 h as not useful (Oe 1938). Decades later, studies with to be commercialized delivery devices from industrial manufac turers (Pfizer/Nektar, Ely Lilly, NovoNordisk) did confirm exactly his findings. However, in contrast to Oe's conclu sion this time it was considered a viable approach (Patton and Byron 2007).

C.7.6.2.2 Inhaled Insulin

After decades of inattentiveness, two developments revoked the idea of insulin inhalation, the epidemic growth of type 2 diabetes treated with insulin and the possibility gained with bioengineering to produce any wanted quan tity of pure human insulin. In addition, medical standards in the largest and leading market, the USA, for many years saw vial and syringes preferred to pens and initiation of injections perceived with pain or even fear. Therefore, inhalation of insulin became the focus of intense industrial efforts and investigations as it was known to be the only realistic alternative route to injectable insulin (Patton and Byron 2007). All major insulin providers developed insu lin formulations and devices for inhalation in alliances with device manufacturers. Pfizer took the lead with Inhaled (now Nektar) and asked Hoechst (renamed Hoechst Marion Roussel (HMR), than Aventis) initially to partner as insulin supplier than to join in for co development for inhaled insulin. Pfizer succeeded and was the first manufacturer to be rewarded with approval for market authorization of an inhaled insulin product (Exubera) (White et al. 2005) in both the USA and Europe in 2006 (U.S. Department of Health and Human Services, Food and Drug Administration 2006b).

NOVO entered into an alliance with AERx liquid inhaled insulin system from Aradigm and Lilly with Alkermes (Garg and Kelly 2009; Siekmeier and Scheuch 2008; Skyler 2007).

However, when ailing market penetration prompted Pfizer, which eventually after the acquisition of Aventis by Sanofi synthelabo, had gained exclusive control over Exubera, the human insulin production plant (Diabel) for this the product included, to withdraw as they "faced the combination of breaking through the barrier of con ventional insulin therapy and the burden of the product on the medical practices, and this innovation was not accepted," both Lilly and Novo followed suit and aban doned seeking regulatory approval. In reality, it was a mix of too bulky, too complex, more expensive products, requiring repeated lung function tests, and the wrongly perceived injection fear. This was summarized by a competitor as "Fast acting inhaled insulin in the form it is known today is unlikely to offer significant clinical or convenience benefits over injections of modern insulin with pen devices." Nevertheless, it was for all who partic ipated in the development a huge scientific challenge mastered with pride, and experience galore gained.

It is worth noting, that inhaled insulin was not taken off the market for safety reasons.

In 2010, solely the Technosphere approach employing a different carrier, N^{ϵ} (Fumaroyl) diketopiperazine of L Lys, FDKP; MannKind, and fostered by a sole entrepre neur is still being persued (Kaur et al. 2008; Renard et al. 2010). Technosphere claims delivery of monomeric insulin entrapped in FDKP, which is questioned as inha lation of monomeric insulin lispro displays similar char acteristics as regular human insulin (Pfützner et al. 2002; Rave et al. 2008; Rave et al. 2009; Steiner et al. 2002).

Methods

There are detailed reviews on inhaled insulin (Harper et al. 2007; Patton and Byron 2007; Siekmeier and Scheuch 2008; Weers et al. 2007). The lung provides for a large surface for alveolar absorption, in the order of 100 m^2; it is well perfused with thin diffusion barriers and low concen trations of degrading enzymes and little mucous in these final branches of the airway. Upon deep inhalation, the aerodynamic diameter determines the deposition of parti cles in the airways. The appropriate size for alveolar depo sition is 1 3 μm, while particles of smaller diameter (< 1 μm) remain suspended in air and larger particles (>5 μm) are caught in mucous of upper airways and

removed by the mucociliary escalator. Other determinants are inherent to the physicochemical nature of the molecules, the breathing maneuvers, and alterations in lung permeability due to disease, for example, chronic obstructive pulmonary disease (COPD), smoking (Becker et al. 2006).

In order to offer sufficient quantities for absorption, the required dose can be delivered with one or several puffs. Powdered insulin carries larger amounts per puff than any liquid formulation. For example, 3.56 mg pure human insulin is 100 IU. Formulated as mannitol embraced spray dried micro beads and 60% fill, 5 mg inhalation powder delivers about 100 IU, of which <10% is systemically available. At an about 70% absolute bioavailability of subcutaneous insulin, the relative bioavailability of inhaled insulin is about 10%, or about 7% absolute. The same amount delivered from a solution of 100 U/mL requires dispersion of 1,000 μL per puff, which is technically demanding. Higher concentrated insulin solutions and more frequent puffs per administration are the consequences.

Inhalation of insulin needs to be highly reproducible in order to match the requirements for a narrow therapeutic margin. The complexity of aerosol aerodynamics and device engineering are extreme challenges almost exclusively to improve convenience vis a vis established subcutaneous administration, which fulfills all medical needs (Hirsch 2007). While Pfizer/HMR (later Aventis) and Lilly favored dry powder insulin, aqueous solutions (Novo, Aerogen) or suspensions in volatile liquids (KOS/Abbott) were used as well.

As for any insulin product, first studies in man used the euglycemic clamp technique to characterize the pharmacokinetics and pharmacodynamics of inhaled insulin in comparison to subcutaneous insulin (Rave et al. 2005b; Rave et al. 2008; Rave et al. 2009). Inhaled insulin is a fast and short acting insulin product for prandial substitution, and therefore results are little confounded by endogenous insulin. Exposure and effect profile of inhaled insulin combines the rapid onset of fast acting insulin analogue products with the duration of action of short acting human insulin products. Exposure is proportional to dose, while glucose lowering activity increases less than proportional, like for subcutaneous insulin. Characterization can be in healthy subjects or subjects with diabetes mellitus type 1 or type 2 (see Sect. C.7.2), as appropriate. In addition, contrary to subcutaneous insulin products, inhaled insulin may be dosed per mg or in in house defined units.

Contrary to subcutaneous insulin, inhaled insulin shows greater bioavailability with some acceleration in absorption and onset in activity in subjects with greater pulmonary permeability such as smokers (Becker et al. 2006; Himmelmann et al. 2003; Pan et al. 2008). Sizable and clinically meaningful changes in absorption with smoking occur within days. In particular, resuming smoking leads to unintentional overdosing which is why patients who wish to use inhaled insulin need to abstain from smoking. Such studies require institutionalization of subjects in order to control smoking habits.

Studies in patients follow the same objectives as for subcutaneous insulin.

C.7.6.2.3 Antibody Formation

Antibody formation, which was substantial prior to purification of animal insulins, decreased with the use of human insulin, regained interest with inhalation and more so with intraperitoneal use. However, the low affinity binding found to be associated with inhalation was without clinical consequences (Fineberg et al. 2007a; Fineberg et al. 2007b).

C.7.6.2.4 Pulmonary Function

Pulmonary function tests were a key element of inhaled insulin development and treatment. Inhalation caused an initial, reversible small decline in pulmonary function presumably related to irritating powder inhalation but without morphological substrate. As such, inhalation was well tolerated and saved injections (Rosenstock et al. 2009; Skyler et al. 2007).

C.7.6.3 Oral, Buccal, Sublingual, Nasal, and Dermal Insulin Delivery

Inconvenience of early short acting insulin preparations with many injections prompted the search for retardation of insulin absorption and action. Protamin and zinc and eventually both were added to yield NPH insulin and lente insulins (Heller et al. 2007; Rinke et al. 1983). An alternative was surfen insulin, an acidic insulin solution precipitating upon subcutaneous injection, which afforded only twice daily injections (Umber et al. 1938).

Rhythmically delivered insulin into the portal vein by an enteral entry is supposed to be the ideal research is striving for (see intraperitoneal insulin). Oral insulins would first pass the hepatic circulation, reducing hepatic glucose production, and hence be more physiologic than subcutaneous injection, which raises peripheral insulin concentration beyond needs. However, enteral delivery

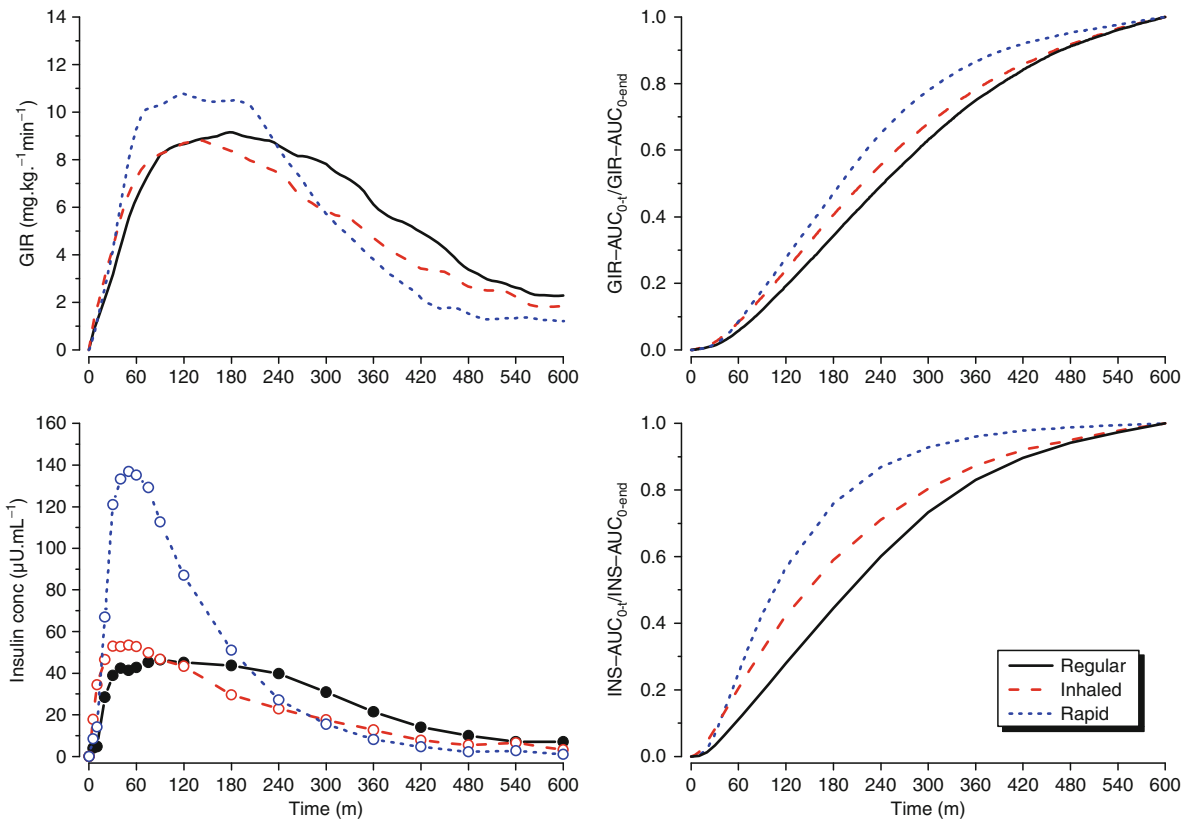


Figure C.7-6

Inhaled insulin (Exubera). *Left upper panel*: Glucose infusion rate profiles for subcutaneously injected regular human insulin and rapid-acting insulin analogue product compared to inhaled human insulin. *Left lower panel*: Serum insulin concentrations. *Right upper panel*: Ratio of actual (at time *t*) to total glucose infusion rate and *right lower panel*: Ratio of actual (at time *t*) to total insulin exposure (Rave et al. 2005a)

of insulin suffers from enzymatic degradation and imper meability of membranes due to high molecular weight and low lipophilicity. Enhancing absorption by facilitating agents also enhances entry of any protein with possible adverse consequences (Aungst et al. 1988). Irrespective of oral availability, there remains the quest for a reproducible and timely absorption. Insulin has to be present with about 60 fold variation in concentration when needed at and between meals. Oral, buccal, and sublingual insulin would not be an achievement on its own; it requires predictable pharmacokinetics in order to be of value (Heinemann and Jacques 2009).

C.7.6.3.1 Oral Insulin

Berger in 1992 describes the history of oral insulin as one of continuous ambition and failure, and as of today nothing has changed to the contrary (Berger M. Oral insulin 1922; Khafagyel et al. 2007). There are attempts with absorption enhancers, enzyme inhibitors, carrier sys tems, and polymer coupled insulins, changing the mucosa permeability or creating new chemical entities (Iyer et al. 2010; Logtenberg et al. 2009). Either approach is associ ated with no or poor availability, and if any, of great variation.

C.7.6.3.2 Buccal Insulin

The buccal and sublingual mucosa is reported to be acces sible for absorption of insulin products, which is facili tated by additives. An example is the RapidMist (Oral Lynn, Oralin) system, which deploys large sized droplets to the mucosa of the mouth and throat (Bernstein 2008; Modi et al. 2002; Morishita et al. 2001; Pozzilli et al. 2010).

Per hub 10 IU are delivered of which 10% are reported to be absorbed. This requires 20 hubs per administration as compared to 1 injection. Human insulin is put into a liquid formulation of GRAS (*g*enerally *r*egarded *a*s *s*afe) ingredients and delivered via a spray device.

C.7.6.3.3 Nasal Insulin

Nasal administration of insulin with among others lecithin or glycochylate based enhancers was successfully applied to nondiabetic subjects and patients with type 2 diabetes (Bruce et al. 1991; Coates et al. 1995; DeVries et al. 2002; Holman RR. Intranasal insulin in type I diabetes. In: Frontiers in Insulin Pharmacology. Berger M, Gries FA (eds) et al. 1993; Kimmerle et al. 1991; Nolte et al. 1990). Availability was low but defined, appearance in blood was quick and short lasting and, so, required repeated admin istrations to cover the meal. Long term studies verified the possibility to replace short acting injected insulin, although advantages to subcutaneous injections are not apparent (Frauman et al. 1987; Hilsted et al. 1995; Salzman et al. 1985). Intranasal delivery allows insulin easier access to the brain, which is subject of investigations.

C.7.6.3.4 Dermal Insulin

Attempts have been made to drive insulin through the skin without penetration applying ointments, liposomes, and iontophoresis (Langkjaer et al. 1993). The results are disappointing as to bioavailability and confirm the skin as natural barrier against the environment (Cecv 1993).

C.7.6.4 Intraperitoneal Insulin

Intraperitoneal administration is an approach to better mimic portal delivery, which is closest to physiology, and which combined with self controlled blood glucose read ing and closed loop algorithms would create the desired artificial pancreas (El Khatib et al. 2010; Klonoff 2003). However, it requires surgery for implantation, interference with intermittent complications and refill. High concen trated insulin, which is required as the reservoir, has to serve for up to 45 days, being delivered with correspond ingly low volumes.

Intraperitoneal administration was introduced prior to the availability of insulin analogues. Currently continuous intraperitoneal infusion is reserved for difficult to control hyperglycemia for any reason (including patients with neurotic disorders) and predominantly confined to certain centers (France, The Netherlands) (DeVries et al. 2002; Liebl et al. 2009; Logtenberg et al. 2009; Renard et al. 2010).

C.7.6.5 Verification of Insulin Delivery

In any case, in order to verify that delivery of insulin is from exogenous sources, studies in subjects with type 2 diabetes require assays specific for the insulin. It is recommended to use insulin analogues, which can be assayed in the presence of human insulin. Studies in subjects with type 1 diabetes may be useful for detection of human insulin provided subjects are rendered euglycemic under close supervision by intravenous insulin infusion, which is switched off immediately prior to administration of test insulin. Onset and duration of action as well as effectiveness relative to subcutaneous insulin can be assessed with the euglycemic clamp technique (*Fig. C.7 6*). Meal studies may follow to characterize the effect on prandial glucose disposition in comparison to subcutaneous administration. Lower C peptide, nonesterified fatty acid (NEFA), and glucagon concentrations may verify additional exogenous to endog enous insulin effects despite unchanged postprandial glu cose profiles and little altered insulin concentrations (Bruce et al. 1991).

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C.8 Pharmacodynamic Evaluation: Inflammation/Immunology

Martin M. Schönharting

C.8.1 General Considerations

The aim of any immune intervention is to modulate the immune response that triggers a disease, intending to change the underlying pathological process. This change may be a result of either immune stimulation (aimed in, e.g., oncology), or of immunosuppression (aimed in, e.g., autoimmune diseases like rheumatoid arthritis, or in trans plantation). As the oncology indication will be addressed in a separate section, emphasis will be laid in this chapter on immunosuppression.

Immunosuppression can be achieved by suppression of immune function cells, by prevention of their homing to lymphoid organs and inflammatory sites, or by induc tion of clonal anergy or depletion of these cells. Actually, a number of points can be considered where the immuno logical process may be modulated. There is increasing evidence that inflammatory mediators such as cytokines play a crucial role in any kind of immune modulation, as they are directing overall lymphopoiesis including differ entiation and activation of lymphocytes. Indeed, cytokines are involved not only in T cell subset differentiation but also in B cell and NK cell development, indicating that interference with cytokine action is critical in the modu lation of the immune response. For example, the pro inflammatory cytokines IL 1 and TNF α are known to be key mediators in inflammation and joint damage that occurs in rheumatoid arthritis (Arend and Dayer 1995), and targeting these cytokines has become an established therapy for the treatment of this autoimmune disease (Gabay 2002).

The function of a cytokine can be manipulated in sev eral ways: (i) inhibition of its production at the transcrip tional level (i.e., interfering with the signal transduction); (ii) inhibition of its production at the translational level; (iii) direct interception by specific monoclonal antibodies (mAbs); (iv) soluble cytokine receptors, which prevent the interaction between the cytokine and its membrane bound receptor; (v) cytokine receptor antagonists, which compete with the cytokine for binding to its receptor but fail to activate it; and (vi) administration of other cytokine(s) with an effect, which is antagonistic to the target cytokine.

An early pharmacodynamic (PD) test in healthy vol unteers (HV) based on the mode of action of the test compound and hinting on its possible effect in the targeted indication is of tremendous importance for the pharmaceutical development of immune suppressants. In view of the various interactions of immunological factors, some selected PD end points in immunopharmacology are described in this chapter.

C.8.2 The Cytokine Stimulation Assay

PURPOSE AND RATIONALE

In order to test whether a compound presents with an immunosuppressive activity, human blood or cellular components of it can be stimulated by different stimuli, and the resulting cytokine concentrations in the superna tant can be measured. As established stimuli either phythemagglutinin (PHA) or endotoxin (lipopolysaccha ride, LPS) may be used, which qualitatively act in a similar way but quantitatively present with a different outcome (Henderson and Rippin 1995). In the following, two pos sible experimental settings for the cytokine stimulation assay will be given, which can equally be applied for in vitro and ex vivo conditions.

C.8.2.1 In Vitro Cytokine Stimulation Assay

For in vitro investigations, typically isolated peripheral blood mononuclear cells (PBMC) will be used for cyto kine production, which are the very targets of either stim ulus. PBMC comprise a collection of a subset of blood cell types including lymphocytes, monocytes, stem cells, and progenitor cells that is defined by the density gradient centrifugation procedure used to isolate the cells. PBMC are defined as cells that do not sediment into buffers of

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density >1.077 g/mL, instead collect just above the interface between physiologic fluids and buffers of that density. They can then be isolated from more dense cells in peripheral blood, such as erythrocytes and granulocytes (PBMC are also known as "buffy coat" cells because of the fuzzy white visual appearance of the cells).

PROCEDURE

First, PBMC have to be prepared from human whole blood: Venous blood is obtained by any standard phlebotomy technique from a peripheral access point, or from a central line by trained personnel into a specialized Vacutainer tube known as a CPT ("Vacutainer Cell Preparation Tube"). The CPT is processed according to approved procedures by density gradient centrifugation through Ficoll Paque (for protocol see, e.g., www.miltenyibiotec.com) to isolate PBMC. The final PBMC concentration will be adjusted to 250,000 cells/mL either by dilution with tissue culture medium (TCM: RPMI 1640 medium supplemented with 1% glutamine (200 mM) + 1% penicillin (5,000 IU/mL) / streptomycin (5,000 µg/mL)) or by concentration of the cell suspension by further centrifugation at 500 × g for 10 min at room temperature.

For each concentration of the test compound, 1 mL cultures will be set up in triplicates with/without 10 µg PHA/mL (final concentration in culture; stock solutions of the mitogen to be made in TCM; controls receive TCM alone) in 24 well culture plates and cultured at 37°C in a humidified, 5% CO_2 atmosphere. After 72 h of culture with the mitogen, supernatants will be obtained by centrifuging the 1 mL cultures at 500 × g for 10 min. The supernatants may be adequately aliquoted (preferably still as triplicates) and stored frozen at −20°C until further analysis.

Cytokine levels in the supernatant of PBMC cultures will be assessed depending on the expected activity of the test compound following validated procedures (e.g., IL 2: Boehringer Mannheim, MAB B G5, LOQ = 20 pg/mL; IL 4: R&D, MAB 604, LOQ = 0.25 pg/mL; IL 5: R&D, LOQ = 7.8 pg/mL; TNFα: Boehringer Mannheim, MAB 199/1, LOQ = 10 pg/mL; IFNγ: R&D, LOQ = 15.6 pg/mL; sIL 2R: Boehringer Mannheim, MAB 3G10, LOQ = 20 pmol/L). For compounds, which interfere with the signal transduction, alternatively, the polymerase chain reaction (PCR) can be used for cytokine analysis, which is a technique to amplify some few copies of a piece of DNA across several orders of magnitude, generating more than millions of copies of a particular DNA sequence. This method of selective and repeated amplification of the target region coding for a specific cytokine allows the analysis of changes in cytokine production, which otherwise may be too small to be detected.

C.8.2.2 Ex Vivo Cytokine Stimulation Assay

Under ex vivo conditions, it is more convenient to use diluted whole blood as the biological specimen, which may produce comparable results to PBMC cultures but is prone to additional implications. Thus, not only the anticoagulant but also the kind of dilution of the blood may influence the outcome (Mayringer et al. 2000). However, when assessing the relative change in cytokine concentration as compared to baseline for each individual HV, it is possible to get information on the immunomodulatory potential of the test compound, dependent on the applied dose and on time of last dosing.

PROCEDURE

For the ex vivo cytokine stimulation assay, human peripheral blood samples (3 mL each) may be collected in monovettes containing lithium heparin at adequate time points after baseline, depending on the pharmacokinetics of the test compound. All blood samples will be processed within 2 h following established methods for peripheral blood mononuclear cells but modified for whole blood samples (Henderson and Rippin 1995). Briefly, 2.2. mL of heparinized blood will be incubated in crushed ice for 30 min, aiming at standardization of the different blood samples for the following processing. Then each individual blood sample will be divided: the first one (2,000 µL whole blood) remains undiluted, the remaining 200 µL of the pre incubated blood will be diluted in the ratio 1:10 with buffer (AIM V medium, [GIBCO, cat. no. 12030 029] supplemented with 2 mM glutamine). Both samples will then be spiked with LPS from *E. coli* (e.g., Sigma, cat. no. L 2630) to a final LPS concentration of 10µg/mL, transferred to a v shaped microtiter plate (200 µL/well) in eightfold (eight wells for each blood preparation) and incubated for 24 h at 37°C in 5% CO_2. At the end of the incubation period, microtiter plates are to be centrifuged at 1,500 × g, and the cell free supernatant is harvested. The supernatants will be pooled for each dilution and may be kept frozen (−20°C) until further analysis. A dose and time dependent decrease in cytokine production hints to an immunomodulatory effect of the test compound in vivo.

MODIFICATIONS OF THE METHOD

Both the PBMC culture and the whole blood can be stimulated by PHA or LPS. Likewise, further stimulants like pokeweed mitogen (PWM), concanavalin A (con A), or phorbol myristate acetate (PMA) may be used depending on the intended pattern of cytokines, which are the result of such a stimulation. Care has to be taken in preceding validation tests to use the most adequate stimulus in a suitable

concentration (note: even different batches of the same stimulus may present with different biological activity!).

Besides the in vitro and ex vivo cytokine stimulation discussed above, LPS challenge has also been reported in man in vivo (Michie et al. 1988; Zabel et al. 1989). Need less to mention that this kind of controlled cytokine prov ocation in humans requests specific expertise and a fully equipped intensive care unit.

C.8.3 The Proliferation Assay

Stimulation of T cells results not only in the generation of various cytokines as described above but goes also along with cell proliferation. Thus, assessment of T cell prolifer ation after stimulation reflects T cell activity and can be used to test the effect of potential immunosuppressants, which should result in downregulation of T cell activity. The following two experimental settings may be used to address this question:

C.8.3.1 Mixed Lymphocyte Reaction (MLR) In Vitro

PURPOSE AND RATIONALE

MLR is a specific in vitro model for the activation of T cells and for the recognition of antigens by T cells, making use of the effect that T cells of different donors are able to stimulate each other. In an MLR, the PBMC of donor "A" will be co cultivated with the PBMC of donor "B" resulting in an allogeneic challenge and proliferation of the stimulated T cell population. The MLR can be performed in a 2 way stimulation if the PBMC of both donors are active, or in a 1 way stimulation if only the PBMC of 1 donor (e.g. donor A) are active whereas the PBMC of donor B are inactivated, e.g., by irradiation. In both cases of such an allogeneic MLR, the active CD4+ and CD8+ T cells will be stimulated as both subpopulations will recognize the human lymphocyte antigens (HLA) such as major histocompatibil ity complexes MHC I and MHC II, and react against it by proliferation (besides, also cytokine production can be measured with this experimental setting). The extent of proliferation is dependent on the difference in HLA types of the two donors, and on the presence of potential immu nosuppressive agents in the incubation mixture. The 1 way MLR will be used to check the tissue compatibility of donor and recipient for transplantation but can also be applied to test the effect of potential immunosuppressive compounds in a concentration dependent manner.

PROCEDURE

PBMC of donors A and B will be prepared as described above for the cytokine stimulation assay. Whereas the PBMC of donor B will be inactivated by irradiation (30 Gy) via a Cs 137 source, the PBMC of donor A will be labeled with 2.5 µM carboxy fluorescein diacetate, succinimidyl ester (CFDA,SE; this pro dye is able to pen etrate the cells by diffusion but is only fluorescent after splitting off of the acetate groups by internal esterases, which enables the resulting anionic dye CFSE to couple with intracellular amines). Aliquots of 100 µL of both cell preparations (5×10^6/mL each) will be mixed, and either TCM or different concentrations of the test com pound dissolved in TCM will be added. The assay will be carried out in triplicate wells of 96 well microtiter plates at 37°C in 5% CO_2 for 5 days. At the end of the incubation period, cells will be washed by centrifugation at 500 × g for 10 min and analyzed for their proliferation rate by standard fluorescence activated cell sorting (FACS). A concentration dependent decrease in proliferation indi cates an immunosuppressive effect of the test compound.

MODIFICATIONS OF THE METHOD

An alternative method for inactivation of PBMCs of donor B is incubation with mitomycin (40 µg/mL cell suspension) for 20 min prior to co culturing with PBMCs of donor A. Furthermore, instead by FACS the cell proliferation can also be determined by measuring the incorporation of [3H] thymidine into the active PBMCs of donor A.

C.8.3.2 Whole Blood Proliferation Assay Ex Vivo

Lectins like PHA are mitogenic for T cells. Binding of PHA to the CD3 complex of T cells is crucial for the stimulatory effect of PHA but has to be supported by cross linking with surface markers on other cell types, notably monocytes. Indeed, accessory monocytes are necessary for PHA induced T cell proliferation. Due to these cell cell interactions, pro liferation of T cells can easily be measured in diluted whole blood ex vivo, which will be the method of choice especially when it is unclear whether a metabolite of the test compound will (also) exert immunosuppressive activity.

PROCEDURE

Peripheral heparinized blood, which is collected at differ ent time points after dosing of the test compound will immediately be diluted in the ratio 1:5 with TCM (TCM composition as described in Section 1.1). For the whole blood proliferation assay, samples of 1 mL of such 1:5

diluted heparinized blood will be set up in triplicates with 10 µg PHA/mL (final concentration in culture; stock solutions of the mitogen to be made in TCM) in 24 well culture plates and cultured at 37°C in a humidified, 5% CO_2 atmosphere. After 96 h of culture, 0.5 µCi [^{3}H]thymidine will be added for a further 24 h period (to be thoroughly mixed after administration!). After a total of 5 days of culture, proliferation of the cells will be assessed following established laboratory practices.

C.8.4 Assessment of the Immune Status by Phenotyping

PURPOSE AND RATIONALE

Suppression of the immune system is critical in patients with autoimmune diseases or transplant recipients. However, immunosuppression has to be balanced in order to reduce drug side effects and to maintain/restore immunity against common infectious agents. Especially the relative amount of CD8+ T helper cell subpopulations has been shown to reflect the amount of immunosuppression. Other lymphocyte subsets such as CD3+, CD4+ (T helper cells, with differentiation into TH1, TH2 and T suppressor cells), CD16+ (NK cells), and CD19+ (reflecting the B cell population) are contributing to the overall immune response. Various assays have been developed in order to assess the immune status of patients, some of which can also be transferred to HV in Clinical Pharmacology in order to test whether a developmental drug may exert an immunomodulatory effect. In the following, an easy to handle protocol will be described, which allows an adaptation to individual requirements:

PROCEDURE

Two mL blood each will be collected in EDTA vacutainers at three time points in intervals of several days relative to start of multiple dosing of the test compound. One mL of the anticoagulated blood each will be transferred into a prepared immune status tube (Becton Dickinson TruCOUNT™ tube, which contains a pellet that dissolves during sample preparation and releases a known number of fluorescent beads), sealed, and kept at room temperature until rapid analysis or shipment to a certified laboratory, respectively (shipment has to be performed such that the samples will be analyzed within 1 week after blood collection at the latest). Immunophenotyping will be performed using BD MultiTEST reagent (Becton Dickinson GmbH, Heidelberg). Two MultiTEST reagents provide the full range of most important lymphocyte subsets: the one assessing CD3, CD4, CD8, and CD45; a second CD3, CD16, CD19, and again CD45. Analysis by flow cytometry has to follow the instructions given by the manufacturer. Briefly, it consists of 3 simple steps: (i) to mix the anticoagulated whole blood collected in the immune status tube with the adequate MultiTEST reagent and incubate at room temperature; (ii) to add BD FACS lysing solution; and (iii) to analyze the prepared samples on a flow cytometer (FACSCount, Becton Dickinson) compatible with BD MultiSET software. The result will be given in absolute counts for each lymphocyte subset.

MODIFICATIONS OF THE METHOD

Depending on the lymphocyte subset of interest, different MultiTEST reagents may be used. Indeed, by applying the same experimental protocol, further MultiTEST reagents are able to cover additional immunological factors such as CD38, CD45RA, CD62L, etc., all of them playing a specific role in the immune response and may be of interest for an immunomodulatory agent with a very selective mode of action.

Needless to mention that there exist also other methods for the assessment of the immune status. Thus, it has been proposed, e.g., to determine the α/β ratio of thymosin in whole blood for early detection of changes in the immune status of humans (Kolb Bachofen 1990), or to analyze the urine for perforin and granzyme B mRNA (Li et al. 2001). The actual method that is most suited for early PD assessments in HV has to be defined according to the immunological target, which is intended to be affected by the test compound.

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C.9 Pharmacodynamic Evaluation: Endocrinology

Jürgen Sandow

C.9.1 Introduction

The methods in clinical pharmacology for compounds with endocrine activity (hormones, hormone analogues, and drugs affecting the system) are directed at evaluating the function of target organs and endocrine systems. In relation to the general scope of clinical pharmacology (Roden 2008), development of endocrine compounds for clinical use is now a very specific approach closely linked to a regulatory requirement for marketing autho rization and postmarketing follow up (Mucklow 2002; Herman 2005; Maxwell and Webb 2006; Cohen 2008; Aronson et al. 2009). For many hormones, there are clearly defined biomarkers to be measured for the pharmacody namic evaluation. The hypothalamic hormones stimulate the secretion of pituitary hormones (LHRH, TRH, and CRH) or inhibit the release of pituitary hormones (somatostatin). The primary investigation is for the dose response range and time action profile.

Studies on therapeutic efficacy are much more demanding because the response may change during repeated dose administration, by adaptation of receptor regulation.

Methods in clinical pharmacology related to the endo crine system are remarkably complex due to the different hormone systems involved. In the group of hypothalamic and pituitary hormones, there are many similarities in the approach to early methods in clinical pharmacology (phase I and early phase II), whereas the methods directed at assessing efficacy and safety (phase III) are much more diverse due to the different disease condition that is targeted.

In the first group of hypothalamic hormones, there are some peptides with predominantly diagnostic applica tions such as TRH, GHRH (sermorelin), and CRH, and others that have been modified to obtain analogues with remarkably diverse therapeutic indications and applica tions, for example, the therapeutic groups of LHRH ago nists (buserelin, goserelin, leuprorelin, etc.), LHRH antagonists (detirelix, ganirelix, etc.), and somatostatin analogues (octreotide). In each group, the methods frequently applied will be described by example. Due to the increasing number of compounds with established indications, there will be a considerable amount of simi larity in the process of clinical pharmacology evaluation. The clinical utility at least in the group of LHRH and somatostatin is determined by the development and avail ability of long acting injectable dosage forms, which require extensive pharmacokinetic monitoring as well as clinical efficacy and safety investigation.

In the second group of pituitary hormone prepara tions, clinical pharmacology methods are now related to the clinical exploration of the recombinant hormones, which have replaced hormone extraction from biological materials almost entirely. In reproductive medicine, prep arations of follitropin and lutropin have replaced gonad otropins of animal origin and are extracted from menopausal urine. In pediatrics and growth related dis orders, recombinant human growth hormone is the established therapeutic agent. In a very limited indication, recombinant human thyrotropin is used for follow up in thyroid cancer. There are guidelines and recommenda tions for the development of recombinant hormone preparations referred to as "biosimilars," a general devel opment including human insulin preparations and eryth ropoietins. Some hormones with limited application, for example, human corticotropin are replaced by synthetic partial sequences with full biological activity, clinical phar macology being restricted to bioequivalence studies.

C.9.2 Hypothalamic and Pituitary Hormones

C.9.2.1 Somatropin

Preparations of purified growth hormone (somatropin, somatotropic) have been used for treatment of growth disorders but where replaced by recombinant somatropin preparations to eliminate the risk of viral contamination, which occurred when human pituitary glands were extracted. The clinical pharmacology studies then focused

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on the bioequivalence of biosynthetic preparations and on pharmacokinetic studies of the absorption after subcutaneous injection. In growth hormone deficient children, one approach was to suppress the secretion of endogenous growth hormone by administration of a somatostatin analogue (octreotide). The phase III studies on clinical efficacy were performed for 6 9 months to evaluate the effect in growth hormone deficient children. In placebo controlled phase I single dose studies by subcutaneous injection, to investigate pharmacokinetics of somatropin, endogenous growth hormone (GH) was suppressed by a continuous infusion of octreotide over 25 h (from 1 h before to 24 h after the injection of somatropin or placebo).

In the pharmacodynamic studies, measurement of IGF 1, IGFBP 3, and NEFA at predefined time points until 96 h was included, and these biomarkers were included in the evaluation of bioequivalence comparing somatropin formulations of different provenience. In the efficacy studies, children with predefined short stature and a decreased spontaneous growth rate monitor over an interval of at least 6 months were eligible for inclusion. Patients with chronic systemic diseases or evidence of tumor growth, with skeletal or chromosomal abnormalities as well as patients on medication known to affect growth were excluded from the study. In one study program (EMEA Omnitrope 2004), primary endpoints were the height and the height standardized for age and sex (Height Standard Deviation Score, HSDS) at month 9, and the height velocity as well as the height velocity standard deviation score (HVSDS) between month 0 and 9. Secondary efficacy endpoints included IGF 1 and IGFBP 3 serum levels at months 1, 3, 6, and 9. It was pointed out that these measurements are not suitable as surrogate endpoints for the assessment of clinical efficacy.

There is now an increasing number of applications for biosimilar hormone preparations (EMEA Guideline 2006) supported primarily by bioequivalence studies (Wiecek and Mikhail 2006; Ranke 2008; Gottlieb 2008; Pavlovic et al. 2008). There is a wide range of compounds including erythropoietin, gonadotropin preparations, human insulin, somatropin, and other biotechnology products.

C.9.2.2 Follitropin

In the treatment of reproductive disorders, follicle stimulating hormone preparations (follitropin) are injected to stimulate maturation of ovarian follicles. Biosynthetic preparations have replaced the PMSG/FSH/LH preparations of animal provenience. Test procedures are bioequivalence studies for recombinant human follicle stimulating hormone preparations (Voortman et al. 1999; EMEA Puregon 2005). Phase I evaluation includes the comparison of subcutaneous injections and intramuscular injection, and the pharmacokinetics of absorption. The endogenous FSH (follitropin) secretion may be suppressed by injection of an LHRH agonist or antagonist. This is one of the general problems in the assessment of recombinant hormone preparations. Measurement is on the background of residual endogenous hormone secretion, even if only low concentrations are present, it is necessary to suppress the endogenous hormone secretion when suitable agents are available. Consistent suppression may require intravenous infusion of the reagent, which is selected to suppress endogenous hormone suppression (LHRH agonist, somatostatin, thyroid hormone).

In the clinical efficacy studies for the treatment of female infertility, the indications addressed were anovulation (including polycystic ovarian disease, PCOD), and controlled ovarian hyperstimulation in medically assisted reproduction programs. In the studies it was found that recombinant follitropin (Puregon) had similar pharmacodynamic activities to the urinary FSH comparator in terms of inducing ovum growth and maturation. Methods of assessment were by ovarian ultrasound and laparoscopic harvesting of oocytes. Phase III studies in clinical conditions of impaired pituitary ovarian function women with chronic anovulation (WHO group II pituitary/ovarian dysfunction) were targeted at synchronized secretion of both gonadotropin and estrogen (in the presence of normal lactotrophic function, absence of prolactinoma).

The primary efficacy parameters for this study were (1) the number of cycles needed to achieve ovulation, and (2) the cumulative ovulation rate after three cycles (using the life table method). Further studies were added for extension of indication in male infertility: Treatment of males with deficient spermatogenesis due to hypogonadotropic hypogonadism was added as a new indication for follitropin (Puregon, all strengths and presentations), namely, treatment of male subjects who suffer from deficient spermatogenesis due to hypogonadotropic hypogonadism. Infertility caused by deficient endogenous production of FSH may be treated by administration of exogenous gonadotropin. The clinical pharmacology studies were performed to confirm that a biotechnologically prepared medicinal product can serve as a substitute for natural FSH, and may replaced previously used urinary hormone preparations. Clinical data based on a large randomized group comparative study were accepted to support the use of

follitropin (Puregon) in Assisted Reproductive Technologies in women.

C.9.2.3 Lutropin Alfa

Recombinant human luteinizing hormone (LH) is used for the treatment of reproductive disorders (hypogonadotropic hypogonadism), replacing in part the previously applied extractive gonadotropin preparations, human chorionic gonadotropin (HCG), and human menopausal gonadotropin (HMG) (Huisman et al. 1997). Preparations of human urinary gonadotropins (menopausal) have been compared for their bioequivalence after subcutaneous and intramuscular injection. Specific pharmacokinetic methods are available, however, the problem is to measure exogenous gonadotropins on the background of residual endogenous secretion. In the evaluation of the human recombinant LH preparation (EMEA Luveris 2004), the treatment with a GnRH analogue was used to achieve suppression of endogenous LH, which was necessary to assess the pharmacodynamic effects of lutropin alfa in women. However, a complete suppression was difficult to achieve and residual LH remained at detectable levels.

The clinical indications in reproductive disorders are well defined. In clinical pharmacology studies (EMEA 2004, Luveris lutropin alfa), no quantitative evaluation of pharmacodynamic effects in humans was performed. During combined r hLH and r hFSH treatment (150 IU of each hormone daily), the effects on estradiol, progesterone, and inhibin levels were investigated. Follicular development was monitored by ovarian ultrasound.

Three studies were conducted in healthy women (18 35 years) in whom endogenous LH secretion had been down regulated by administering a GnRH analogue. These studies examined pharmacokinetics and safety of exogenous lutropin, endogenous LH concentrations in serum and urine were measured using a specific immunoradiometric assay. Pharmacokinetics comprised terminal half life after IM and SC administration (about 18 h), and absolute bioavailability of r hLH after IM or SC administration.

Lutropin alfa is the recombinant human form of luteinizing hormone (LH) developed for use in the stimulation of follicular development (Dhillon and Keating 2008). Dose finding studies revealed a significant dose dependent increase in the rate of optimal follicular development among women with hypogonadotropic hypogonadism and profound LH deficiency (<1.2 IU/L) who received subcutaneous lutropin alfa 0 225 IU/day plus follitropin alfa. Similarly, in a double blind, randomized study, the rate of optimal follicular development was significantly higher in women with hypogonadotropic hypogonadism and profound LH deficiency receiving subcutaneous lutropin alfa 75 IU/day plus follitropin alfa.

Precise methods are available for the monitoring of follicular development; the primary endpoints in studies were follicle size by ultrasound examination and measurement of estradiol and progesterone concentrations in plasma. These combined surrogate endpoints are accepted as an indication of the clinical pregnancy rate to be achieved in women with profound hypogonadotropic hypogonadism.

C.9.2.4 Thyrotropin Alfa

The recombinant human TSH preparation (EMEA Thyrogen 2004) has a specific limited indication in thyroid cancer follow up, to stimulate the uptake of 131 I in the whole body scan procedure and find possible indications from metastatic disease. Bioequivalence of recombinant preparations was determined by pharmacokinetics using a human TSH assay. Methods in clinical pharmacology related to thyrotropin alfa are exclusively related to this oncology indication (EMEA Thyrogen 2004). The whole body scan procedure is now being replaced by the measurement of serum thyroglobulin, as an indicator of thyroid tissue remaining after total thyroidectomy and radioiodine therapy.

C.9.2.5 Corticotropin

The human adrenocorticotropic hormone (ACTH) sequence consists of 39 amino acids, the first 24 amino acids are the biologically active sequence (N terminal corticotropin sequence). The clinical pharmacology procedure is stimulation of cortisol secretion, for dose finding and bioequivalence studies. The synthetic (1 24) corticotropin is used for diagnostic purposes (Nye et al. 1999). Therapeutic trials with ACTH have been performed in neurological disorders, for example, multiple sclerosis with glucocorticoids as the comparator but no evidence for therapeutic utility has been established. In adrenal insufficiency, substitution therapy with adrenal steroids is indicated, whereas corticotropin is not sufficiently effective.

C.9.2.6 LHRH Analogues

There is a dose related to effect on the release of follitropin and lutropin, followed by stimulation of gonadal steroids

secretion. In clinical practice, LHRH (gonadorelin) is used for diagnostic purposes, in reproductive endocrinology for treatment of cryptorchidism, for stimulation of ovarian function by pulsatile administration (infusion pumps), and in assisted reproduction protocols. The clinical pharmacology of LHRH agonists and antagonists is much more complex. The agonists have a "paradoxical" inhibitory effect on the secretion of gonadal serum hormones, by inducing pituitary down regulation upon repeated dosing. As a consequence, their clinical pharmacology is studied by repeated dose administration (e.g., 2–4 weeks) and much more effectively during controlled release from injected depot formulations.

C.9.3 Calcitropic Hormones

In this section, the hormones and related compounds affecting calcium metabolism and the related therapeutic indications, for example, in osteoporosis, tumor hypercalcemia, and end stage renal disease are discussed. In the group of calcitropic hormones and related drugs, clinical pharmacology has been evaluated for salmon calcitonin, parathormone (1–84), a partial sequence of parathyroid hormone (teriparatide), several calcitropic steroids (vitamin D derivatives, calciferols), the bisphosphonates, and more recently calcium analogues (cinacalcet).

C.9.3.1 Calcitonin

The clinical pharmacology of salmon calcitonin is described in several reviews (Stevenson and Evans 1981). There are several established indications for the prevention of bone demineralization, nasal application of calcitonin has been evaluated repeatedly (Grigoriou et al. 1997), and a formulation for oral administration has been described (Tankó et al. 2004; Karsdal et al. 2009). Calcitonin has been recommended for the treatment of postmenopausal osteoporosis (Body 2002). Several formulations are marketed in Europe and North America, including nasal spray formulations (Chesnut et al. 2000).

One indication is the hypercalcemia of malignancy, associated with tumor effects on bone. Another indication is postmenopausal osteoporosis.

Calcitonin acutely inhibits parameters of bone resorption. Single dose injection in healthy volunteers is followed by an increase in serum calcium and plasma cAMP. For bioequivalence studies, the response to repeated dose injection can be measured by urinary concentration of deoxypyridinoline and urinary excretion of the C terminal type I collagen telopeptide (CTX) after 5–7 days of treatment. There are several biomarkers, which have been used for the quantification of the pharmacodynamic effect (bone resorption marker serum CTX I and the cartilage degradation marker urine CTX II). In long term therapy, the effect may be monitored by suitable markers of bone resorption such as alkaline phosphatase or urinary hydroxyproline or deoxypyridinoline. The pharmacokinetics can be followed by specific radioimmunoassay. Studies on bioequivalence were performed in healthy postmenopausal women.

When the calcitonin was tested in hypercalcemia of malignancy, the fall in serum calcium following administration of sCT is rapid and occurs within 2 h. There is also evidence that intravenous infusion rather than a bolus injection gives a more complete response. The maximum response is observed after approximately 2 days; the duration of response persists for 7–14 days. For long term control of metastatic disease, however, bisphosphonates have been evaluated in many studies and appear to be superior (Gürlek et al. 1997; Dursun et al. 2001).During high dose treatment with calcitonin, the most frequent side effect reported in publications is nausea followed by facial flushing, local pain at the injection site, diarrhea, and vomiting. The benefit/risk profile of salmon calcitonin was considered favorable in the treatment of Paget's disease and hypercalcemia of malignancy by EMEA. Several calcitonin preparations have been approved by the FDA (including recombinant salmon calcitonin). Clinical pharmacology studies have established that calcitonin can prevent postmenopausal or postovariectomy bone loss, increase trabecular bone mass among patients presenting an established osteoporosis, and increase lumbar spine bone mineral density (BMD). The effect of calcitonin on the reduction of fracture risk and the relative risk of developing new vertebral fractures were assessed with lateral radiographs of the spine, measuring lumbar spine bone mineral density.

C.9.3.2 Parathormone

Preparations of parathormone [rPTH(1–84)] are available in Europe and North America, including recombinant human PTH (EMEA Preotact 2004), and the recombinant partial sequence preparation [PTH (1–34)], which has full biological activity (EMEA Forsteo 2004; FDA Forteo 2009). Parathormone has an immediate and dose related effect on the serum concentrations of calcium and phosphate. In clinical pharmacology of the recombinant product, bioequivalence studies are performed based on

single dose administration (intravenous and subcutaneous) and repeated dose injection by subcutaneous injection. There are several established biomarkers, changes in serum phosphate, 1,25 dihydroxyvitamin D, osteocalcin, bone specific alkaline phosphatase, and tartrate resistant acid phosphatase were measured in clinical studies along with urinary calcium (Ca/creatinine ratio), phosphate, cyclic AMP, deoxypyridinoline, and hydroxyproline in selected studies. PTH injection is followed by a dose dependent increase in serum 1,25 dihydroxyvitamin D concentrations 12 h after treatment and increases in cyclic AMP/creatinine ratio were detected in 12 urine samples. The clinical indication is established as postmenopausal osteoporosis, with treatment periods up to 2 years, combined treatment by adding alendronate, or co medication with estrogens has been evaluated. In the studies, primary analysis was the change from baseline in lumbar spine L1 L4 BMD until month 12, in subsequent studies the primary efficacy endpoint was the incidence of new and/or worsened vertebral fractures as assessed by spinal radiographs (X rays). Secondary efficacy variables were incidences of vertebral fractures: hip and wrist. Other secondary variables were clinical fractures; changes in height; changes in BMD, BMC, and BMA of the lumbar spine, total hip, regional hip, whole body, and forearm assessed by DXA; changes in cortical and trabecular bone compartments assessed by quantitative computed tomography (QCT/pQCT) at lumbar spine, hip, forearm, distal femur, and central tibia (QCT substudy); and by bone histomorphometry at the iliac crest. In the studies, changes in bone turnover markers are helpful but the critical question is prevention of fractures. During treatment with parathormone alone, the primary endpoint was the mean percentage change from baseline in lumbar spine BMD measured by DXA at month 24. Secondary end points included mean percentage change from baseline in BMD at month 12, bone quality as measured by QCT, height, and biochemical markers of bone turnover. In further clinical pharmacology exploration, the efficacy of PTH and alendronate (ALN) as monotherapy and in combination for the treatment of postmenopausal osteoporosis were assessed in women between 55 and 85 years of age, with a bone mineral density scan (DXA). Safety evaluation was performed in non osteoporotic men and women with either renal or hepatic impairment.

The duration of PTH treatment was severely limited by the finding that in one rat toxicology study, formation of osteosarcoma was found. Even though a second toxicology study in rats did not confirm the initial alert, treatment remains limited to 2 years. In an extension study on sequential treatment by PTH for 1 year followed by alendronate, osteoporotic women who were treated for 12 months with PTH injections entered a 1 year open label extension study of daily alendronate (ALN) 10 mg administration. The primary objective was to determine whether ALN would preserve or enhance lumbar spine, femoral neck, and whole body BMD in patients previously treated with PTH. One study was conducted in women with low bone mass on stable estrogen replacement therapy. Another study evaluated the PTH with a bisphosphonate after PTH in the Phase II study (Rittmaster et al. 2000), and found a prolonged and lasting effect. No consistent trend for the reduction in the risk of fractures was observed in patients having received previous treatment with either bisphosphonates or estrogens.

C.9.3.3 Teriparatide

The recombinant human parathyroid hormone analogue (1 34), [rhPTH(1 34)] is indicated for a treatment period of up to 2 years in patients with specific risks:

1. Treatment of postmenopausal women with osteoporosis at high risk for fracture.
2. Increase of bone mass in men with primary or hypogonadal osteoporosis at high risk for fracture.
3. Treatment of men and women with osteoporosis associated with sustained systemic glucocorticoid therapy at high risk for fracture. The restricted indications are due to the effect observed in rat toxicology studies, where the incidence of osteosarcoma was increased after long term treatment, even though the relevance for human therapy is not clear. Clinical pharmacology studies closely followed the precedent set by the evaluation of the full sequence hormone [rPTH(1 84)].

In the European evaluation, teriparatide is indicated for the 'treatment of established osteoporosis in postmenopausal women. A significant reduction in the incidence of vertebral, but not hip fractures has been demonstrated. There was obvious concern about the acute changes in serum calcium concentrations induced after teriparatide injection. A safety pharmacological program was conducted, which includes specific studies of the teriparatide effects on calcium homeostasis in healthy postmenopausal women, on cardiac conduction, and on reporalization in healthy volunteer (EMEA Forsteo 2004; EMEA Preotac 2006). The program compromised one dose ranging study, three studies performed in women: one placebo controlled pivotal study and two comparative supportive studies (teriparatide versus alendronate, and teriparatide versus placebo in women receiving hormonal replacement therapy (HRT)); and one

pivotal study performed in men targeted at the indication of osteoporosis.

C.9.3.4 Calcitriol and Vitamin D Analogues

Several analogues of vitamin D have been evaluated in clinical pharmacology studies, their differences being onset of action and duration of the effect on calcium regulation. The indications for systemic treatment are rickets (prophylaxis and therapy), osteomalacia, some cases of hypoparathyroidism, secondary hyperparathyroidism in end stage renal failure (ESRF) and chronic kidney disease (CDK stage 5), and the local administration of vitamin D derivatives in psoriasis (calcipotriol and paricalcitol).

In CKD, the effect of vitamin D on calcium reabsorption is increasingly lost and there is a compensatory rise in parathormone secretion (secondary hyperparathyroidism). Clinical pharmacology was investigated in patients with uremia (Malluche et al. 2002; Brandi 2008). Alfacalcidol is indicated for the treatment of hypocalcemia, secondary hyperparathyroidism, and osteodystrophy in patients with chronic renal failure (Alfacalcidol Monograph 2000). Clinical pharmacology of alfacalcidol on the development of renal bone disease in patients with renal failure not yet undergoing dialysis was evaluated in a large, randomized, placebo controlled study. The effect was monitored by biomarkers and bone biopsy. Long term oral administration improved bone histology and halted the progression of changes in serum alkaline phosphatase activity and parathyroid hormone levels compared to placebo. One advantage of alfacalcidol is its rapid onset of action due to hepatic activation.

Calcitriol is indicated in the management of secondary hyperparathyroidism and resultant metabolic bone disease in patients with moderate to severe chronic renal failure, and in the management of hypocalcemia and its clinical manifestations in patients with postsurgical hypoparathyroidism, idiopathic hypoparathyroidism, and pseudohypoparathyroidism (Rocaltrol 2008). There are preclinical studies that indicated potential for the use in oncology (Krishnan et al. 2008) based on the anti inflammatory activity of calcitriol and other vitamin D analogues (Vanoirbeek et al. 2009). Paricalcitol is indicated for the prevention and treatment of secondary hyperparathyroidism associated with chronic kidney disease Stage 5 (Zemplar 2008). In the clinical studies of 12 weeks off, the reduction in intact parathyroid hormone (iPTH) was measured as the surrogate bio marker of clinical efficacy.

Recent studies on the application of vitamin D analogues in dermatology have shown the utility of calcipotriol (Clareus et al. 2009) in a fixed combination with betamethasone, and of tacalcitol (Prignano et al. 2009).

C.9.3.5 Cinacalcet

Cinacalcet is an organic small molecule that acts as an allosteric modulator of the calcium sensing receptor on the parathyroid cell surface. Its clinical pharmacology and the efficacy in therapy of hyperparathyroidism (HPT) associated with end stage renal failure and of parathyroid carcinoma has been extensively reviewed (Torres 2006; Messa et al. 2008) and documented (Sensipar cinacalcet 2008a, b, EMEA Mimpara 2004). Established indications are secondary hyperparathyroidism in patients with end stage renal disease (ESRD) on maintenance dialysis therapy, and reduction of hypercalcemia in patients with parathyroid carcinoma. In summary, 47 clinical studies in normal volunteers and patients with primary or secondary HPT were performed. Cinacalcet is a first in class calcimimetic that modulates the activity of the calcium sensing receptor (CaR). Cinacalcet acts to reduce circulating PTH concentration through activation of the CaR by increasing its sensitivity to extracellular calcium. In view of the marked effect on calcium concentrations, the preclinical program included an evaluation of the effect of cinacalcet on ECG, in particular, the QT/QTc interval. The primary efficacy studies were four randomized, double blind, placebo controlled Phase II dose titration studies in ESRD patients on hemodialysis with secondary HPT, three randomized, double blind, placebo controlled main Phase III studies in hemodialysis or hemodialysis/peritoneal dialysis patients with secondary HPT in end stage renal disease (ESRD), and two randomized, double blind, placebo controlled phase II studies in patients with chronic kidney disease (CKD) and secondary HPT not receiving dialysis. There were several extensions added to the studies. A specific randomized, double blind, placebo controlled study examined the effects of cinacalcet on renal osteodystrophy in ESRD patients with secondary HPT on dialysis, and one ancillary placebo controlled study was conducted in patients with osteitis fibrosa. The surrogate marker measured throughout the studies was the reduction in mean intact parathormone (iPTH) levels. This biomarker was lowered in a dose dependent manner when monitored as the primary endpoint in the phase III

studies. A significantly higher proportion of subjects in the cinacalcet group versus the placebo group achieved the predefined target iPTH level (iPTH < 250 pg/mL).

The biochemical marker of bone turnover, bone specific alkaline phosphatase (BALP) was measured in the phase III ESRD studies and a reduction in median BALP levels was noted among the cinacalcet treated patients. In a double blind placebo controlled phase II study in subjects with ESRD, bone mineral density (BMD) was measured at various sites by dual X ray absorptiometry scanning at baseline and after 54 weeks of treatment. In a multicenter, open label, single arm, dose titration study evaluating the efficacy, safety, and pharmacokinetics of cinacalcet in subjects with parathyroid carcinoma or intractable primary HPT (failed or contraindicated for parathyroidectomy) with increased serum calcium at screening, the primary endpoint was the proportion of subjects showing a predefined reduction in serum calcium at the end of the titration phase.

In the EMEA evaluation, it was stressed that the capacity of cinacalcet to reduce iPTH levels in patients with secondary HPT in CKD with ESRD had been convincingly demonstrated. The effect was sustained over time and is consistent in different subgroups and in different degrees of disease severity. Moreover, the reduction of iPTH was accompanied by moderate but significant and consistent reductions in calcium, phosphate, and the calcium x phosphate (CaxP) product levels. The primary biochemical endpoint iPTH was considered to be a relevant surrogate marker, which is used in clinical routine today to balance currently available therapy for secondary HPT in CKD. Concerning the pathophysiology of bone, it was discussed that consistent application of the current advanced treatment possibilities for secondary HPT in CKD (more effective phosphate binders, active vitamin D) has contributed to a shift from the classical high turnover bone pathology (osteitis fibrosa) toward a low turnover bone pathology ("adynamic bone disease," Frazão and Martins 2009). Indications accepted by the CHMP were secondary hyperparathyroidism in patients with end stage renal disease (ESRD) on maintenance dialysis therapy, and the reduction of hypercalcemia in patients with parathyroid carcinoma.

C.9.4 Adrenal Steroid Hormones

The clinical pharmacology evaluation of adrenal steroid hormones (glucocorticoids and mineralocorticoids) is related to their general hormonal activity (receptor binding profile, activation of nuclear receptors, cellular signalling) followed by definition of the clinical condition to be treated and assessment of efficacy and safety. There is a wide range of applicable methods directed at basic mechanisms (to find and consolidate suitable biomarkers), and for the specific clinical conditions. Frequently, glucocorticoid treatment is part of a therapeutic regimen together with other drugs substances (co medication), for example, in asthma and chronic obstructive pulmonary disease (COPD), in hematology and in oncology indications. High dose glucocorticoids have application in emergency medicine; long term administration at clinically effective dose above the physiological range requires particular care. The mineralocorticoid antagonists have found applications, for example, in internal medicine, to treat heart failure, ascites in patients with liver disease, low renin hypertension, hypokalemia, Conn's syndrome, and have also found indications in emergency medicine, for example, following myocardial infarction. The glucocorticoids and mineralocorticoids are vitally important compounds secreted by the adrenal glands under physiological conditions (dose range for substitution therapy) and during enhanced requirements for adrenal activation in many disease conditions (medium and high dose pharmacotherapy). The doses required under these conditions may be far in excess of physiological requirements, megadose treatment by injection may be necessary in emergencies. Adrenal insufficiency is a life threatening condition, which requires emergency treatment; substitution of prolonged insufficient secretion is required using, for example, oral hydrocortisone as the glucocorticoid and fludrocortisone as the mineralocorticoid.

C.9.4.1 Exploratory Studies, Mechanisms and Biomarkers

There are many ongoing studies related to the physiological effects of adrenal steroids and the related mechanisms in receptor activation, directed at exploring mechanisms of molecular biology and establishing biomarkers related to the therapeutic activity in specific disease conditions. The structure and function of glucocorticoid and mineralocorticoid receptors and their isoforms has been explored and characterized in great detail (Funder 1979; 1992; Buckbinder and Robinson 2002; Bray and Cotton 2003; Sheppard 2003; Hu and Funder 2006; Lu et al. 2006; Pippal and Fuller 2008; Kino et al. 2009; Nicolaides et al. 2010). These systematic studies are helpful for the understanding and classification of synthetic corticoids and

their signalling mechanisms, both genomic and non genomic. For instance, studies are ongoing at the National Heart, Lung, and Blood Institute (NHLBI), to characterize the effect of combinations of a beta 2 agonist and a cor ticosteroid (beclometasone) administered to patients with asthma depending on their genotype of the beta 2 adren ergic receptor gene. Other ongoing studies are designed to identify a biomarker or biomarker set for the adverse metabolic effects of various doses of prednisolone treat ment (dose finding, safety assessment). This is of consid erable clinical relevance because the initial doses in prednisolone treatment of emergency conditions may be rather high, and it is also of practical interest to be able to monitor the potential for adverse effects on the organ systems often affected by long term corticoid therapy (e. g., ulcer formation, osteoporosis). One such biomarker is identified, which is of course applicable to all synthetic glucocorticoids currently introduced to therapy.

C.9.4.2 Compounds in Clinical Use

There is a large group of glucocorticoid compounds cur rently in clinical use for their anti inflammatory and/or immunosuppressive activity by a systemic administration over a wide dose range, and by local application for anti inflammatory activity. The systemic effects may relate to connective tissue diseases, severe asthma and COPD, severe allergic reactions including anaphylactic shock, prevention of organ transplant rejection, acute lymphatic leukemia, thrombocytopenic purpura, co medication with cytostatics and immunosuppressive drugs in oncol ogy, central nervous system disorders (acute spinal cord injury, multiple sclerosis) and severe conditions in derma tology. Anti inflammatory medication may be required in some cases of rheumatoid arthritis, ankylosing spondy litis, ulcerative colitis and regional enteritis (Crohn's disease). For replacement therapy in adrenal insufficiency, hydrocortisone as the glucocorticoid and fludrocortisone as the mineralocorticoid (replacing aldosterone) may be administered orally.

Prednisone is frequently used because it is marked anti inflammatory and has little sodium retaining activity; it can be orally or intramuscularly administered. 6 Methyl prednisolone is similar to prednisone; there is an inject able dosage form for megadose treatment in emergency medicine. The fluorinated corticosteroids (triamcinolone, fludrocortisone) are used extensively, triamcinolone for its anti inflammatory activity, fludrocortisone for mineralo corticoid substitution. Triamcinolone acetonide has been frequently used in dermatology viz. contact dermatitis, psoriasids, and by topical application in allergic and perennial rhinitis (Jeal and Faulds 1997). Some of the safety studies are in children below the age of 6 years, to investigate the effect on prepubescent growth velocity, including secondary outcome parameters, for example, the global efficacy of triamcinolone nasal spray, and the rate of treatment emergent adverse events in children. There are a number of ophthalmological preparations currently under investigation for glaucoma, diabetic reti nopathy and age related macular degeneration (Kiernan and Mieler 2009; Jonas 2007; el Matri et al. 2010; Bressler et al. 2009; Baath et al. 2007; Batioglu et al. 2007). Dexametasone and betamethasone are anti inflammatory steroids used for longer time periods, and their effect may be suppression of adrenal cortical function with careful adjustment and read your dose reduction to restore adre nocortical responsiveness (Helfer and Rose 1989). Adrenal suppression may also be observed after long term use in dermatology (Goa 1988; Levin and Maibach 2002). The atrophy of connective tissue in these indications is well known, it is found both in experimental models and in clinical studies (Frauman 1996). Clinical pharmacology studies have been performed by nasal and pulmonary administration of corticoids (fluticasone, budesonide, beclometasone) in the treatment of allergic rhinitis, asthma, COPD and local infections, for example, Pneumocsystis Carinii pneumonia (Holliday et al. 1994; Dhillon and Keating 2006; Frois et al. 2009). Clinical outcome measures in COPD were clinical symptom related, time to first moderate or severe on treatment COPD exacerbation and number of on treatment COPD exacerbations, systemic administration in pulmonary fibrosis disease progression was studied in combination therapy with immunosuppressants, for example, predni sone and azathioprine. In persistent asthma, fixed combi nations for inhalation have been found to be more effective (budesonide/formoterol or fluticasone/ salmeterol), and in COPD the fixed combination of fluticasone/salmeterol together with tiotropium has been studied. Pharmaceutical formulations with new propel lants have been developed (HFA134a), device technology has been improved requiring clinical pharmacology stud ies with new inhalers and inhalation powder applicators. The Methacholine challenge has been used for single dose and repeated dose administration, together with spirom etry evaluation methods. Research on such methods is ongoing to differentiate the response of non asthmatic controls and asthmatic patients in a cross sectional, case control study measuring the sensitivity and specific ity of methacholine challenge testing, followed up by

a cross over, randomized, double masked trial designed to evaluate the impact of high dose versus low dose inhaled corticosteroids (ICS) on bronchial hyperreactivity (BHR) in asthmatics (NCT00705341). Pharmacology studies have established the permissive action of glucocorticoids on beta 2 adrenergic receptors, compounds in this group are beclometasone, fluticasone, budesonide, mometasone and ciclesonide (Nathan et al. 2010; Bousquet 2009; Tomillero and Moral 2010).

There is a wide range of synthetic derivatives of adrenal steroids which have been characterized with regard to their systemic and/or local administration, for example, in dermatology and ophthalmology (Bennett and Brown 2008; Schimmer and Parker 2006; Chrousos 2009). There is also an increasingly wide spectrum of pharmaceutical presentations, including metered dose inhalers and powder dispensers for asthma and COPD. Frequently, the studies are directed at characterizing contribution of adding an adrenal steroid compound as part of the therapeutic regimen, for example, in oncology and hematology. They may also be directed at the clinical use of fixed combinations of drugs in the treatment of asthma and COPD, namely, studies on lung deposition after a single dose administration.

C.9.4.3 Studies for Guidelines and Health Economics

There are many ongoing studies in the established indications, increasingly based on the concepts of evidence based medicine, to establish clinical efficacy in randomized controlled studies based on clinical outcome. Such studies are essential for the development and updating of guidelines, frequently also for cost assessment in the insurance system (health economics, pharmacoeconomics), as described in Health Technology Assessment [HTA] reports (Collins et al. 2007; Main et al. 2008). An overview of ongoing and completed studies is available at the website maintained by the U.S. National Institute of Health (www.clintrials.gov). This website is a large database providing information on study details such as medication and dose range, test compound and comparator in case of RCT studies, clinical indication, specific study purpose, study design, primary and secondary study outcomes. Information about study medication is available from the "Drug Information Portal" of the website, providing an overview of the pharmacological class of compounds to which the study medication belongs. More detailed information is available from links to the literature database www.pubmed.gov which is maintained by the U.S. National Library of Medicine and National Institutes of Health.

Substitution therapy at physiological doses is required for adrenal insufficiency and related clinical conditions. The pharmacotherapy is performed with much higher doses to address a variety of inflammatory conditions (Swartz and Dluhy 1978), autoimmune diseases, transplantation (EBPG Expert Group 2002), rheumatoid arthritis and central nervous systems conditions. For each of these studies, the dose range may be far in excess of physiological secretion, leading to involution of the adrenal gland, long term dependence on therapeutic corticoid administration, and a variety of dose related adverse reactions both as a reaction to acute very high dose administration, and more frequently as a consequence of glucocorticoid dependence (Schimmer and Parker 2006). For clinical diagnostic evaluation, and in the course of long term studies with glucocorticoid administration (risk of adrenal suppression), the response of the adrenal gland by an increase of cortisol secretion can be tested by injection of synthetic corticotropin analogue (Synacthen test), and by injection of corticotropin releasing hormone (CRH test, Schuermeyer et al. 1987). These tests are applied to differentiate Cushing's disease of hypothalamic origin from that due to adrenal tumors, for Cushing's syndrome due to ectopic ACTH secretion of tumors, and for changes due to acute brain injury (Chatha et al. 2010; Maguire et al. 2008; Wijesurendra et al. 2009). They are also suitable for assessing the extent of adrenal suppression and recovery due to long term glucocorticoid therapy (Paton et al. 2006; Gupta et al. 2009).

Under physiological conditions, the secretion of corticosteroids is stimulated by corticotropin, and to a lesser extent by vasopressin. The secretion of aldosterone is stimulated by angiotensin. There is an endogenous circadian rhythm of cortisol secretion which needs to be considered when prescribing substitution therapy with adrenal steroids. Due to the physiologic rise of cortisol secretion in the morning, a higher fraction of the total dose needs to be administered at this time, and the smaller fraction in the afternoon (chronotherapy).

C.9.4.4 Pharmacotherapy and Study Design

In humans, the major endogenous glucocorticoid is cortisol and the important mineralocorticoid is aldosterone. Furthermore, dehydroepiandrosterone (DHEA) and two other adrenal androgens are secreted (androstenedione and androstenedione) under physiological conditions, the

adrenal androgens are the major endogenous precursors of androgens in women after the menopause Pharmacotherapy with corticoids is different from physiological secretion because higher doses and chemically modified compounds are applied in indications which make use of the potent anti inflammatory and immunosuppressive potential. There is a wide range of pharmaceutical presentations comprising corticoids for injection (megadose therapy), oral formulations for a number of indications including allergies and rheumatoid arthritis, metered dose inhalers, for example, for asthma and COPD, numerous presentations for dermatological indications, solutions for local injection, and topical application in ophthalmology. Frequently, corticoid preparations are part of a therapeutic regimen comprising, for example, cytostatic agents and other antiproliferative compounds in the oncology indications, and in hematology. Obviously given the wide range of indications and presentations, methods for evaluating the effect of corticoids and corticoid combinations depend largely on the clinical indication, which may require systemic effects (e.g., oncology and hematology, rheumatoid arthritis) or specific local action (e.g., aerosols for asthma, perennial rhinitis) with limited potential for systemic undesired effects. High dose glucocorticoids have central nervous effects and have been used in neurosurgery, traumatology, and psychiatric conditions.

Frequently studied compounds comprise prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone, beclomethasone, fluticasone and budesonide in many dosage forms and presentations, often in fixed combinations and in regimens requiring co medication with other drugs. There are numerous studies in asthma and COPD, frequently based on clinical outcome (e.g., severe asthma related events, asthma related events and health care utilization, and symptom free days [SFD]) and on spirometry before and after bronchodilator administration. Other studies target the established safety issues, such as the effect of inhaled corticosteroids on the risk of diabetes, impaired glucose tolerance and characteristics of glucose regulation in adults with asthma (University of Hon Kong). Studies in COPD focus to a large extent on the inflammatory response and progression of the disease. One biomarker study is for the exhalation of nitrous oxide and related metabolites, intra and inter subject variability of exhaled (alveolar and bronchial) and nasal NO in smoking subjects with mild and moderate COPD on or off steroid treatment, and smoking healthy volunteers and non smoking mild asthmatics off steroids to be investigated. An explorative study is on the expression of genes in sputum to measure drug response in COPD, to determine whether analysis of genes in sputum is a useful non invasive technique for measuring response to drugs in patients with COPD (NCT00233051). In a number of studies, the clinical outcome is the primary objective, Health related quality of life (HRQL) being one of the methods to assess change and improvement (NCT00728715).

C.9.4.5 Inhibition of Adrenal Steroids Synthesis

In tumors of adrenal origin and in Cushing's disease of adrenal origin, the pharmacological approach is to inhibit the synthesis of adrenal steroids by a number of compounds (Rainey and Nakamura 2008; Igaz et al. 2008; Jansen et al. 2009; Schteingart 2009). The options for tumor treatment are surgery, chemotherapy and pharmacological inhibition of adrenal steroidogenesis. Metyrapone inhibits the enzyme, steroid 11 beta hydroxylase and blocks cortisol synthesis more than the synthesis of aldosterone. Trilostane blocks the 3 beta hydroxysteroid dehydrogenase and inhibits the synthesis both of cortisol and aldosteroneAminoglutethimide prevents the conversion of cholesterol to pregnenolone; it therefore blocks the synthesis of all subsequent steps of steroidogenesis, including hydrocortisones, aldosterone, and gonadal sterol and is (also including the conversion of androgens to estrogens). This compound has been used in oncology for treatment of breast cancer. Each of these inhibitors of adrenal steroidogenesis has been explored in a limited number of instances, selection largely depending on the individual circumstances and experience of the clinical center (Veytsman et al. 2009; Igaz et al. 2008). The experience with treatment of ACTH dependent Cushing's syndrome and Cushing's syndrome of adrenal origin is more extensive and has been summarized (Biller et al. 2008; Diez and Iglesias 2007; Nieman 2006; Alexandraki and Grossman 2010).

A recent exploratory approach to Cushing's syndrome by an ectopic ACTH secreting tumor has been the use of the progesterone receptor antagonist mifepristone (Baulieu 1991; Agarwai 1996; Raudrandt and Rabe 2003) previously used as an antiprogestagen. The antiglucocorticoid activity is being tested in Cushing's syndrome using the change in glucose control as the Primary Outcome Measure, and the change in clinical presentation ("features of Cushing's syndrome") as the Secondary Outcome.

C.9.4.6 Mineralocorticoid Antagonists

Aldosterone has been used by intramuscular injection for acute adrenal insufficiency. For practical substitution

therapy, fludrocortisone is administered orally. Spironolactone has been used in the treatment of primary hyperaldosteronism being a competitive antagonist of aldosterone (McInnes et al. 1981; 1982). In clinical terms, spironolactone is a potassium sparing diuretic in resistant hypertension. It has found important clinical indications for secondary hyperaldosteronism in cirrhosis of the liver and congestive cardiac failure. Currently ongo ing clinical studies are directed at indications in cardiology (low renin hypertension, atrial fibrillation, cardiovascular disease, diastolic heart failure, endomyocardial fibrosis), hepatology (alcoholic cirrhosis, steatohepatitis, portal hypertension), nephrology (renal failure, idiopathic hypercalciuria, hypokalemia caused by thiazide diuretics). Another group of indication is related to the anti androgenic activity of spironolactone (e.g., hirsutism, female pattern baldness, polycystic ovarian disease [PCOD]). Several of the studies aimed at hypertension and cardiac failure address the combination of spironolactones with new therapeutic entities such as aliskaren and satavaptan.

Eplerenone is another aldosterone antagonist which is found to have considerable application in resistant hyper tension and cardiac failure (Keating and Plosker 2004; Muldowney et al. 2009). For the clinical pharmacology of the aldosterone antagonist, eplerenone a study on the endocrine mechanism was performed. Primary outcome measures were the AUC for serum aldosterone and plasma renin activity (PRA) before treatment (day 0), on the first day of treatment (day 1) and on the 10th day of treatment (day 10) after daily single dose oral administration of Eplerenone 100 mg. Secondary outcome measurements were the single dose and multiple dose pharmacokinetics for Eplerenone; the safety and tolerability of Eplerenone is determined by adverse event with porting, the clinical laboratory results, and the changes in vital signs (supine blood pressure and heart rate) and changes in the electro cardiogram (ECG). To this evaluation of the secondary outcome measures, the molecular biology evaluation on day 0 and day 8 was added based on exploratory mRNA gene expression biomarkers, and changes of the mineral ocorticoid receptor and beta actin after administration of Eplerenone or placebo (NCT00990223).

A comparative study of 30 weeks duration is being performed to determine whether eplerenone is more effec tive than doubling the dose of ACE inhibitor in reducing urinary protein (albumin) loss in diabetes mellitus (NCT00315016). Primary Outcome Measures are the changes in proteinuria and blood pressure by home mea surements. Secondary Outcome Measures are clinical chemistry changes in serum, hamoglobin, urinary excretion of the biomarkers CTGF, TGF b, collagen IV, changes in renal function assessed by inulin and PAH clearance, changes in cardiovascular biomarkers measured during the studies are plasma aldosterone, renin, plasma angiotensins and bradykinins. Quality of Life is added as a secondary outcome measure for this 30 weeks study.

Recent studies with eplerenone have addressed the indications of hypertension and cardiac failure (George and Struthers 2007), and comparative efficacy of spironolactones and eplerenone (Struthers et al. 2008). As a topic of increasing interest for clinical practice, the pharmacoeconomics of eplerenone in post myocardial infarction heart failure have been evaluated (Croom and Plosker 2005).

C.9.4.7 Clinical Summary

The physiological effects of adrenal steroid hormones (glucocorticoid and mineralocorticoid) and their clinical relevance has been studied very extensively for many years, leading to a large array of synthetic derivatives with more specific action, in an ever increasing number of clinical indications.

The methods to be applied in investigation of their clinical pharmacology are extremely diverse, because of their wide dose range covered in many indications, and the risk of inducing long term adrenal involution/insuffi ciency by high dose therapy. There is a large number of ongoing clinical trials covering a wide array of topics. This is due to the fact that many of the early studies with adrenal steroid hormones are now extended to basic clin ical pharmacology, to address topics such as identification and validation of new biomarkers both for efficacy (sur rogate endpoints), and for safety assessment. There are also several studies which address genomics, in an early attempt toward individualized therapy. Many of the ste roid compounds are in the generic domain; numerous proprietary studies are ongoing on new pharmaceutical presentations.

A new and widening field for clinical pharmacology studies has been opened by the health economics and phar macodynamics aspects, health technology assessment and the requirement for randomized clinical trials, including clinical outcome measures rather than surrogate parame ters to firmly establish the place of specific adrenal steroid products in therapy. These RCT trials are frequently sum marized in meta analysis publications (Cochrane database system) and build the foundation for guidelines and rec ommendations, for example, in internal medicine, and by healthcare providers. The focus is changing from

investigating the clinical pharmacology of steroid drug substances and pharmaceutical presentations toward establishing the relevance of specific groups of adrenal steroids in therapy, when used as part of the clinical regimen together with other drugs, for example, in cardiovascular medicine, transplantation medicine, and oncology.

C.9.5 Thyroid Hormones

The thyroid hormones thyroxine (3,5,3′,5′ tetraiodothyronine, T4, and 3,5,3′ triiodothyronine, T3) are iodinated derivatives of thyronine. Thyroid specific enzymes are responsible for the iodination of tyrosine. Numerous thyroid hormone analogues have been synthesized but did not have practical benefits for therapy (Pittman and Pittman 1974). New methods of receptor pharmacology (Laugwitz et al. 1996) and mechanism based research on thyromimetics interacting with isoforms of the thyroid hormone receptor (Kraiem 2005; Grover et al. 2007; Scanlan et al. 2010) have been identified and established new compounds with the spectrum of activities different T3 and T4, designated as thyromimetics. The thyroid hormone receptor and its isoforms are well characterized (Latif et al. 2009), the involvement of the thyroid hormone receptor in proliferation of tumors is being investigated (Aranda et al. 2009).

Thyroid hormone preparations have been a mainstay of substitution therapy in hypothyroidism for many years, frequently the problems of bioavailability of different pharmaceutical presentations have been addressed. The clinical evaluation was based on improvement of symptoms caused by thyroid hormone deficiency, for example, fatigue, bradycardia, goiter, weight gain, cold intolerance, etc. The biochemical evaluation (Saravanan et al. 2007) is now based on, for example, serum free T3 and free T4, serum TSH (preferably by a high sensitivity assay), lipid profile (hypercholesterolemia) and is particularly relevant in case of subclinical hypothyroidism with the diagnosis difficult due to the absence of overt symptoms of thyroid hormone deficiency (Villar et al. 2007; AACE Guideline 2007). There has been a debate about the need for thyroid hormone substitution, based on the risk of rapid initiation of treatment, risk inherent to selection of T3 instead of T4, and penetration of the suitable dose range and avoiding myocardial ischemia, atrial fibrillation, and heart failure (Ochs et al. 2007). It is now standard of care that slow titration with thyroxine (T4) up to clinically required dose is preferable to initiating therapy with liothyronine (T3) due to the rapid onset of action when using T3, which may precipitate atrial fibrillation and heart failure in patients with ischemic heart disease.

A clinical trial is ongoing in patients who have had their thyroid gland removed (post thyroidectomy, NCT00106119) to examine how the available thyroid preparations levothyroxine (T4) and liothyronine (T3) affect fat and cholesterol metabolism, blood sugar regulation, and thyrotropin secretion. Results of the study may help to optimize blood sugar and cholesterol levels in some patients (individualized therapy). Patients included in the study are screened with a medical history and physical examination, blood tests, electrocardiogram (EKG) and neck ultrasound to visualize any remaining thyroid tissue. Thyroid medications are assigned and adjusted, if needed. Follow up visits are scheduled until the patient's thyroid hormone levels have been stabilized and they have maintained the same dose for at least 30 days. This study is a good example for a comprehensive evaluation program comprising all currently available options in methodology.

After dose adjustment, patients are then hospitalized for 5 days for the following tests and procedures: Blood tests to analyze thyroid hormones, lipids, glucose, electrolytes, clotting factors, kidney function, red cells, and DNA (Day 1), DEXA scan to determine percentage of body fat tissue (Day 1), and investigation directed at the fat mobilizing activity of thyroid hormones, thyrotropin releasing hormone (TRH) stimulation test to assess the response of serum T4 or T3. A test dose of TRH (5 ug) is intravenously administered to measure the response of TSH (thyrotrophic stimulating hormone). Blood samples are collected immediately before and after the TRH injections. This test is done three times over 3 days with increasing doses of TRH (Days 1, 2, and 3). A basic research question is addressed by subcutaneous fat tissue microdialysis to understand how T3 and T4 affect the activity of fat tissue (lipolytic activity). The test compound isoproterenol is injected subcutaneously in fat tissue of the abdomen, and fluid samples are collected from the area over a 2 h period (Day 2).

The cardiovascular activity of thyroid hormone substitution is addressed by tests, including exercise on a stationary bicycle with ECG recording; and repeat echocardiogram, ECG, and vascular endothelial function evaluation (Day 3).

An approach different from the biochemical physical investigations is a subject of change during therapy: Questionnaires on well being and eating habits are applied for this purpose (Day 3).

The effect on glucose regulation is addressed by an euglycemic hyperinsulinemic clamp to measure the effects of insulin. Insulin is infused through the catheter in the

arm, glucose is measured every 5 min from the catheter in the hand and adjusted by infusion to maintain levels in the normal range (Day 4). As a classical test for the metabolic effect of thyroid hormone substitution, indirect calorime try is included to evaluate how the body uses sugar to generate energy. Skeletal muscle biopsy is performed to find out how T3 and T4 affect muscle strength and its ability to store glucose (intramyocellular glycogen deposits and their mobilization) (Day 5). Fat tissue biopsy is done to find out how substitution with either T3 or T4 affects fat tissue size and its ability to store glucose optional (Day 5).

At the conclusion of these tests, patients are discharged from the hospital and enter the second phase of the study, in which all the procedures described above, from thyroid stabilization through the 5 day hospitalization, are repeated. This time, however, patients who were taking T3 now take T4, and vice versa. The time interval between the two hospitalizations depends on how quickly the thy roid hormone medical dose can be adjusted. The study is defined as Study Type: Interventional, Study Design: Randomized Clinical Trial (RCT), Intervention Model: Crossover Assignment and Masking: Double Blind (Subject, Caregiver, Investigator).

The predefined Primary Outcome Measures are insulin mediated glucose disposal (evaluation after 1 month of therapy), with Secondary Outcome Measures being cholesterol, triglycerides, and apolipoproteins; energy expenditure by indirect calorimetry; muscle strength by graded exercise tolerance test; and cardiovas cular function by echocardiogram, and vascular endothe lial function (evaluation after 1 month of therapy). Details for this unusually comprehensive study by the NIDDK are available from using the identifier NCT00106119 (Official Study Title: Peripheral Thyroid Hormone Conversion and Glucose and Energy Metabolism).

The pituitary hormone thyrotropin (TSH) stimulates synthesis of thyroglobulin and release of thyroxine and liothyronine into the blood stream (secretory function). TSH secretion is inhibited by elevated levels of blood T4 or T3 (negative feedback mechanism). Thyroxine (T4) is stored in the thyroid follicles in the form of thyroglobulin (a glycoprotein with iodinated tyrosyl residues). In the bloodstream, T4 and T3 are transported bound to serum proteins, principally thyroxine binding globulin, about 0.03% of T4 and 0.3% of T3 are present in free (physio logically active) form. The biological half life of T3 is 1 day, whereas that of T4 is 8 10 days. Thyroxine is the prohormone of the biologically more active triiodothyro nine, T3 has approximately ten times the biological activity of T4. Further formation of T3 (approximately 30 μg/day) occurs in the peripheral tissues as a result of the deiodination of T4. A second isomeric iodoamino acid, reverse T3, (L 3,3′,5′ triiodothyronine, rT3) is formed from T4 in the periphery but has no apparent physiological activity (Chopra et al. 1973, Farwell and Braverman 2006).

C.9.5.1 Recombinant Human Thyrotropin

Recombinant human TSH (rhTSH) is used for transient stimulation of iodine uptake, in preparation of radiother apy with 131 I (EMEA Thyrogen 2004; Emerson and Torres 2003; Molinaro et al. 2009). It is also used as a diagnostic procedure after thyroid ablation, for the absence of met astatic tissue if there is no response of thyroglobulin to TSH (Chen et al. 2010; Paz Filho and Graf 2008; Kraenzlin and Meier 2006; Bonnema and Hegedues 2009), and is being tested in goiter (Woodmansee and Haugen 2004).

The thyroid hormones regulate the adaptation of body temperature and metabolic rate to exogenous conditions, basal metabolic rate has been used as a clinical test (energy expenditure by indirect calorimetry). Levothyroxine (L T4) and triiodothyronine (T3) affect metabolism in a variety of ways. They regulate energy turnover (basal metabolic rate and stress related increments), water balance, and contrib ute to the metabolism of carbohydrates, proteins, fats, and minerals. The thyroid hormones also affect growth and maturation in children, with particular relevance for fetal and postnatal development. During pregnancy, there may be need for monitoring of thyroid function and thyroxine substitution; neonatal screening for hypothyroidism is essential, and immediate initiation of substitution is man datory for postnatal brain development. Monitoring of maternal and neonatal thyroid function is of particular importance in areas with endemic iodine deficiency. Pri mary Outcome Measures may be maternal thyroid function (time frame: 3 month intervals during pregnancy, at deliv ery), and Secondary Outcome Measures may be birth out come (time frame: at delivery), infant thyroid function (time frame: regular intervals up till 2 years of age), infant cognitive and motor development (time frame: regular intervals up till 2 years of age), maternal and infant urinary iodine (time frame: regular intervals during pregnancy up till 2 years after delivery) and breast milk iodine (time frame: 3 and 6 months after delivery) . These investigations are part of a study "Iodine Supplementation in Pregnant Women Living in Mild to Moderately Iodine Deficient Areas in India and Thailand: Effects on Pregnancy Outcome and Infant Development (NCT00791466)", as an example of clinical pharmacology for the time period from preg nancy until 2 years after delivery. A similar study of mater nal neonatal thyroid function is ongoing in China

(NCT00505479) to provide evidence about Iodine Status in Pregnant Women and Their Newborns: is Congenital Hypothyroidism Related to Iodine Deficiency in Pregnancy? This study was to determine whether pregnant women show evidence of iodine deficiency, and to examine the correlation between maternal urine iodine concentration and newborn thyroid function. Another study on "Thyroid Function Throughout Pregnancy With and Without Iodine Supplementation (NCT00831402)" was performed (1) to study maternal thyroid function during pregnancy with or without supplementation with pregnancy tablets fortified with iodine, (2) to establish reference values of thyroid function at different stages of pregnancy (3 trimesters), and (3) to design and implement a screening strategy of iodine deficiency in the French population and suggest recommendation for its prevention. Primary Outcome Measures were the intra individual variation of maternal thyroglobulin between the first trimester and the delivery and cord blood thyroglobulin with comparison of the two groups (control and supplementation with iodine) (time frame: every 3 months up to the give birth, the day of a give birth, and after 3 months) . Secondary Outcome Measures were they comparison of control and treated groups for : ● frequeny of miscarriage, duration of gestation, birth weight, APGAR, neonatal complications, maternal thyroid function, frequency of post partum thyroiditis,cord blood thyroid function (time frame: every 3 months up to the give birth, the day of a give birth, and after 3 months). Such studies in particular age groups at risk for iodine deficiency and benefiting from adequate supplementation are an important task for clinical pharmacology studies in preventive medicine. The study results contribute to guidelines and recommendations for obstetrics and neonatology.

The parafollicular C cells of the thyroid produce calcitonin (thyrocalcitonin), a peptide hormone, which participates in the regulation of calcium metabolism and also contributes to diagnostic evaluation of post treatment follow up after radiotherapy of thyroid cancer, in particular for metastatic disease. Another peptide hormone, parathormone, which also influences calcium metabolism, is secreted by the parathyroid glands, detailed are addressed in the section on calcitropic hormones.

Several synthetic compounds that inhibit the biosynthesis of thyroid hormones have been found, they act primarily by inhibiting thyroid hormone iodination, or by restricting the uptake of iodine into the thyroid gland (Murad and Haynes 1980; Farwell and Braverman 2006; Bennett and Brown 2008; Dong 2009). Of practical relevance are methimazole and its active metabolite carbimazole, and propylthiouracil. This group of the thyroid study drugs has a marked potential for adverse effects including urticaria, arthralgia, fever anorexia and nausea, the major effects include agranulocytosis, thrombocytopenia, acute hepatic necrosis, cholestatic hepatitis and glucose like syndrome.

C.9.5.2 Physiology of Thyroid Hormones

Free thyroxine and especially free triiodothyronine bind to the nuclear and mitochondrial receptors, thereby activating protein synthesis via adenosine triphosphate production. The primary effects of the thyroid hormones are (1) stimulation of carbohydrate turnover, growth, and maturation in the central nervous system, skeletal and muscular systems, and genital organs; stimulation of heat production, oxygen consumption, cholesterol metabolism, turnover of free fatty acids, muscle contraction, heart rate, and cardiac output; and (2) inhibition of glycogen and protein synthesis (at supra physiological concentrations). The amount of thyroid hormones required for substitution in hypothyroidism varies between 50 and 300 μg/day, depending on the individual metabolism.

C.9.5.3 Pathophysiology

Thyroid diseases can affect the morphology (goiter, nodules, carcinoma) or the function (hyperthyroidism, hypothyroidism) of the thyroid. In many cases, thyroidal autoantibodies or iodine deficiency is the cause of the thyroid disorder. Hyperthyroidism is characterized by tachycardia, sweating, weight loss, diarrhoea, etc. An adequate supply of thyroid hormone is essential for postnatal intellectual development hypothyroid children show abnormal physical and mental development. Hypothyroid adults tend to react slowly or to be depressed; additional symptoms are dry skin, constipation, and sensitivity to cold. Specific studies on the effect of liothyronine in the central nervous system, in particular in depressive disorders are ongoing, for example, Liothyronine (T3) for Bipolar Depression (NCT00790738), Triiodothyronine (T3) Supplementation in the Treatment of Bipolar and Unipolar Depression (NCT00158990), and Biochemical Brain Changes Correlated With The Antidepressant Effect Of Thyroid Hormones (NCT00562367).

The diagnostic evaluation of thyroid function and the detection of thyroid disease is based on clinical symptoms and is confirmed by the measurement of serum concentrations of free T4, free T3, and serum TSH (basal or stimulated in the TRH test). The clinical relevance of dynamic function tests based on stimulation by TRH

injection or nasal administration is now being disputed, to some extent the result of high sensitivity serum TSH assays have supplemented information for the diagnostic evaluation of low serum TSH. Relevant clinical information is obtained by methods of measuring on thyroid uptake of radioiodine, iodine turnover, and detailed exploration of the pituitary thyroid feedback system. More importantly in clinical terms is the measurement of thyroid autoantibodies, which are frequently detected in disorders of thyroid function.

Substitution therapy in thyroid hormone deficiency (subclinical or overt hypothyroidism, myxoedema) is provided by oral administration of thyroid hormones, preferably by L thyroxine (Farwell and Braverman 2006; Bennett and Brown 2008; Dong 2009). Treatment with levothyroxine is preferred because of the long half life when compared with triiodothyronine. Levothyroxine is absorbed to the extent of 70–85%, depending on the pharmaceutical formulation. The daily dose of levothyroxine varies between 50 and 200 μg, depending on the severity of thyroid deficiency. The daily dose of liothyronine is correspondingly lower (10–100 μg). Injectable thyroid hormone solutions are used in cases of severe hypothyroidism (myxoedema coma). Preparations containing both thyroid hormones at a fixed rate are also available, until now no clinical benefit for comedication of thyroxine plus liothyronine has been confirmed by clinical trials, although such studies are still ongoing.

The marked effect of hyperthyroidism (Graves' disease) on weight loss has prompted studies on the use of liothyronine/thyroxine as antiobesity drugs. No evidence whatsoever has been obtained for a consistent therapeutic effect of weight reduction, whereas the cardiovascular risk was soon evident, and atrial fibrillation as well as heart failure of the obvious complication at higher dose. For sometime, dextronine was advocated as a lipid lowering agent; there are today much better options, for example, in the group of statins.

The pathophysiology of obesity however remains to be understood and presently the surprising results of markup and consistent weight reduction by bariatric surgery are being followed up with regard to the effects on glucose regulation and lipid lowering. There is currently an ongoing study on "The Effect of Bariatric Surgery on Thyroid Function and Morphology". The study is designed to to evaluate the effect of dramatic weight loss after bariatric surgery on thyroid function (thyroid hormone levels and particulary morphological changes) in the short and long term setting of a cross sectional study. This is a longitudinal study with an intent to establish a correlation of preoperative thyroid function abnormalities and postoperative changes over time with respect to the type of bariatric intervention carried out (there are several methods of different technique and impact). Primary Outcome Measures are the measurement of change in thyroid hormone levels preoperative, and 3, 6 and 12 months postoperative. Secondary Outcome Measures of the morphological changes of the thyroid gland followed up to 12 months postoperatively.

C.9.5.4 Thyroid Cancer

There are several ongoing clinical trials addressing particular aspects of thyroid cancer, for example, types of thyroid cancer that seem to cluster in families. Non medullary thyroid cancer accounts for the vast majority of all types of thyroid cancer, but little is known about possible genes that may cause the cancer. More research is needed to develop the best ways to screen for familial non medullary thyroid cancer (FNMTC) so that it can be diagnosed and treated at an early stage. In an ongoing study, the study objectives are to evaluate the natural history of FNMTC, to determine the best screening strategy for FNMTC, and to identify genes that may indicate susceptibility to FNMTC (Official Title: Clinical and Genetic Studies in Familial Non Medullary Thyroid Cancer, NCT01109420, National Cancer Institute NCI). It is currently not possible to envisage or predict the impact that this research on genetic background (genomics and proteomics) of thyroid cancer may have on future therapies in oncology, always involving, of course, thyroid hormone substitution once the primary and metastatic cancer has been down sized or eradicated. There is obviously a wide field of basic studies in clinical pharmacology to be covered, before individualized therapy can be designed and implemented. Treatment of thyroid cancer (primary are metastatic) by radiotherapy with 131 I is a highly specialized clinical specialty. There are two options of preparing patients for radiotherapy, either by increasing iodine uptake after temporarily removal of thyroid medication (hypothyroid conditions), or by stimulating iodine uptake during thyroid medication by injection of recombinant human thyroid stimulating hormone (rhTSH, thyrotropin). Several studies have been performed comparing these protocols. In "A Dosimetry Study of Radioiodine (131 I) Uptake Following the Administration of Thyrogen and Hypothyroid States During Thyroid Hormone Withdrawal" (Phase IV, NCT00001730 by the NIDDK), the study was described as a multi centered, open labeled, randomized, two parallel arm study designed to compare quantitative radiation dosimetry assessments obtained during thyroid hormone suppression therapy with recombinant human TSH (Thyrogen® (Registered

Trademark)) and hypothyroidism in thyroid cancer patients preparing for post surgical radioiodine ablation. By protocol: The primary endpoint of this study is to identify the ratio of administered activity of radioiodine (131I) to deliver a targeted dose of 30,000 rad to the thyroid remnant when patients are euthyroid on Thyrogen® (Reg istered Trademark) and hypothyroid after hormone with drawal. Secondary endpoints are to identify and compare effective 131 I clearance and cumulated activity in the whole body and blood during euthyroid and hypothyroid states. Participants will undergo two 131 I whole body scans: one after injection of rhTSH (Thyrogen®) while taking thyroid hormone suppressive therapy and the sec ond after withdrawal from thyroid hormone. 131 I abla tive therapy will be given under hypothyroid conditions at the completion of the dosimetry study.

Observational studies have targeted the exposure to radioactive iodine from military resources and after acci dents. Among the ongoing studies are those on the pathological effects of radioisotope exposure after the Chernobyl plant accident (Studying Thyroid Cancer and Other Thyroid Diseases in Participants Who Were Children in Ukraine During the Chernobyl Nuclear Power Plant Accident), in this study, measuring changes in the thyroid gland after radiation exposure may help doctors learn about the long term effects of radiation exposure and help the study of thyroid cancer and other thyroid diseases. This clinical trial is studying thyroid cancer and other thyroid diseases in participants who were children at the time of the Chernobyl nuclear power plant accident, to carry out valid and credible assessments of the early and late morphologic and func tional changes in the thyroid glands of individuals exposed to radiation from radioactive materials released in 1986, and examine other possible risk factors, including dietary iodine intake during and after 1986 and the ingestion of potassium iodide for thyroid protection shortly after the accident. Similar study objectives are directed at "Studies of Thyroid Abnormalities in Northeastern Kazakstan Associated With Nuclear Weapons Testing" (National Cancer Institute NCI), applying a wide spectrum of methods for assessment of the effect of high dose radia tion on a large population. The prevalence of thyroid nodules and cancer in relation to radiation dose is inves tigated, in a defined cohort of Kazakstan residents exposed as children to radioactive fallout from atomic bomb tests at the neighboring Semipalatinsk Test Site (STS). The population near the STS is believed to have received radi ation doses from fallout that were much higher than that experienced by any population of comparable size in the USA. The study population is a defined cohort of 20,000 residents, half of whom, in 1960, resided in heavily exposed villages; the other half lived in lightly exposed villages. The population is rural, with a diet that was and is heavily dependent upon fresh milk from household or local cows and therefore likely to have led to ingestion of radioactive iodine from fallout. The first part of the study involves a cytogenetic assay for radiation biodosimetry purposes of peripheral lymphocytes obtained from blood samples donated by 40 cohort members with individuals radiation dose estimates. Blood samples will be collected from 25 putative high dose and 15 low dose cohort mem bers and processed or cytogenetic assay using fluorescent in situ hybridization (FISH) for stable chromosome aber rations in peripheral lymphocytes. The second part will involve thyroid screening by ultrasound in selected villages. The population to be screened will comprise 100 1,500 members of the study cohort exposed as young children to high fallout levels, and equal numbers of comparable ages exposed to little or no fallout. Fine needle aspiration biopsy will be performed, under separate informed consent, if the palpation and ultrasound results suggest presence of a tumor. Presence and malignancy of tumor will be deter mined by cytopathology. The most sensitive statistical com parisons are expected to be dose response analyses with respect to prevalence of thyroid nodules, which are common and known to be associated with radiation dose. Compari sons in terms of thyroid cancer, and benign and malignant neoplasms combined, are likely to be less sensitive but of acceptable power if risks associated with chronic radiation in this population are similar to those associated with acute exposure to X ray or gamma radiation in other populations.

There is an increasing array of clinical pharmacology studies contributing to the understanding of basic mech anisms in oncology, related to the development and pro gression of thyroid cancer, and also to the exploration of genomics and proteomics related to specific patient populations. A study by the NCI on "Clinical and Genetic Studies in Familial Non Medullary Thyroid Cancer" is ongoing, the NIDDK has started a large investigation enti tled "Studies on Thyroid Nodules and Thyroid Cancer" with inclusion of numerous specific oncology methods The purpose of this study is to evaluate methods for preoperative diagnosis and therapy of thyroid cancer and to screen patients for participation in other protocols. Study subjects will include adults and children with thyroid nodules or cancer requiring diagnostic fine needle aspira tion biopsy, surgery, radioiodine scanning or therapy for persistent or recurrent disease.

The use of methods for follow up of patients using radiopharmaceutical tracers such as 131 I, 123.I, 201T1 chloride, (99)mTc Sestamibi, (111) Inpentetreotide, and

18 FDG PET will be evaluated. All radionuclides will be administered according to standard clinical practice indi cations and published guidelines. The limitations and significance of serum thyroglobulin (Tg) measurement for diagnosing tumor recurrence will be assessed. The study will permit a continued evaluation of the risk/ben efit ratio of already established methods of administering 131 Itherapy including the impact of pre treatment dosi metric calculations and administration of lithium (a well established, yet not widely used, adjuvant to 131öI treat ment), especially in selected cases of thyroid cancer in which high dose (greater than 150mCi) 131 I therapy is clinically indicated. Samples of benign nodules and cancer tissue specimens for research studies will be collected to assess new immunohistochemical markers, and other techniques to characterize tumors for correlation with response to therapy and prognosis. Blood specimens will be collected for future clinical and research studies in both the hypothyroid and euthyroid state.

Details for another large scale study "Multicenter Study Differentiated Thyroid Carcinoma" (University Hospital Muenster, Germany, NCT00144079) address the clinical benefit of adjuvant external beam radiotherapy (RTx) for locally invasive differentiated carcinoma (TNM stages pT4 pN0/1/x M0/x; 5th ed. 1997) of the thyroid gland (DTC). Patients are treated with surgery (thyroidec tomy and lymphadenectomy), radioiodine therapy (RIT) to ablate the thyroid remnant tissue, and TSH suppressive L thyroxine therapy with or without RTx after documented elimination of cervical I 131 uptake. The study is based on clinical outcomes. Primary Outcome Measures are time to local or distant failure, and cancer related mortality. Secondary Outcome Measures are acute toxicity of radiotherapy (RTOG), chronic toxicity of radio therapy (RTOG), and quality of life. One specific focus is the post radiotherapy substitution with thyroxine with regard to dose finding and measurement of serum TSH, hTG, and anti Tg antibodies.

C.9.5.5 Antithyroid Drugs

In the treatment of hyperthyroidism there are two clinical options: (1) Thionamides which block the synthesis of thyroid hormone, (two) radioiodine therapy with 131 I used to destroy the hormonally active cells of the thyroid gland after selective uptake of 131 I. For short term inhi bition of the production of thyroid hormones, high doses of iodide can be administered.

Thyrostatic compounds are the option when radiother apy is not immediately considered or when there are contraindications for radiotherapy. The major action of thionamides is to reduce the formation of thyroid hormone by inhibiting oxidation and organification incorporation into Ireland of thyroid means. There is a lag phase in reaching the maximum effect until existing hormone stars are exhausted, this period may require weeks of treatment. When using high dose, the reduced hormone synthesis may lead more rapidly to hypothyroidism. Propylthiouracil dif fers from carbimazole and methimazole in that it also inhibits peripheral conversion of T4 to T3. This occurs however only at the higher doses used in the treatment of thyroid storm, as a complication of thyrotoxicosis.

A specific condition that may require thyroidectomy is the complication of Graves' orbitopathy sometimes associ ated with Graves' disease. This is an example of emergency treatment ("The Effect of Early Total Thyroidectomy in the Course of Graves' Orbitopathy, University of Ankara, NCT01056419)". The relationship between the method of the treatment of hyperthyroidism due to Graves' disease and the course of Graves' ophthalmopathy is debated. The inves tigators aimed to compare the results of total thyroidectomy done in 6 months following the appearance of the symptoms of ophthalmopathy and the antithyroid drug therapy in patients with moderate to severe Graves' ophthalmopathy.

The inclusion criteria described are hyperthyroidism and moderate to severe Graves' ophthalmopathy within 6 months, thyroid volumes greater than or equal to 15 ml in thyroid ultrasonography, patients taking no treatment except local medications for Graves' ophthalmopathy, clinical activity score of 3/7 or more, proptosis greater than or equal to 21 mm in one eye or 2 mm difference between two eyes, presence of diplopia, the opening of the eye lid greater than or equal to 9 mm.

All patients will be treated with antithyroid drug until TSH levels of the patients are between 0.4 1 . During this period all the patients will take pulse methyl prednisolone treatment of a total dose of 4.5 gr. After pulse steroid treatment the patients will be randomized to two groups: one group will be sent to surgery for total thyroidectomy, and their TSH levels will be kept between 0.4 and 1 with levothyroxine treatment; the other group will be followed under antithyroid drug treatment and their TSH levels will be kept between 0.4 and 1. Primary Outcome Measure is the improvement in the proptosis and activity of Graves' ophthalmopathy (assessed after 12 months).

C.9.5.6 Thyromimetics

Selective thyromimetics have been designed and shown to exhibit some of the beneficial effects of thyroid hormones,

such as lowering of cholesterol and weight reduction, without the adverse thyroid hormone action on muscle, bone, and heart rate (Kraiem 2005).

The search for new compounds with hormone activity related to the physiological model of thyroid hormones has focused on identification of new molecules by their affinity for and binding to isoforms of the thyroid hormone receptor (Taylor et al. 1997; Latif et al. 2009; Aranda et al. 2009). Extensive structure activity studies have been reviewed (Hirano and Kagechika 2010), covering a wide range of molecules. The structures of most thyromimetic compounds are based on those of endogenous thyroid hormones, which consist of a biaryl ether skeleton substituted with iodine, alpha alanine moiety and hydroxyl group at two benzene rings.

These thyromimetics are subtype or tissue selective TR agonists and antagonists, and their potential to become novel therapeutic agents is being investigated, especially in the field of metabolic diseases where the indication envisaged is related to hypercholesterolemia and obesity. Thyromimetic compounds selective for the liver or for the thyroid hormone receptor isoform beta 1 may constitute a novel approach for the treatment of dyslipidemia (Tancevski et al. 2009). In preclinical studies, selective thyromimetics significantly reduced plasma cholesterol levels and provided protection from atherosclerosis by upregulating the hepatic LDL receptor and promoting reverse cholesterol transport. Data from ongoing clinical trials have provided the first evidence that selective thyromimetics may also reduce the levels of plasma cholesterol in humans (Scanlan 2010). Sobetirome,(GC 1, QRX 431) is a member of a class of compounds known as selective thyromimetics. These compounds are synthetic structural analogues of thyroid hormone that have tissue specific thyroid hormone actions. Many of the compounds in this class, including sobetirome, have been identified as being subtype selective thyroid hormone receptor (TR) agonists. Sobetirome selectively binds to and activates the TRbeta over TRalpha isoform of the thyroid hormone receptor (TR).

C.9.5.7 Clinical Summary

Studies on the physiology and pathophysiology of thyroid hormones indicate their relevance in metabolic regulation, internal medicine, ophthalmology (Graves orbitopathy), oncology, obstetrics, neonatology and pediatrics, as well as affective disorders. In addition to the established therapy, there is an increasingly wide range of clinical pharmacology studies focusing on the genetic background of thyroid disease including tumors. The results of a new development of compounds interacting with thyroid hormone receptor isoforms (thyromimetics) may have their place in regulation of lipid metabolism.

C.9.6 Gonadal Steroid Hormones

The clinical pharmacology of the gonadal steroid hormones is unusually complex. Steroid hormones share cholesterol as their common precursor, the gonadal steroids produced in the ovary (estrogens and progestagens) and in the testis (androgens) are important throughout life: for the development of the female reproductive system and secondary sex characteristics, for cyclic function of the ovaries throughout the reproductive phase of life, and for maintenance of organ function during the postmenopausal phase of life (estrogen dependent organs, e.g., bone), for the development of the male reproductive system and its function throughout the reproductive phase, and for maintenance of function during aging (androgen dependent organs). The pharmacology of gonadal steroid hormones and their synthetic derivatives, as well as that of drugs acting on the reproductive system has been extensively reviewed, with references to the nuclear hormone receptors involved, agonists and antagonists at sex steroid receptors, inhibitors of gonadal steroid synthesis acting on specific steps in steroid biosynthesis (Bennett and Brown 2008; Chrousos 2009; Loose and Stancel 2006). The sex steroids have important psychological effects on behavior at different periods of life, and their psychological effects in affective disorders are increasingly being recognized. Clinical pharmacology of natural estrogens, progestagens and androgens has been extensively studied based on their physiological effects (estrogens and androgen dependent organs), biochemical effects induced by these steroids, biomarkers activated as a consequence of their action, and the secretion profiles throughout life have been measured and describes (e.g., cyclic changes of estrogens and progestagens during the menstrual cycle, diurnal patterns of testosterone secretion in men. There is an extensive array of methods for measurement of the gonadal steroid hormones, with references values for the different stages of the menstrual cycle, and for the range of values found throughout reproductive life, after the menopause and in aging men during the late period of life.

Estrogens and *progestogens* are endogenous hormones with physiological actions on the gonadal system and on other organ systems, behavioral and effects on the central nervous system.

In women, these include developmental effects, neu roendocrine actions involved in the control of ovulation, the cyclical preparation of the reproductive tract for fer tilization and implantation, and major actions on mineral, carbohydrate, protein, and lipid metabolism (Loose and Stancel 2006). The pharmacological preparations of gonadal steroid hormones, their agonists and antagonists are used for substitution of hormone deficiency (post ovariectomy, menopause), for regulation of gonadal func tion (contraception, Sherif 1999), in reproductive disor ders (e.g., endometriosis, leiomyoma), and increasingly for indications relying on their specific receptor affinities e.g., for bone protection of osteoporosis, and as neoadjuvant or adjuvant therapy in oncology (selective estrogens modula tors, selective progesterone modulators).

C.9.6.1 Estrogenic Compounds

There are now numerous clinical research trials ongoing, to characterize specific detail of hormone action in healthy volunteers and in patients with disease conditions requir ing treatment. Such studies are directed at finding suitable biomarkers, establishing baseline parameters for clinical studies with existing and new drugs (active pharmaceuti cal ingredients, and in many cases new specific pharma ceutical formulations).

For the substitution therapy in hypogonadism, there are a number of pharmaceutical presentations of estrogens which may be considered based on their convenience for the patient, e.g., by depot injection, transdermal patch (Cisternino et al. 1991), Suitable outcome measures may be changes in body composition, rates of lipid oxidation, changes in plasma lipids, and concentrations of growth related Insulin like Growth Factor (IGF I). In a study planned for 1 year duration with estrogens administered orally or by a patch, changes in body com position and bone mineralization will be assessed by DEXA scan, and the dose titration of estrogens will be based on measuring serum estrogen concentrations achieved during therapy (patients with Turner's syndrome).

A much more controversial topic is the substitution of estrogen deficiency after menopause. Numerous studies have addressed the benefit/risk evaluation of hormone substitution during the early post menopause and at later stages of life, with particular references to cardiovascular effects (Humphrey et al. 2002; Garbe and Suissa 2004; Bromley et al. 2008; Sare et al. 2008), components of the metabolic syndrome (Salpeter et al. 2006), effects on bone and connective tissue due to estrogen decline (Torgerson and Bell Syer 2001; Wells et al. 2002) or estrogen suppression as found in endometriosis (Sagsveen et al. 2003), and on cognitive function with advancing age, including Alzheimer's disease (Hogervorst et al. 2009). The clinical problem of hormone substitution may be particularly important in cancer survivors after the men opause, some studies were performed in patients after surgical removal of breast tumors (Biglia et al. 2004). There are some studies in clinical research which address specific aspects of developing methods for evaluation. One such study is based on the effect of 17ß estradiol adminis tration on inflammatory immune cells, namely antigen presenting cells (monocytes/ dendritic cells), and their acti vation by inflammatory stimuli. This study by the University of Toulouse will address menopausal women with regard to their propensity to develop e.g., rheumatoid arthritis, and will allow to investigate a protective effect of estrogens substitution.

In estrogen substitution studies (Centro A.F. de Estudios Tecnologicos NCT00775242) , primary outcome measures may be the change of frequency and intensity of vasomotor symptoms within predefined time period of, for example, 1 year, with secondary outcome measures being the frequency and intensity of vulvar and vaginal atrophy symptoms, vaginal pH, index of vaginal matu ration (surface, intermediate and parabasal cells), karyopyknotic index, lipids profiles, and in addition spe cific assessment by quality of life inventories (quality of life scale, Green climacteric symptoms scale). In this particular study, the injection of microspheres containing estradiol and progesterone is being evaluated. In another study comparing 17 beta estradiol/ dihydrogesterone versus tibolone (NCT00145522), the primary outcome measure is the concentration of insulin like growth factor.

(IGF 1) values during a study period of 1 year, the Secondary Outcome Measures being other breast metabolic markers (IGFBP 1 and 3, SHBG, free estradiol, fasting insulin), breast tenderness and breast density, cardiovascu lar metabolic markers, menopausal symptoms and bleeding pattern (study duration 1 year).

A different study with transdermal versus or estrogenic therapy in healthy menopausal women will investigate the concentration of free testosterone, with Secondary Out come Measures being changes in thyroid binding globulin concentrations and tests of thyroid function, as well as changes in cortisol binding globulin and total and free cortisol concentrations. Such interventional studies with the design of a randomized control trial and crossover assignment are particularly suitable for development of pharmaceutical presentations containing drug substances with established effects on biomarkers and menopausal

symptoms, in the long term they need to be followed by clinical outcome studies.

In the *Midlife Cholesterol Study* (Northwestern Univer sity, NCT00361075), the effect of estrogens substitution on lipid profiles is explored, comparing an oral presentation of estrogens and progesterone with a transdermal oestradiol patch. The study design is based on previous evidence that the postmenopausal state is associated with an increase risk for heart disease. The increase in risk may be due to the effect of decreasing estrogens on blood lipids (blood fatsEstrogen replacement therapy seems to have a beneficial effect on lipid levels. The purpose of this randomized controlled trial is to understand (1) how menopause affects lipids and (2) how hormone replacement therapy affects the lipid metabolism of postmenopausal women. The detailed study description states that women with the met abolic syndrome (central obesity, insulin resistance, and dyslipidemia) are at especially high risk for coronary heart disease (CHD). The prevalence of the metabolic syn drome increases with menopause and may partially explain the acceleration in CHD after menopause. Men opause is associated with increased central adiposity, insulin resistance, dyslipidemia (hypertriglyceridemia, increased low density lipoprotein (LDL), reduced high density lipoprotein (HDL) and small dense LDL parti cles), and increased thrombotic/inflammatory states, but there are no studies investigating the mechanisms that mediate these changes. The objective of the study is there fore to investigate features of the metabolic syndrome in women followed prospectively through the menopause and determine if these features can be reversed with transdermal estrogen. Study how does this is that the increase in central adiposity with menopause will be a major contributor to the increased prevalence of the Metabolic Syndrome. The study is intended to investigate the effects of menopause and their correction by estrogen replacement therapy (ERT) (oral vs. transdermal). The increase in central (intraabdominal) fat with menopause will be monitored and the associated changes in lipids, insulin resistance, adipocytokines, and fibrinolytic/inflammatory markers. Due to the particular focus on lipids, the primary outcome measure will be LDLparticle size and density; with second ary outcome measures being total body adiposity (dexa scans); intra abdominal fat (CT scans); lipid profile; inflammatory factors; and adipocytokines. The study design is obviously in line with and based on similar ongo ing studies in diabetes type II and obesity, where the bio chemical parameters and biomarkers are quite similar to those selected for the *Midlife Cholesterol Study*.

In a clinical research study entitled "Biological Mech anisms of Arterial Stiffening With Age and Estrogen Deficiency" performed in perimenopausal women of the National Institute on Ageing (NCT00608062), the pri mary evaluation is for changes of the arterial system and their prevention by transdermal oestradiol patch substi tution. The primary outcome measures is arterial stiffness (carotid artery compliance) during saline and ascorbic acid (measured before and after treatment) (designated as safety issue: no), and the secondary outcome measures are those from cardiovascular research studies, namely endothelial function brachial artery flow mediated dilation , endothe lial cell protein expression, and biochemical parameters from serum or plasma blood samples endothelin 1, catechol amines, angiotensin converting enzyme, oxidative stress markers, IL 6, CR, sex hormones, ascorbic acid, glucose, insulin (as in other studies), and the carotid and brachial blood pressures will be monitored before and after treatment.

The psychological effects of estrogens substitution are of significant interest for clinical research, in the context of affective disorders and premenstrual dysphoric syndrome. Similar clinical research exploration for the effects of tes tosterone substitution on mental status and affective dis orders will yield much additional information of interest.

C.9.6.2 Progestagens, Progestins

There are a number of progestational steroid used either in combined oral contraceptives (COC), by a single dose administration for specific indications, or for their thera peutic effect in specific patient populations (examples are gestodene, desogestrel, medroxyprogesterone acetate, megestrol acetate Drospirenone).

C.9.6.3 Contraceptives

There is now a wide selection of contraceptive methods and pharmaceutical presentations available (Bennet and Brown 2008), based on very extensive clinical pharmacol ogy evaluation, both by clinical research on mechanisms of contraceptive action of estrogens and progestagens, and by large scale postmarketing studies. The selection is from combined oral contraceptives (COC) containing an estro gens and progestagens, progesterone only preparations (POP), depot injections, locally acting preparations (trans dermal patches), vaginal rings (NCT00455156), and intra uterine devices (e.g., copper T380A), and post coital emergency contraception by a single dose administration. For long term satisfaction of users, a study in Canadian women is being performed (NCT00653016) with the offi cial title: "An Open Label Multicentre Study to Evaluate Patient Satisfaction and Preference for the EVRA

Transdermal Contraceptive System Compared to Previously Used Contraceptive Method," and similar studies have been conducted for most of the combined oral contraceptives.

The search for a male contraceptive is ongoing, a number of clinical research projects are underway. In one example, healthy male volunteers receive a new hormonal contraceptive consisting of an implant releasing a hormone and hormone injections in order to investigate the suppressive effect on sperm production and reversibility of sperm production after end of treatment (NCT00403793). The evaluation is based on a primary outcome measure for percent of men who had a sperm concentration of 1 million/ml or less at week 16, the secondary outcome measures being semen parameters at several timepoints during treatment, the reversibility of suppression (a recovery of spermatogenesis after treatment), measurement of hormone concentrations including pituitary hormones and endogenous steroid hormones, pharmacokinetics of test compounds and monitoring of general tolerance (adverse event profile, assessment of well being by questionnaires).

A particular clinical condition that presents specific questions is polycystic ovarian syndrome (PCOS). The condition is often associated with obesity and metabolic syndrome, the addition of metformin was found to be effective in restoring fertility. In a contraceptive trial "Combination Metformin and Oral Contraception for PCOS (NCT00682890), the effect of adding metformin in a combined oral contraceptive is being explored. The purpose of this research study is to determine if adding metformin to birth control pills will reduce the risk of developing type 2 diabetes, high blood pressure. high lipid levels and heart disease in women with PCOS. Inclusion criteria for the study are PCOS women between the ages of 18 45, with less than eight periods annually, elevated serum free testosterone, normal thyroid function tests and serum prolactin, exclusion of late onset adrenal hyperplasia, acceptable health based on interview and medical history, physical exam and lab tests, ability to comply with the requirements of the study, and to provide signed, witnessed informed consent (the latter requirement being, in general, for clinical studies, in particular those for clinical research).The primary outcome measure in this study, in a similar manner as for other studies on metabolic syndrome is improved insulin sensitivity after a treatment period of 3 months. Similar questions are addressed in trials with specific populations of test persons requiring contraception. In a multicenter, open label, uncontrolled study the impact of weight and body mass index (BMI) on inhibition of ovulation of a transdermal patch formulation containing ethinylestradiol and gestodene in young female volunteers is assessed over a period of three treatment cycles.

C.9.6.4 Testosterone

Testosterone supplementation is being investigated in men and to some extent also in women. The pharmaceutical presentations for men are testosterone undecanoate for injection and to a limited extent for oral administration, testosterone patches and gels for local administration. The indications named in the numerous clinical trials are erectile dysfunction; hypogonadotrophic males, aging; frail elderly; rehabilitation, and hypoactive sexual desire disorder, all referring to substitution where the serum concentrations of free testosterone have been measured and found to be in the lower range. In order to restore fat free muscle mass, and change the pattern of body composition in the elderly, the state in aging man is referred to as sarcopenia. A large study of clinical research, "The Testosterone Trial" is underway supported by the National Institute on Aging (NIA), National Institute of Neurological Disorders and Stroke (NINDS), Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Heart, Lung, and Blood Institute (NHLBI), and Solvay Pharmaceuticals (NCT00799617). The Testosterone Trials are a multi center set of trials involving 12 clinical sites of the USA. The primary specific aims are to test the hypotheses that testosterone treatment of elderly men whose serum testosterone concentrations are unequivocally low and who have symptoms and objectively measured abnormalities in at least one of five areas that could be due to low testosterone (physical or sexual function, vitality, cognition, and anemia) will result in more favorable changes in those abnormalities than placebo treatment. For this randomised clinical trial of 1 year duration, the primary outcome measures will be physical improvement (improved walking speed), improvement in sexual activity, improvement on the vitality scale and verbal memory test, and anemia correction. The test medication is a locally applied testosterone gel. Restoring sexual activity is the aim of a study (NCT00244023) co administering testosterone with phosphodiesterase PDE5 inhibitors in patients with erectile dysfunction who have previously not responded to the treatment with PDE5 Inhibitors alone (20 mg Tadalafil and Vardenafil, 100 mg Sildenafil). Other similar studies are ongoing, primary and secondary outcome measures being questionnaires for erectile function (Orgasmic Function, Sexual Desire, Intercourse Satisfaction and Overall Satisfaction Domains). Clinical research is directed at physiological effects on aging men

(Testosterone and Lipolysis, Insulin Sensitivity and Protein Metabolism). Pharmaceutical presentations of a particular relevance are injections of testosterone undecanoate. In one of the studies (NCT00613782) particular aspects of the metabolic syndrome are being addressed. The rationale is that in men with type 2 diabetes, low testosterone levels have been associated with insulin resistance, truncal obesity and symptoms such as fatigue and erectile dysfunction. Low testosterone may impair cardiac function and increase cardiovascular risk and cause osteoporosis. In this study questions to be addressed are whether in men with type 2 diabetes mellitus and low testosterone levels, testosterone replacement improves insulin resistance, body composi tion, bone density, cardiac function symptoms associated with low testosterone level. The primary outcome measure is the effect on insulin resistance (study duration 40 weeks), secondary outcome measures are based on bone microarchitecture. Laboratory studies will consist of blood tests for total testosterone, fasting glucose, C peptide, and HBA1c. Imaging studies (at baseline and after 40 weeks of treatment) are the body composition and bone mineral density by DEXA, body composition by magnetic reso nance imaging, bone micro architecture by high resolution quantitative computed tomography [HR pQCT]), and car diac dimensions and function by transthoracic doppler echocardiography. A study of 1 year duration on "The Effect of Testosterone Therapy on Angina Threshold and Atheroma in Patients With Chronic Stable Angina" (NCT00131183) will apply to both cardiovascular investi gation and psychological inventorying, the Primary Out come Measure being the Change in time to ST segment depression of >1 mm during exercise , with Secondary Outcome Measures including the change in carotid ather oma assessed by media:intimal thickness ratio of the carotid artery, change in time to exercise induced chest pain as judged by a single observer, change in frequency of attacks of angina as recorded in the patients' angina diary, change in high sensitivity C reactive protein (hs CRP), change in scores on the Seattle Angina Questionnaire (SAQ), scores of quality of life (Euroquol), and scores of depression using the Beck Depression Inventory.

New presentations for local administration in men are a nasal preparation of testosterone (Nasobol) to be tested in hypogonadal men for substitution (effects and pharma cokinetics), and topical vaginal administration in women ("Vaginal Testosterone Cream For Atrophic Vaginitis in Women Taking Aromatase Inhibitors for Breast Cancer").

A number of studies are underway to develop a male contraceptive raised on the combined administration of LHRH peptide for pituitary suppression together with testosterone supplementation by a testosterone gel, providing predefined levels of serum testosterone. Clinical research to elucidate details of the effect of testosterone on the prostate, in preparation of developing a male contra ceptive are underway with combined investigational pro tocols for a 28 day study to use pituitary suppression by an LHRH antagonists to reduce endogenous testosterone to a minimum, and testosterone supplementation to identify the specific effect on prostate function (primary outcome measure being the hormonal regulation of prostate gene expression and tissue hormone levels, tissue protein expression and apoptosis, and secondary outcome mea sures being cellular immune function).

One approach to provide physiological testosterone substitution while avoiding undesired exposure to dihydro testosterone (DHT) causing enlargement of the prostate has been the combined use of testosterone together with the 5 alpha reductase inhibitor, dutasteride. In a clinical research study in hypogonadal men (NCT00194675), tes tosterone will be administered alone or as a fixed combina tion of testosterone with dutasteride, primary outcome measure being effects of testosterone alone or in combina tion with dutasteride on prostate volume in hypogonadal men with BPH (study of six months duration), the second ary outcome measures being the effects on symptoms and signs of BPH, serum and intraprostatic hormone levels, androgen responsive gene expression and proliferation in the stromal and epithelial compartments of the prostate, and psychological inventories for spatial and verbal memory to assess the psychological effects of the supplementation.

C.9.6.5 Antiestrogens

Antiestrogens in the strict sense are clomiphene and fulvestrant (Robertson et al. 2003). Both compounds are fewer estrogenic antagonists in all tissues studied. Fulvestrant is used for the treatment of breast cancer in women with this is progression after initial tamoxifen treatment (Osborn et al. 2004; Robertson and Harrison 2004). The antiestrogens tamoxifen and toremifene have been investigated in great detail for adjuvant therapy in early stage breast cancer (Coombes et al. 2004; Joensuu et al. 2000; Tiitinen et al. 2004; Ellmaen et al. 2003; Holli K 2002; Milla Santos et al. 2001; Holli et al. 2000; Lewis et al. 2010; Pagani et al. 2004). Toremifene has been explored for premenstrual mastalgia, and in androgen deprivation therapy for prostate cancer, with an improvement in lipid profile being shown (Lewis et al. 2010; Kusama et al. 2004; Tiitinen et al. 2004; Ellmaen et al. 2003; Holli 2002; Milla Santos et al. 2001; Joensuu et al. 2000; Holli et al. 2000). Currently ongoing clinical trials in oncology

are in ovarian cancer, and in prostate carcinoma (neoadjuvant therapy). One topic of considerable interest has always been the prevention of prostate cancer and of the preneoplastic conditions, prostatic intraepithelial neo plasia (Prostate Cancer Prevention Study for Men With High Grade PIN). The proposal is to determine if toremifene citrate is effective and safe in the prevention of prostate cancer in men who have been diagnosed with high grade prostatic intraepithelial neoplasia (PIN). A specific study by invitation is ongoing on extended adjuvant treatment with letrozole in breast cancer who complete 5 years of toremifene (NCT01072318). A randomised controlled trial is presently underway to compare postoperative adjuvant therapy using sequential administration of the hormone, toremifene citrate (TOR) or anastrozole (ANA), after chemotherapy in breast cancer (NCT00437359). Anastrozole is an aromatase inhibitor which has been extensively profiled in adjuvant therapy of cancer (Freedman et al. 2010; Murray et al. 2009; Campos et al. 2009; Robertson et al. 2003; Howell et al. 2005; Ewer and Glueck 2006).

C.9.6.6 Antiprogestins

The first anti progestogen (mifepristone, RU 486) became available for studies in reproduction for termination of pregnancy (McDonnell and Goldman 1994). Mifepristone also has significant anti glucocorticoid activity which is however not reflected in a specific therapeutic indication.

C.9.6.7 SERM

Selective estrogen receptor modulators (SERMs)that dis play tissue selective agonist or antagonist activities are tamoxifen, raloxifen, and toremifen. Raloxifene has been evaluated for the prevention and treatment of osteoporo sis (Cummings et al. 1999; Delmas et al. 2002), often in combination with bisphosphonates. Tamoxifen and toremifene have been evaluated for treatment of breast cancer. The classification of SERM or as an antiestrogen is largely dependent on the prevalence of antagonistic activity, or that of other pharmacological effects different from the antagonism antiestrogen receptors.

C.9.6.8 Aromatase Inhibitors

Anastrazole is an aromatase inhibitor used to treat breast cancer after surgery and for metastases in post menopausal women (Robertson et al. 2003) . Exemestane, letrozole and anastrozole are currently approved in the United States for adjuvant treatment of breast cancer. Exam ples of compounds in this class with established therapeutic efficacy, or being evaluated in newer clinical studies are Anastrozole, Letrozole, exemestane, Formestane, vorozole, and fadrozole.

C.9.6.9 5-Alpha-Reductase Inhibitors

The 5 α reductase inhibitors were initially developed for the treatment of benign prostatic hyperplasia and found to be effective, alone or in combination with alpha adrenergic blockers such as doxazosin and terazosin (Wilde and Goa 1999; Edwards and Moore 2002; Wilt et al. 2002; Clark et al. 2004; Roehrborn et al. 2004; Bhardwa et al. 2007; Smith and Carson 2009). Recent studies with dutasteride have confirmed this concept (Djavan et al. 2005; Dolder 2006). The clinical studies were then extended to the treatment of prostate carcinoma by reducing or eliminating androgen receptor stimulation by dihydrotestosterone. Finasteride (De Nunzio et al. 2008) and dutasteride (Crawford et al. 2010) were applied to the adjuvant treatment of prostate cancer, and studies on the prevention of prostate carcinoma by early treat ment with 5 α reductase inhibitors are ongoing (Rittmaster 2008; Reed and Parekh 2009). The relevance of blocking androgen receptors in metastatic breast cancer is being explored (Nicolás Díaz Chico et al. 2007).

An important indication for 5 alpha reductase inhib itors is now their cosmetic effect in delaying male pattern baldness (androgenic alopecia) caused by endogenous testosterone. Finasteride has an indication in oncology for prostate carcinoma, and a different indication for hirsutism and androgenic alopecia.

C.9.6.10 Antiandrogens

Androgens have received considerable attention due to their anabolic effects, however the more interesting development has been in antiandrogens which have an important place in the adjuvant treatment of cancer, in particular cancer of the prostate. In women, adrenal androgens are secreted throughout life and during reproductive age from the corpus luteum (gonadal androgens). The testosterone precursors androstenedi one and dehydroepiandrosterone (DHEA) are weak androgens.The antiandrogens nilutamide, flutamide, bicalutamide, and cyproterone acetate have been

extensively evacuated in the treatment of prostate carcinoma, either alone or in combination with testosterone suppression by LHRH agonists (neoadjuvant or adjuvant therapy regimens).

For the inhibition of testosterone secretion and inhibition of testosterone action at the receptor site there are different groups of compounds. At the pituitary level, control of testosterone secretion is blocked by LHRH agonists when administered at supraphysiological doses and/or by controlled release from depot injections (Sandow et al. 1988; Iversen et al. 1990; Waxman 1985; Gommersall et al. 2002). In some preclinical studies, Leydig cell hyperplasia was found in rats but has no equivalent in human physiology and pathology (Prentice and Meikle 1995). Inhibition of testosterone secretion is of particular clinical importance in the adjuvant treatment of prostate carcinoma at early stages (Wilbert et al. 1983), tumors that are initially androgen dependent become progressively independent of androgens support (Balk 2002; Chatterjee 2003; Heinlein and Chang 2004; Taplin and Balk 2004). A frequently used regimen is based on blocking the androgen receptor by antiandrogens (Schroeder 1990, 1998) as monotherapy or in combination with pituitary control. The flare phenomenon during the early phase of LHRH agonists depot injections was compensated by treatment with cyproterone acetate (Goldenberg and Bruchovsky 1991; Neumann 1994). Early compounds for combined androgen blockade were *nilutamide* (Moguilewsky et al. 1987; Dole and Holdsworth 1997) and *flutamide* (Brogden and Chrisp 1991; Labrie 1993). Numerous studies then followed on the preclinical profile (Furr 1996) and clinical efficacy of monotherapy with *bicalutamide* in prostate carcinoma (Goa and Spencer 1998; Iversen 2003; Wellington and keam 2006), as well as combined androgen blockage with bicalutamide (Schellhammer et al. 1995; Klotz and Schellhammer 2005; Klotz 2008).

C.9.6.11 Clinical Summary

The clinical pharmacology of gonadal steroid hormones and their synthetic derivatives, in particular of their numerous pharmaceutical presentations for local or systemic use is best explained by the development and extensive clinical application of contraceptive steroid. Another field of great importance is the use in oncology either alone or for neoadjuvant and adjuvant therapy e.g., of breast cancer and prostate cancer.

An overview of ongoing and completed clinical studies is available at the U.S. National Institute of Health website (www.clintrials.gov). This website is a large database providing information on study details such as medication and dose range, test compound and comparator (for RCT studies), clinical indication, specific study purpose, study design, primary and secondary study outcomes. Information about study medication is available from the "Drug Information Portal" of the website, providing an overview of the pharmacological class of compounds to which the study medication belongs. More detailed information is available from links to the literature database www.pubmed.gov which is maintained by the U.S. National Library of Medicine and National Institutes of Health.

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C.10 Pharmacodynamic Evaluation: Dermatology

Clinical Pathways for CE Marking of Novel Dermal Fillers in Europe

Holger Köhler

PURPOSE AND RATIONALE

In addition to "classical" treatment of neoplastic, inflam matory, and allergic diseases, aesthetic dermatology has grown to become an important field of dermatology. The desire for beauty and youth is regularly expressed by patients themselves and such patients seek advice on the improvement of their appearance and look (Steinkraus 2006). Developed countries' population is aging and preserving a juvenile look is considered normal and desirable in our present society and reflects the current way of life. Thus, the demand for aesthetic treatments is high and respective markets are continuing to grow.

Biological aging of the skin involving the loss of hyaluronic acid, subcutaneous fatty tissue, as well as collagen and elastic fibers differs between individuals. The aging process and the process of facial wrinkle forma tion depend on genetic predisposition (intrinsic aging) and on exposure to ultraviolet (UV) radiation, environ mental factors, nicotine, or alcohol (extrinsic aging) (Rauch and Ruzicka 2004). The mimic activity of the fine, small muscles under the skin also contribute to wrinkle formation.

Unwanted wrinkles can be corrected by a variety of methods both surgical and nonsurgical, depending on the nature and causation of the lesion. To select a suitable method, it is necessary to accurately define the wrinkles (depth, classification into dynamic folds, gravity induced folds, etc.) and evaluate the facial fat distribution.

Besides surgical procedures and neurotoxin treatment, a very effective method of treating facial folds is the aug mentation procedure in which absorbable or nonabsorbable biomaterials (fillers) of various types are implanted or injected.

Aesthetic dermatology has developed enormously in recent years with the arrival of many new outpatient aesthetic techniques that help to improve patients' appearance. Although wrinkles remain the major sign of aging, the restoration of facial volume and contours and the creation of a natural look must also be considered when treating the aging face. Injectable filler products have greatly evolved and are used worldwide. However, it must be emphasized that these techniques should be used according to the results of evidence based science to prevent distorting results, bearing in mind that successful aesthetic treatments also require a minimum of "artistic" talent in order to achieve an overall natural look.

It has been over 20 years since the approval of the first dermal filler device, with the majority of the currently marketed dermal fillers approved in the last 10 years.

The types of materials, which are US food and drug administration (FDA) approved for dermal fillers, vary from biologic to synthetic materials and absorbable to nonabsorbable compounds (FDA 2008) as illustrated in *Table C.10 1*.

Outside the United States, including the European Union a wide variety of additional fillers are registered/ marketed:

Widely used brands of absorbable fillers (besides others):

- Collagen: autologous (Isolagen®), isogenic (Cymetra®)
- Hyaluronic acid: nonanimal derived (Belotero®, Hydrafill®, Surgiderm®, Hyaluderm®, Matridex®, Teosyal®, Captique®, Idune®, Isogel®)
- Gelatin powder and aminocaproic acid: Fibrel®
- Human cadaver tissue: Fascian®
- Polyoxyethylene, polyoxypropylene: Profill®
- Polyvinyl alcohol: Bioinblue®
- Synthetic calcium hydroxyapatite: Radiesse®

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Table C.10-1

FDA approved dermal fillers (2008) are both permanent and absorbable; of both synthetic and natural origin (Niamtu 2005; Smith 2007; Lambros 2007; Jansen and Graivier 2006; Man et al. 2008)

	Permanent	Absorbable			
		Synthetic		Natural	
Major component	Poly(methylmethacrylite) (PMMA) Microspheres	Hydroxylapatite	Poly(L-lactic acid)	Hyaloronic acid	Collagen
Brand name	Artefill®	Radiesse®	Sculptra®	Restylane®, Perlane®	Zyderm®, Zyplast®
				Hylaform®	
				Hylaform® Plus	Cosmoderm®, Cosmoplast®
				Juvederm® 30	Evolence®
				Juvederm® 30 HV	
				Juvederm® 24 HV	
				Elevess®	

Widely used brands of nonabsorbable fillers (besides others):

- Polydimethylsiloxane: Fluid silicone 350cs, Silskin® 1,000cs
- Dimethylsiloxane: Bioplastic®
- Polymethyl metacrylate in collagen: Artecoll®
- Polymethyl metacrylate in hyaluronic acid: Dermalive®, Dermadeep®
- Polyacrylamide gel: Aquamid®, Outline®
- Polyalkylimide gel: Bio alcamid®
- Polyacrylamide and polyvinyl acid gel: Evolution®

If permanent implants (nonabsorbable silicone or substances containing acrylate) are used, the effects are virtually irreversible. This includes those potential adverse effects of permanent implants such as dislocation, sensiti zation and nodule formation and may remain lifelong or have to be remedied by interventional measures.

Although the overall incidence of severe complications is low, aesthetic practice is still seeing too many patients with complications due to filler injection (Andre et al. 2005). Often, the exact pathophysiology of the reaction and the physicochemical nature of the product used are unknown to the aesthetic physician. Histopathology can be of assistance (Zimmermann and Clerici 2004) in iden tifying the underlying processes.

Since an ideal, risk free filler is not available and appears only theoretically conceivable, difficulties in treating side effects caused by non resorbable fillers should encourage aesthetic physicians to utilize biodegradable products and motivate both industry and academia to establish reliable testing systems before marketing such devices. Upcoming next generation fillers may improve safety and tolerance of dermal fillers and also regulatory demands concerning testing systems increase.

PROCEDURE

From a regulatory standpoint, aesthetic dermatology deploys procedures that are monitored by different types of administration. Thus, food (e.g., additives), cosmetic (e.g., peelings, moisturizers), drug (e.g., neurotoxins), and device (e.g., fillers) regulation may apply.

Although, a specific regulation applies to fillers as medical devices and pharmacological effects as the main mechanisms are excluded for medical devices *per defini tion* in both European and US legislation, clinical testing systems and overall philosophy of testing are often adopted from methods of clinical pharmacology, drug research, and drug development.

Before marketing and distribution, fillers and any other medical devices pass through a procedure of clinical evalua tion. In contrast to drug development and respective pro cedures, it is not safety and efficacy that have to be shown, but safety and function/effectiveness (Krummenauer 2003). In a retrospective view, the number of clinical trials conducted by the medical device industry has been far less than the volume performed by pharmaceutical industry. This observation could mainly be attributed to the different regulatory structures and development processes of drugs and devices. In Europe, the regulatory framework under the Medical Device Directives is relatively emerging. The first of these Directives, the Active Implantable Medical Device

Directive, became enforceable in 1993, besides previously established national controls prior to common European legislation. In the pharmaceutical industry, the equivalent European Directive was introduced in 1965 (Wright et al. 2002). Meanwhile, more typical Pharma players invest into the development of devices and also in aesthetic dermatology. Thus, influence of "Pharma philosophy" in device research and development grows.

There are three key differences between product development in the medical device and pharmaceutical industries, which define their differing approaches to clinical research (Wright et al. 2002):

- The processes of concept discovery
- Device classification
- Iterative development

Since drug discovery to a large extent represents a trial and error process, it involves massive screening programs to identify candidate compounds for further exploration in animal and human models through an extensive multiphase preclinical and clinical trial development program. In contrast, devices are designed to deliver a particular intended performance with specific safety requirements and regulatory systems throughout the major markets are designed to ensure safety and effectiveness through design quality and manufacturing principles.

While device regulation in the United States is to a greater extent centralized and FDA processed, there is a decentralized process throughout the countries of the European Union. Since 1998, the CE mark ("Conformité Européenne") is required on medical devices marketed in Europe. The CE mark is a proof of compliance with the "Essential Requirements" of the Medical Device Directive 93/42/EEC. The CE mark indicates that the device is readily designed for its intended purpose and meets these "Essential Requirements." Once a CE mark has been placed on a device, the product can be freely marketed in the European economic area.

CE marking of new medical devices may require clinical investigation. This is especially relevant for devices that are classified as Class IIb or Class III (higher risk) devices, such as dermal fillers. Such devices may also employ new materials or components without an established history of performance or may target a new indication or a new intended use.

The process of CE marking of devices is regularly accompanied by "Notified Bodies" (NB), which are the only CE marking approval institutions recognized by the European Commission. NB are independent organizations but "notified" (nominated) for the CE marking approval of medium and high risk devices (Welsby 2002) and monitored by "Competent Authorities," according to European and national legislation.

Before starting any type of clinical investigation with exposure of the device to humans, results of all necessary physical, chemical and biological tests have to be available. This battery of tests regularly includes biocompatibility testing according to ISO 10993. Before commencing an actual study, these results must be summarized in an "Investigator's Brochure" and, in accordance with Annex VIII of the Medical Device Directive 93/42/EEC, the sponsor has to provide a provisional declaration of conformity and submit the clinical investigation plan, case report form, informed consent form, etc., to a registered review board (ethics committee, IRB). After receiving their positive vote and notifying the study to the "competent authorities" according to the applicable national laws, a study may be started.

In the following, we present a clinical model, which has to be seen as an exemplary strategy to generate the necessary human data for CE marking of an experimental dermal filler with so far (in the context of the intended use) unknown ingredients. The envisaged intended use may include the general correction of facial wrinkles and folds as well as signs of facial fat loss (lipoatrophy) (Strand and Wolters 2006; Valantin et al. 2003; Jones et al. 2004; Andre 2008; Suryadevara 2008).

The clinical protocol is designed for a prospective, multicenter, uncontrolled clinical study, pursuant to European Medical Device Legislation and International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) (EMEA 1996; MPG 2002; ISO 14155 1 2003; ISO 14155 2 2003; WMA 1964). A comparison of the filling state of the nasolabial folds (NLF) after implantation of an experimental filler over time and the original state of the NLF before the implantation serves as the parameter of primary interest. The focus is to examine the in vivo degradation characteristics, the extent and longevity of aesthetic improvement, and the overall short and long term safety and tolerability of an experimental filler.

The protocol indicates a 144 week study comprising a 72 week (18 months) core phase with effectiveness parameter to be measured and another 72 week long term safety extension phase in order to cover long term safety issues.

The aesthetic effect of an experimental filler (wrinkle filling effect) should be evaluated by the patient and the investigator before and at defined time points during the study. Closed meshed monitoring is guaranteed by ten control visits during the core phase. During the extension phase, three further visits for safety reasons appear useful.

Patients to be included into such a study have to be selected by previously defined inclusion and exclusion criteria.

Such criteria, besides further specific topics relevant to the respective biomaterial or intended use, may include the following:

Main inclusion criteria:

- Men or women 18 years
- Ability to give written informed consent after being told of the potential benefits and risks of entering the trial
- Signed informed consent
- Willingness to use adequate contraceptive measures to prevent pregnancy during the duration of the study (for female patients of childbearing capacity), if necessary
- Patient's written commitment not to have any non allowed treatment to correct the nasolabial folds (NLF) during the study period
- Documented severity rating scale (SRS) score of 2 (moderate) or 3 (severe) for NLF at baseline evaluation

Main exclusion criteria:

- Pregnant women
- Females of childbearing potential without adequate methods of contraception or lactating women
- Diabetes mellitus
- Autoimmune disease
- Clinically relevant coagulation disorders
- Severe psychiatric, neurological, or mental disease
- Known hypersensitivity/allergy to the ingredients of the experimental filler
- Evidence or suspicion that the patient is not willing or unable to understand the information that is given to him/her as part of the informed consent
- Expected noncompliance
- Patients in a poor physical condition
- Patients who are imprisoned
- Patients who are lawfully kept in an institution
- Infections, active dermatological condition, or inflam mation in the nasolabial area
- Scars in the nasolabial region
- Tendency to keloid formation and granuloma history
- Pretreatment with permanent and semipermanent fillers

Considering the scope and nature of such a study, several previous treatments should not be allowed prior to inclusion into the study (and/or during the course of the study):

In the last 6 months before baseline:

- Injections of botulinum toxin of any serotype for wrinkle treatment below the zygomatic arch
- Surgery on the face including facelift and implanta tions (e.g., dermal fillers)
- Surgical treatment in the oromaxillary region

In the last 8 weeks before baseline:

- Laser treatment, thermo lifting, dermabrasion, or chemical peeling (excluding home treatment with cosmetic products) in the nasolabial area
- Immunosuppressive or cytostatic therapy

In the last 7 days before baseline:

- Use of skin irritating topical preparations on the face
- Anticoagulant agents (e.g., heparin, heparinoids, coumarin derivatives)
- Antiplatelet drugs (e.g., acetylsalicylic acid, dipyridamole, clopidogrel, ticlopidine)

Patients should be withdrawn from the study if at least one of the following events occurs, which may affect trial outcome variables and/or ethical issues:

- Treatment of the facial area with botulinum toxin of any serotype below the zygomatic arch
- Laser treatment, thermo lifting, dermabrasion, or chemical peeling (excluding home treatment with cosmetic products) in the nasolabial area
- Touch up or treatment of any facial regions with any other dermal filler besides the experimental device
- Any facial surgery
- Withdrawal of informed consent
- Women getting pregnant during the study

In this design, each study participant would receive injection treatment in the right and left NLF (bilateral application/implantation of the experimental device). The procedure is normally performed in an outpatient environment under aseptic conditions. Thus, after care fully disinfecting the skin at the injection sites and if desired by the patient administering local or infiltration anesthesia, intradermal implantation into the NLF has to be carried out by using common techniques (Pavicic 2009; Dover et al. 2009; Narins and Bowman 2005).

An individually optimal correction should be achieved by injecting the experimental filler without overcorrection of the folds. Touch up treatments within 2 weeks after first implantation may be allowed.

EVALUATION

The aesthetic correction parameters to be recorded in such a design should be standard parameters used in the man agement of nasolabial folds both in aesthetic practice and in clinical trials.

Standardized photography may be helpful before treatment to provide a baseline for any later assessments of the overall aesthetic result by the investigator and the patient, for example, Global Aesthetic Improvement Scale (GAIS), $GAIS_{investigator} + GAIS_{patient}$ with the help of a scale (Table C.10 2). Both patient and investigator should compare baseline photos with the conditions at the respective visit and should rate the aesthetic improvement independently from each other.

A rating of NLF severity represents a basis for an objective assessment of the effect of tissue augmentation in aesthetic medicine. Visual assessment of the severity of the NLF on a severity rating scale (SRS) is a recognized method of standardizing the rater's judgment (Moody et al. 2008; Day et al. 2004). An example for such a severity rating scale is given in Table C.10 3.

The following, main secondary effectiveness parameters may be relevant for further assessment of the experimental filler's function and safety:

- Proportion of responders over time based on the intraindividual change of the NLF SRS score compared to baseline as assessed by the investigator at all time points during the core period.
- The time to the worsening of the severity of the NLF SRS by one score point compared to optimal correction (including touch up, as far as applicable), as assessed by the investigator.
- Global assessment of the aesthetic improvement (GAIS) by the investigator compared to the baseline state (see Table C.10 2).
- Global assessment of the aesthetic improvement (GAIS) by the patient compared to the baseline state (see Table C.10 2).
- The change in the subjective assessment of the filling state in percent by the investigator compared with the optimal correction filling state versus current state by using a visual analogue scale (VAS, see Table C.10 4).

Incidence and severity of adverse events should be recorded by the investigator, including the examination of the implant area at all visits. Global assessment of safety at each visit by the investigator and patient and a safety laboratory with standard parameters should be performed during screening and final study visit, especially when applying biomaterials that are so far unknown in the given context.

Table C.10-2

Global aesthetic improvement scale (GAIS) (Moers-Carpi et al. 2007; Beer 2007; Lindqvist et al. 2005; Narins and Bowman 2005)

Score	Appearance of face	Definition of GAIS
5	Very much improved	Optimal cosmetic result for the implant for this volunteer.
4	Much improved	Marked improvement in appearance from the initial condition,[a] but not completely optimal for this volunteer. A touch-up would slightly improve the result.
3	Improved	Obvious improvement in appearance from the initial condition,[a] but a touch-up or retreatment is indicated.
2	No change	The appearance is essentially the same as the original condition.[a]
1	Worse	The appearance is worse than the original condition.[a]

[a]Findings before implantation

Table C.10-3

Severity rating scale (SRS) for nasolabial folds (SRS)

Score	Folds	Definition of SRS
4	Extreme	Extremely deep and long nasolabial fold; detrimental from facial appearance; 2 4-mm visible V-shaped fold when stretched; unlikely to have satisfactory correction with an injectable implant alone.
3	Severe	Very long and deep nasolabial fold; prominent facial feature;< 2-mm folds when stretched; significant improvement is expected from injectable implant.
2	Moderate	Moderately deep nasolabial folds; clear facial feature visible at normal appearance but not when stretched; excellent correction is expected from injectable implant.
1	Mild	Shallow but visible nasolabial fold with a slight indentation; minor facial feature; implant is expected to produce a slight improvement in appearance.
0	Absent	No visible nasolabial folds; continuous skin line.

Table C.10-4

Filling state of nasolabial folds (NLF) visual analogue scale (VAS)

Filling state of NLF 100%VAS	
0%	100%
The fold depth is identical to the original state (before implantation)	*The folds are optimally filled (=status immediately after Implantation)*

The treatment with an experimental device has to be regarded as successful in terms of this model (patient is a "responder") if a patient has shown an SRS score at the respective time point that is still at least one score point below (i.e., "better") compared to the patient's original SRS score measured before treatment. If this change in score was not achieved, the patient would be counted as a nonresponder.

The calculation of sample size should be justified by biometrical means. Thus, the following assumptions serve as examples:

Binomial test for proportions

- Type I error $\alpha = 0.025$ (one sided)
- Type II error $\beta = 0.1$ (power = 90%)
- Difference to be detected versus 0.40:0.15

would result in a sample size of 122 patients.

Validated systems should be used for statistical analy sis and data management.

For proving the respective longevity of a filler over a certain time frame, confirmatory testing for the primary effectiveness analysis should be conducted, for example, after month 6 post implantation and optimal correction. However, in order to analyze additional timepoints and prolonged function, for example, month 12, month 15, and month 18 further testing might be carried out in a hierarchical manner.

Thus, the first confirmatory analysis would be carried out for month 6. Only if this comparison shows a statistically significant result, the test for the next hypothesis (comparison at month 12) would be interpreted as confirmatory. In line with this practice, the comparisons at month 15 and at month 18 would be declared as confirmatory, only if the former comparisons showed statistical significance.

The primary effectiveness analysis at the first timepoint, for example, month 6 and the comparisons for the primary effectiveness variable at months 6, 12, 15, and 18 would be analyzed testing the following hypotheses:

Hypotheses ($i = 6, 12, 15, 18$):

$$H_0^i : p_{\text{responder}} \leq 0.4$$

vs.

$$H_1^i : p_{\text{responder}} > 0.4$$

where H_0^i means that the proportion of responders is less than or equal to 40% and H_1^i that the proportion of responders is greater than 40%.

The primary effectiveness variable would be analyzed using a one sided binomial test for proportions, with α set at 2.5%.

Descriptive statistics may prove to be sufficient for the evaluation of the secondary effectiveness parameters.

CRITICAL ASSESSMENT OF THE METHOD

A comprehensive regulation and numerous guidelines have to be observed when performing clinical studies with devices such as dermal fillers in the European Union (ISO 14155 1 2003; ISO 14155 2 2003; Directive 2001/20/EC; Council Directive 90/385/EEC; Council Directive 93/42/EEC; European Standard EN 540; MEDDEV 2.12.2; MEDDEV 2.7.1). Additionally, national regulation of Member States has to be observed.

The above described uncontrolled clinical trial design may prove to be sufficient for a comparably fast track clinical development and CE marking of a novel filler material in Europe. It has to be stressed that numerous CE marked fillers (often with "known" ingredients) are marketed based on explorative clinical data only or even on bibliographic assessment. This means that pivotal clin ical data are not mandatory for marketing such a dermal filler in Europe. The European approach on medical devices emphasizes manufacturers' responsibility rather than authority control. As a rule, it is up to the manufac turer to decide which data are sufficient for CE marking. Notified Bodies may provide guidance whether or not the chosen development pathway provides adequate data for CE marking.

As previously mentioned, in case a clinical trial is initiated for CE marking, the common objective of such a trial is to demonstrate safety and performance. There fore, the majority of these trials are single arm feasibility studies involving a double digit number of patients with the primary objective to show general safety and effective ness and to identify frequent adverse reactions.

According to the Medical Device Directive, the clinical data used for CE marking may be derived from a compila tion of the relevant scientific literature currently available on the intended use of the device and a report containing a critical evaluation of the compilation of the results and, if

applicable, conclusions from a specifically designed clinical investigation.

The device approval process in the United States follows a different philosophy, especially in terms of the scope and size of clinical trials required for Class III devices, such as fillers. To receive approval to market a high risk device in the United States, the manufacturer must demonstrate that the device is reasonably safe and effective. Following FDA's philosophy and their regular guidance, this typically requires controlled, prospective randomized trials with biometrically based sample sizes (Sorrel, Medical Device Development).

As a result, most dermal filler devices are available in the EU several years before being marketed in the United States. This results in a delay for patients in the United States who may benefit from improved technologies. On the other hand, a preferred access must be weighed against a less extensive clinical testing program.

The presented design appears to be a commercially reasonable and ethically sound method for targeting the approval of novel filler materials in Europe. A sufficient number of patients selected by common criteria are treated under controlled conditions and observed up to 3 years in a safety extension phase. Besides some modifications, widely used scales for measuring aesthetic outcome parameters are applied (Moody et al. 2008; Day et al. 2004; Moers Carpi et al. 2007; Beer 2007; Lindqvist et al. 2005; Narins et al. 2003). These parameters reflect the desired clinically visible rejuvenation effects, when comparing pretreatment status and aesthetic results from respective timepoints after implantation of such an experimental dermal filler device (*Fig. C.10 1*). From a safety standpoint, respective clinical parameters should verify the clinical relevance of findings from biocompatibility testing (*Fig. C.10 2*).

MODIFICATIONS OF THE METHOD

The scientific basis for CE marking of dermal fillers is not published on a regular basis. Since a bibliographic pathway or a small bridging study approach to approval is feasible in Europe and there is no data exclusivity for the technical documentation of a medical device, manufactures depend on the extent of the respective patent protection and may avoid disclosing detailed data in order to safeguard both their product know how and their investments into clinical studies. Larger published trials may however focus on specific features of a product (Wahl 2008) in a postmarketing environment and may to a certain extent have "seeding trial" intentions.

The main differences in designs between European device studies and the US philosophy are probably determined by the fact that comparator controlled studies are regularly requested by the FDA. Thus, for US registration, such controlled studies are usually performed using a split face design with the test device and the comparator randomly assigned to the one or the other half of the

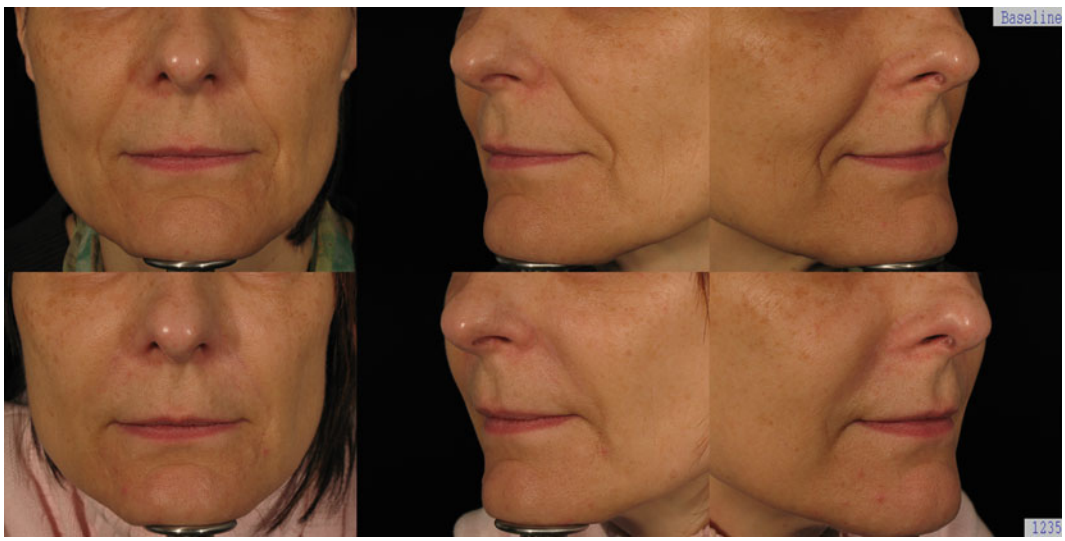

Figure C.10-1

Standardized photography serves as a tool for the documentation of aesthetic outcome parameters: Lower half shows aesthetic improvement of the severity of nasolabial folds 6 months after implantation of a dermal filler device. Compare this to baseline status (upper half)

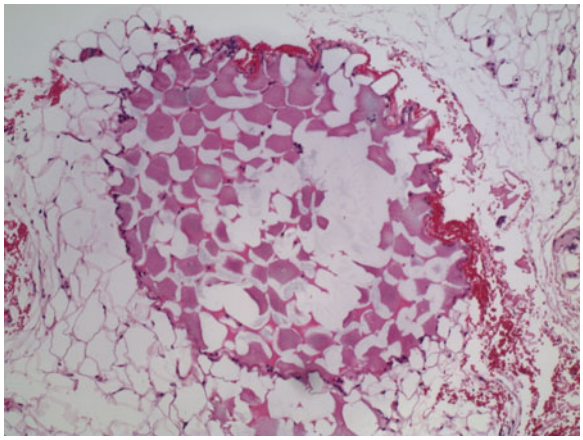

Figure C.10-2
Histological assessment may provide additional data on biocompatibility, function, and safety: Foreign body reaction with only mild fibrous constituent around clearly visible filler material is shown, absence of significant cellular inflammatory response (material from 4-mm punch biopsy, upper arm skin, 3 months after implantation, HE staining)

patients' face. Fillers that are already marketed in the respective indication serve as comparators. Since test and reference fillers are often visually distinguishable, an observer blinded design is applied and rating with the help of aesthetic scales is performed by an investigator, other than the physician who injected the implant.

One may discuss such comparator controlled studies with view to potentially different longevities of test and reference devices and aesthetically unsatisfying results during the course of such split face studies. The latter may include a potential need for retreatment and additional patient burden. Moreover, one might question if and to which extent prolonged in vivo persistence of a filler material actually indicates the statistically asserted "superiority" from a clinical or market standpoint.

Noninferiority and superiority (in terms of longevity of the aesthetic effects) designs are utilized, especially for US registrations (Cosmetic Tissue Augmentation Product P050033; Cosmetic Tissue Augmentation Product P050047).

The interval between the patients' visits and the timepoint for the primary effectiveness analysis and the permission of "touch ups" or retreatments during the course of a study are basically determined by the expected longevity of the respective test device.

Aesthetic rating scales may also differ from the above presented SRS 5 point scale (Lemperle et al. 2001). Nevertheless, for regulatory purposes, such scales should be adequately validated.

GAIS scales may also be adapted to actual study demands and may also be applicable to aesthetic indications other than nasolabial folds (Carruthers and Carruthers 2008; Bechara et al. 2008; Beer et al. 2008; Bugge et al. 2007; Silvers et al. 2006).

The latter draws attention to a further difference between European and US regulatory practice. Thus, aesthetic "indications" such as NLF, cheek volume augmentation, or lip augmentation can be covered by one exemplary study (or other clinical data) and may serve as reference for each other to support a comprehensive intended use in Europe. In contrast, FDA generally requires separate, randomized studies in each of such claimed indications and allows only limited reference. While FDA practice in this context appears to be somehow driven by "Pharma" or drug development philosophy, European Notified Bodies more obviously consider the predominant physical effect of a dermal filler, displacement of dermal, or subcutaneous tissue in terms of the Archimedean principle.

The presented clinical protocol describes a pathway to deliver pivotal data for the clinical evaluation of novel dermal filler materials for the European market according to European regulation. It should be stressed that different market claims, intended performance and physicochemical characteristics of dermal filler devices determine the design of potentially necessary clinical studies. Besides these scientific factors, territorial regulation has a tremendous impact on the design of such a trial and is subject to a continuous flow of legislative activities. Within a growing market in aesthetic dermatology, new filler materials and upcoming demands to the performance of fillers, for example, pain management determine the need for the development of new testing protocols.

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C.11 Pharmacodynamic Evaluation: Oncology

Francois Lokiec

C.11.1 General Introduction

The introduction of pharmacodynamic modeling into clinical oncology has been a slow process. First of all, if it is clear that pharmacokinetic evaluation of anticancer drugs is easy the pharmacodynamic evaluation is more complicated because it is dual. It concerns both the toxic side effects and the efficacy of the treatment. For example, the relationship between toxicity subsequent to high dose methotrexate (MTX) and that of delayed MTX clearance has led to the routine use of therapeutic drug monitoring of plasma MTX concentrations to guide leucovorin dosing (Monjanel et al. 1979). However, studies of other drugs have not clearly resulted in a change in clinical practice although there has been a recent increase in clinical research in this area. Most early pharmacodynamic studies addressed relationships between measurements of drug exposure (AUC, C_{ss}) and toxicity (Calvert et al. 1989; Chatelut et al. 1995; Gamelin et al. 1998; Kitchen et al. 1997). More recently, investigators have modeled toxicity by using novel pharmacokinetic parameters, such as time above a threshold concentration for etoposide and paclitaxel (Lowis et al. 1995; Edick et al. 2003; Rowinski et al. 1999). Other investigators have addressed the importance of active metabolites (Arakaki et al. 2008). This is of particular importance for irinotecan, a drug with both complex metabolism and toxicity patterns (Lokiec et al. 1996; Takeba et al. 2007) (*Fig. C.11 1*). However, studies have suggested that irinotecan induced diarrhea is secondary to relative deficiency in the glucuronidation of SN 38, its active metabolite (Ratain 2000; Yamamoto et al. 2008). Hematologic toxicity has been easier to model than non hematologic toxicity. One of the best characterized drugs is carboplatin, an analogue of cisplatin (*Fig. C.11 2*). Unlike cisplatin, the dose limiting toxicity of carboplatin is thrombocytopenia, which is a function of drug dose, renal function, pretreatment platelet count, and prior therapy (Budd et al. 1999). The platelet nadir produced by a dose of carboplatin is related to the carboplatin clearance, which is directly proportional to creatinine clearance. Thus, patients at high risk of severe thrombocytopenia following carboplatin therapy can be identified prospectively, and the drug doses can be modified by monitoring creatinine clearance. Etoposide has also been the subject of extensive evaluation. Pharmacodynamic modeling of etoposide is complicated by the need to either measure free etoposide directly or estimate its concentration on the basis of measured total plasma concentration of etoposide, albumin, and/or bilirubin (Bo et al. 1995; Aita et al. 1999). Many studies have now demonstrated that the extent of leukopenia/neutropenia is correlated with etoposide exposure (Green et al. 1988; Miller et al. 1998). Furthermore, interpatient pharmacodynamic variability may be significant and needs to be considered in future modeling of etoposide and potentially of other drugs. There is an expanding interest in trying to optimize cancer chemotherapy by individualizing dosing on the basis of measurements of plasma or tissue drug concentrations. One recent example is the titration of carboplatin dosing discussed above. Other investigators have attempted to optimize the dosing of etoposide, teniposide, melphalan, MTX, 5 Fluorouracil (5 FU) by monitoring plasma drug concentrations during treatment, then using the information obtained to modify the total dose of chemotherapy administered in an attempt to avoid severe toxicity (Alnaim 2007; Gao et al. 2008).

Since the use of the new "targeted drugs," the pharmacodynamic evaluation of the classical cytotoxic anticancer drugs could be define as "relatively simple" but as the number of molecular targeted agents under clinical development keeps growing, it is becoming increasingly evident that it will be far more complex to demonstrate clinical benefit with these agents than with cytotoxic agents. The reason for this new paradox in clinical research lies in the own nature of targeted therapy: these agents are predicted to work only in a subpopulation of patients whose tumor growth and/or survival are driven either by the target or target dependent processes. This is best exemplified in the case of erbB2 overexpressing breast tumors that are sensitive to the anti erbB2 monoclonal antibody trastuzumab (Herceptin®) (*Fig. C.11 3*) and with the remarkable success of imatinib (Gleevec®)

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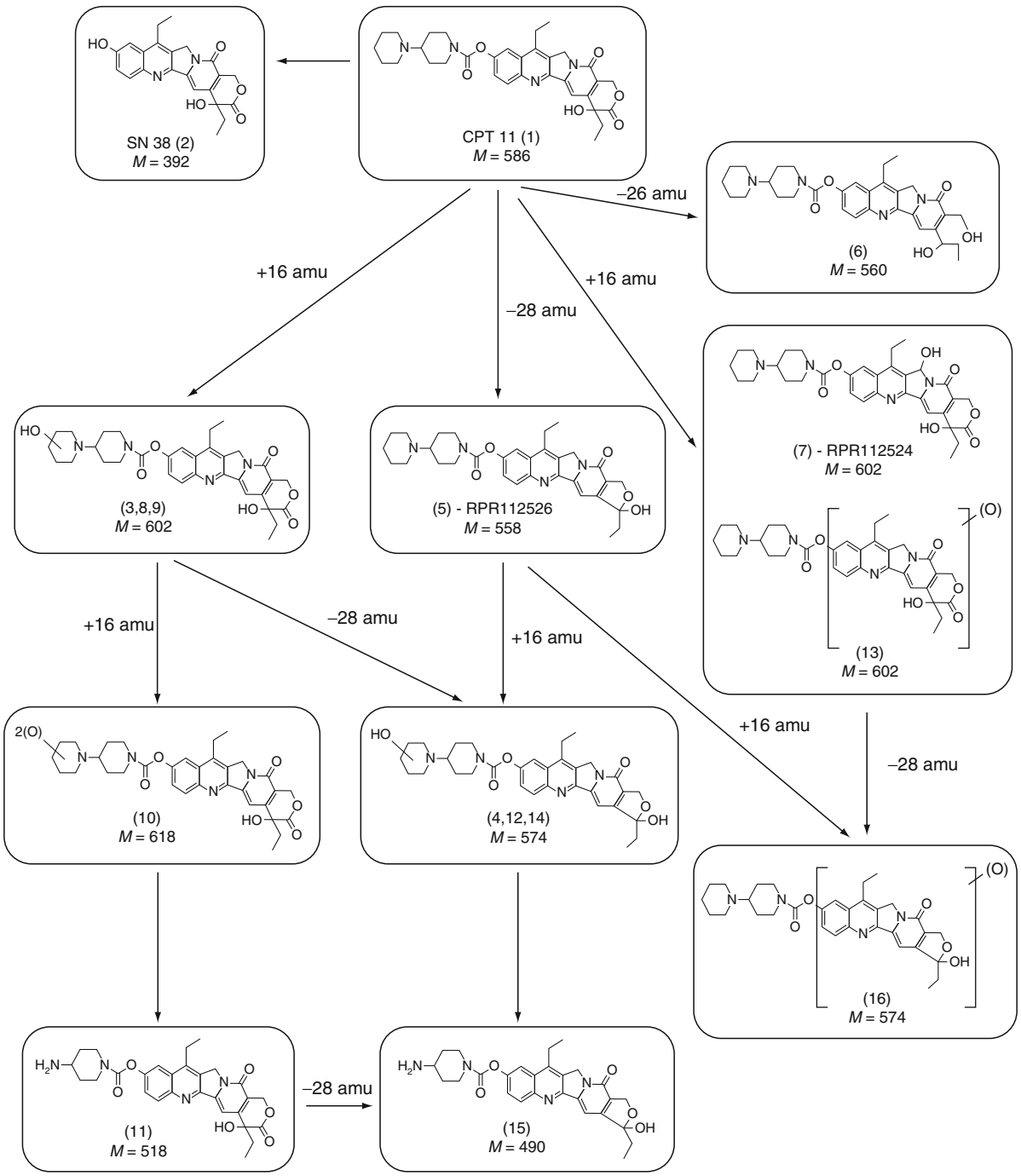

Figure C.11-1
Metabolic pathways of CPT-11 in patients

(*Fig. C.11 4*) in the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors. In addi tion, with these agents, it is difficult to identify the optimal biological dose (Rojo et al. 2007; Van Herpen et al. 2009; Reckamp et al. 2006). In most occasions, there should be no need to dose escalate until reaching the maximally tolerated dose. Pharmacokinetic endpoints alone might also be insufficient to identify the best dose and schedule

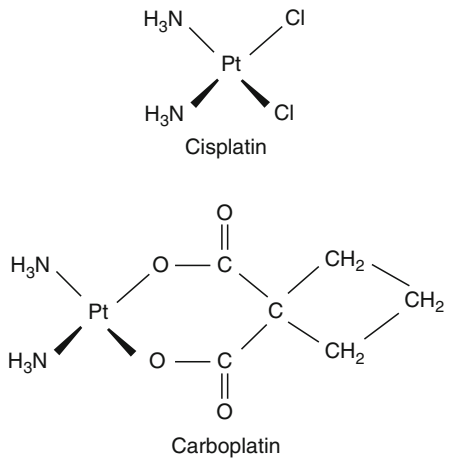

Figure C.11-2
Cisplatin and carboplatin structures

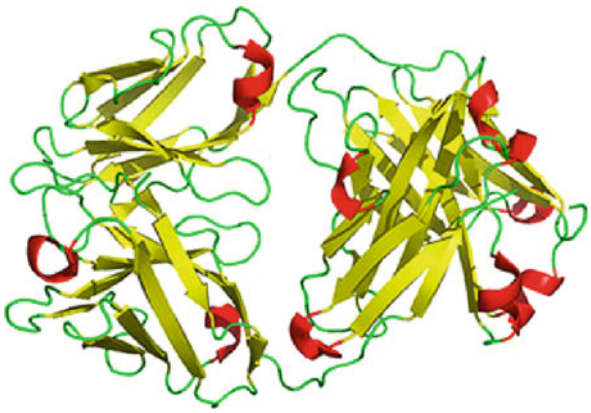

Figure C.11-3
Trastuzumab structure (Herceptin®)

unless a solid correlation could be demonstrated with in vivo effect in the target. There is, therefore, a growing consensus that the definition of the optimal biological dose of a targeted therapy should be based on the demon stration in vivo of the desired biochemical effect on the target molecule (Rojo et al. 2007). So, how are we going to resolve these challenges that could endanger the development of this promising class of agents? First, there is no substitute for good preclinical models as again exemplified with trastuzumab (Fujimoto et al. 2007). Second, there is a need to study in patients the effects of these agents on their targets by performing careful phar macodynamic evaluations in the tumor or in appropriate surrogate tissues (Sledge 2007; Willett et al. 2005; Willett et al. 2007; Majidi et al. 2009). The concept behind phar macodynamic studies is that by (sequentially) analyzing the effects of a given therapy on a patient's tissue, we might be able to determine the expression level of the target and to monitor the effects of a given agent on its molecular target. If in addition, treatment with the study agent results in a modulation of expression of genes related to the target, pharmacodynamic studies could also be instru mental in the identification of the subpopulation of patients that may derive benefit from this therapy.

C.11.2 Conventional Chemotherapy with Cytotoxic Agents

If we refer to the Phase I trials of anticancer agents published in the NIH guide (National Cancer Institute 1996):

> "The increasing number of promising new agents with diverse and novel mechanisms of action makes it desirable to continue NCI support in this area. Institutions responding to this Request for Applications (RFA) should be able to perform Phase I trials and establish the pharmacological characteristics, in parallel with biochemical and other appropriate biological studies, of the effects of these agents on cancer cells and normal tissues. It is expected that pharmacokinetics and, where possible, other laboratory correlative studies will be conducted in real-time, throughout the course of the clinical trial to facilitate optimal utilization of the data in the design and coordination of clinical trials with the agent. Applications from any one institution may focus on studies of one or more classes of agents or therapeutic approaches, reflecting the interest, expertise, and experience of the applicant investigators or a more general approach to the *pharmacokinetic/pharmacodynamic* evaluation of new agents may be developed. . ..
>
> B. Research Goals and Scope
>
> The aims of this initiative are to: (1) provide support for Phase I trials of promising new anticancer agents in cancer patients; and (2) provide support for complete pharmacokinetic, *pharmacodynamic*, and other important laboratory correlative studies in cancer patients receiving these anticancer agents. Phase I clinical trials have as their objectives the characterization of drug toxicity, maximally tolerated dose, pharmacokinetics, and biological effects (*pharmacodynamics*) of drugs. These anticancer agents have traditionally been obtained either from the NCI drug development program or through collaborative drug development agreements with the pharmaceutical industry. Recent advances in understanding of the pathobiology of malignancy are leading to the development of a wide range of novel anticancer therapeutic agents that

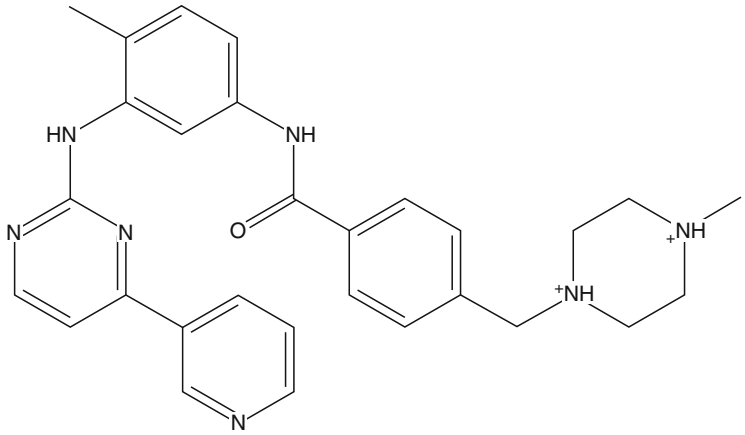

Figure C.11-4
Imatinib structure (Gleevec®)

require Phase I testing. These agents include, but are not limited to, new classes of cytotoxic agents derived from natural products, as well as rationally designed anticancer agents targeted specifically to novel cancer cell targets, including surface receptors, signal transduction molecules, transcriptional factors, and particular DNA and RNA sequences. Furthermore, mechanisms of action of these new anticancer agents available for clinical study include the mediation of anticancer effects not only through classical cytotoxic mechanisms, but also through growth inhibition by interruption of specific oncogene-associated biochemical functions, inhibition of protein synthesis through targeted toxins, induction of differentiation and/or programmed cell death (apoptosis), and through inhibition of tumor angiogenesis and metastasis. In addition, new strategies to overcome resistance to conventional cancer therapeutic approaches are also of interest.

The laboratory studies should be in support of the clinical trial, such that their conduct leads to a greater understanding of the relationship between drug administration and biological changes in patients. Laboratory studies would include pharmacokinetic studies of cytotoxic, differentiation-inducing, targeted and/or other novel anticancer agents, including monitoring of metabolites and intracellular products when appropriate, or other relevant pharmacology correlative studies; and the measurement of relevant indicators of *pharmacodynamic or biologic response* (e.g., changes in signal transduction pathways, induction or suppression of specific gene function, other indications of differentiation induction, or induction of apoptosis). . . ."

It means that the pharmacodynamic evaluation for cytotoxic drugs remains of high importance. Cytotoxic drugs act during the cell cycle, the impact of their action remains on the cell and their specific cellular processes, referred to as cellular pharmacodynamics, may lead to a clearer understanding of drug action and further refine our design of clinical regimens (Huschtscha et al. 1996; Mohr et al. 2004). The potential value of cellular pharma codynamics in cancer therapy is to maximize activity of cytotoxic agents, guide individualized therapy, optimize doses and schedules of drug resistance reversing agent, define mechanisms of resistance, and select patients with high probability of resistance or sensitivity and chemopre vention. The ultimate goal of cellular pharmacodynamic studies is to improve the therapeutic index of drugs.

C.11.2.1 What is Possible and Should Be Possible to Measure?

For the toxic side effect pharmacodynamic evaluation of cytotoxic cancer drugs, the easiest way is to follow the appearance of the toxic side effects, depending on the drugs, but mainly myelosupression, nausea, vomiting, neuropathies, etc. (Chen Hardee et al. 2006; Huria and Lichtman 2008; Saif et al. 2004).

The pharmacodynamic evaluation of the efficacy of the drugs is much more complicated.

C.11.2.1.1 Biomarkers

A number of biological markers are currently assessed in various types of cancer to understand the role of these markers with diagnosis, disease progression, and drug response. Some of the biomarkers are associated with the

diagnosis and disease progression, whereas others are associated with the therapeutic intervention and disease palliation (Ludwig and Weinstein 2005; Duffy et al. 2007). In other words, several clinical assays used routinely in the diagnosis of particular cancers can demonstrate a quantitative or semiquantitative correlation to tumor burden that allows them to be used as biomarkers for monitoring response to treatment. Examples include serum PSA for prostatic carcinoma (Soga et al. 2008) and serum CA 125 antigen for ovarian cancer (Kobayashi et al. 2008). Extraneous factors can affect the levels of these markers making their correlation with tumor type and burden imperfect, so they are most valuable for monitoring clinical response when validated preclinically or used in combination with other markers such as imaging. Evaluating standard assays in preclinical studies using appropriate tumor models can demonstrate how well these markers correlate with tumor growth inhibition produced by a particular compound in early development. Good preclinical correlation between tumor response and biomarker level increases confidence in use of that biomarker for subsequent clinical trials. Use of established, commercially available assays as biomarkers for drug development is highly desirable. They will require minimal technical validation effort, and their use will be relatively inexpensive because certified commercial diagnostic laboratories will be able to measure them with satisfactory confidence. Such importance for biomarkers will also be developed in this chapter concerning targeted therapies. The pathologist engaged in biomarker development most likely will be working with tissue and cell based technologies. Immunohistochemistry (IHC)(Osin and Lakhani 1999; Umemura and Osumura 2004), in situ hybridization (ISH) (Gray et al. 1994), and flow cytometry (FC) (Heydley 1993), three commonly used and well established techniques, now have greatly extended utility for biomarker use because of the continuing development of new reagents. These new reagents include oligonucleotide probes for any sequenced gene and antibodies that detect proteins and phosphoproteins involved in cell signaling cascades and cellular processes important in oncology such as oncoprotein activation, tumor suppressor protein inactivation, cell cycle events, and apoptosis. Many preclinical and clinical investigators use cell proliferation markers such as Ki 67 (Jalava et al. 2006) and apoptosis markers such as Bax (Penault Llorca et al. 1998) and cleaved caspase 3 (Kobayashi et al. 2007) as biomarkers to monitor early antitumor activity by IHC. FC is useful for examining pharmacodynamic responses like phosphoreceptor modulation in surrogate populations of cells such as peripheral blood leukocytes (PBL) that express the same pharmacological target expressed on tumor cells. Additionally, FC applications have expanded with the development of methods for concentrating and quantifying rare cells that can be used as biomarkers, such as circulating tumor or endothelial cells (Melo et al. 1988). For tissue based analyses, numerous types of software tools for morphometric image analysis are now widely available, and technological platforms are emerging for quantification of protein and gene expression in tissue sections and cytology preparations using immunofluorescence (Dardick and Caldwell 1985; Gil et al. 2002). These platforms include the laser scanning cytometer, which operates much like a flow cytometer to measure fluorescent cells on slides, and other automated fluorescence based systems that can quantify protein or RNA expression in cellular and subcellular compartments in tissue sections. The ability to localize and quantify markers such as β catenin and p53 within the tumor cell is becoming more important in understanding cancer biology and determining clinical prognosis. The advent of the tissue microarray and new types of long lived fluorochromes (e.g., quantum dots) give these quantitative IHC and ISH technologies the potential for high throughput analysis and centralized, independent quality control and peer review. A newly emerging tissue based technology of great potential utility is imaging mass spectrometry (Seeley and Caprioli 2008). This technology utilizes matrix assisted laser desorption ionization (MALDI) and time of flight mass spectrometry (TOF MS) for spatial mapping and quantification of proteins and peptides directly within tissue sections, providing a highly informative and integrated proteomic and morphologic analysis (Franck et al. 2009). The use IHC or ISH for biomarker development has several advantages. Foremost is the ability of these in situ methods to demonstrate pharmacodynamic effects, such as decreased receptor phosphorylation or increased apoptosis, within the tumor itself while identifying the cell populations affected. Biopsy and histopathology are routine hospital techniques for tumor diagnosis, often making tissue samples readily available. Preclinical development of assay methods in relevant animal models is highly feasible and allows early evaluation of biomarker robustness for demonstrating drug induced oncology drug development can be optimized by using a tiered set of clinical biomarkers that predict compound efficacy with increasing confidence at each rise in tier. Hierarchical biomarkers can help to support a sequence of increasingly critical corporate decisions about progression of a compound through clinical testing. First level biomarkers, the tier having the lowest level of predictivity, confirm biochemical or pharmacological mechanism of action. These

biomarkers show that the drug is modulating its biochemical/molecular target as desired in vivo, providing pharmacodynamic information that is one step beyond the drug exposure demonstrated by pharmacokinetic analysis.

C.11.2.1.2 Application of Some Technologies to Cellular Pharmacodynamics

Technological advances in analytical chemistry, including improved detection systems, have the potential to increase the sensitivity of drug and metabolite assays, and thus may be applied to the limited number of cells that can practically be collected. Indirect assays for metabolic intermediates (such as phosphoribosylpyrophosphate, PRPP) have been used to elucidate antimetabolite effects (Ghitis et al. 1987). Measurement of cellular effects of a drug, the other needed component in establishing cellular pharmacodynamics, can be difficult. Enzyme assays can prove more troublesome than measurements of gene expression as a result of intrinsic variability of the assay and biological specimens. The use of monoclonal antibodies is sufficiently sensitive and specific to permit semiquantitative assay by immunochemical methods, and these techniques have be applied to the measurement of thymidylate synthase in needle biopsies from patients with colorectal and others cancers (Johnston et al. 1991). Immunohistochemicals are increasingly being used to direct natural product based therapy for tumor with elevated MDR1 expression, and are likely to grow importance as active inhibitors are identified (Meissner et al. 2002). More refined quantification is achieved by western blotting, in this technique, cellular proteins are purified, separated in polyacrylamide gels, transferred to a solid phase support, and detected using specific antibodies (Taipalensuu et al. 2004) (*Fig. C.11 5*). The requirement of starting material for gel electrophoresis may be diminished by either the use of highly specific antibodies or the application of newer luciferase based detection methods (Vilgem et al. 2008). The topoisomerase I quantification by this method is now possible with tumor samples as small as a few milligrams. The most striking increase in sensitivity, however, has come from the application of the polymerase chain reaction (PCR) to detect drug effects in cellular samples. The quantification of gene expression has become feasible in peripheral mononuclear cells (PMN) derived from as little as 3 ml of blood. Following separation of the PMN by density centrifugation and extraction of a total RNA by standard methods, the mRNA is transcribed into cDNA using a reverse transcriptase (RT). Amplification of the cDNA of interest is then accomplished by PCR along with a control cDNA, usually β actin. Following an appropriate number of rounds of amplification, with starting conditions carefully chosen to ensure comparable replication efficiency for both target and control, the cDNA is purified, and transcribed in vitro back into RNA, using a labeled ribonucleotide triphosphate (*Fig C.11 6*). Separation of the products by gel electrophoresis is followed by excision of the labeled band and quantification of the signal by scintillation counting. The RNA content of the gene of interest is expressed as the ratio of its content to that of β actin. This technique is easily applicable for human tissues (Mao and Muller 2003).

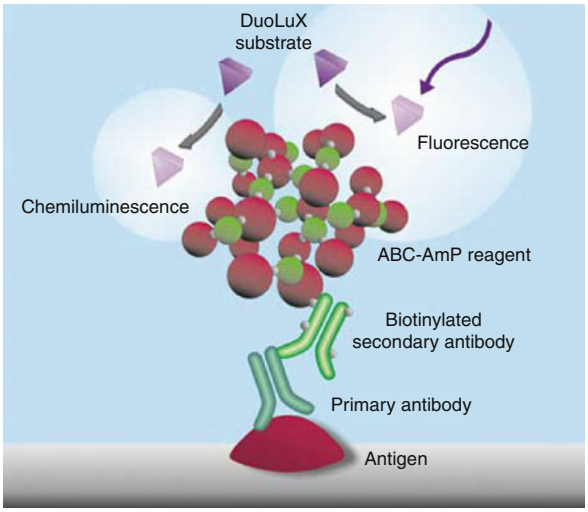

Figure C.11-5
Example of western blotting

C.11.3 Targeted Therapies

In oncology, these proof of mechanism (POM) biomarkers are most reliable when measured within the tumor itself (O'Connell and Roblin 2006; Schuster 2007). This can be technically or logistically difficult, however, and POM is often demonstrated peripherally using blood components or accessible surface tissues in which the pharmacological response has been demonstrated clinically or preclinically to correlate with that in the tumor. For example, clinical investigators used skin biopsies to demonstrate pharmacologic activity of epidermal growth factor receptor tyrosine kinase inhibitors in clinical trials (Albanell et al. 2001; Ranson 2004). Biomarkers advance further into the realm of biological response to confirm that the drug is producing a desired pharmacodynamic effect directly related to its potential for efficacy.

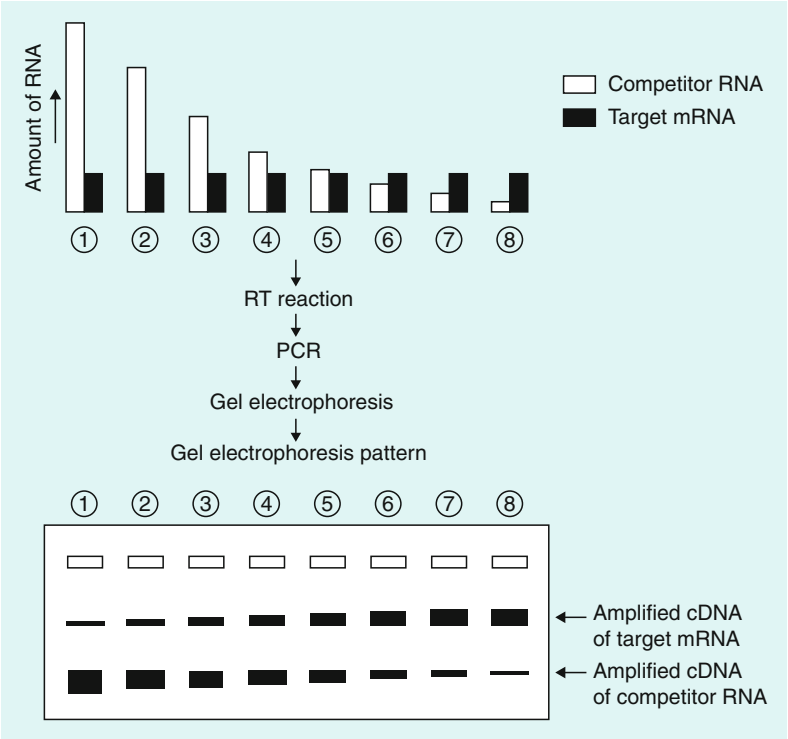

Figure C.11-6
Example of RT-PCR

In oncology, desired biological responses in a tumor include altered downstream cell signaling in pathways related to the target, decreased metabolic activity, decreased cell proliferation, increased cell death, increased cell differentiation, or changes in tumor vascular perfu sion. A number of different techniques and biomarkers are available for measuring each of these responses preclinically and clinically, some of which will be illus trated later in this chapter. A biomarker may have the predictive power for a desired clinical outcome that has been confirmed epidemiologically or by analysis of results from multiple clinical trials. Biomarkers may be surrogate endpoints or endpoints that have been evaluated exten sively enough to be useful for making corporate decisions during late stage development although they are not yet institutionally accepted (Schatzkin 2005; DePrimo et al. 2007). In oncology, the most widely accepted surrogate endpoint of efficacy is objective tumor response, that is, a treatment induced decrease in tumor size measured according to established. Other tumor endpoints are com monly used in clinical trials, but regulatory agencies accept them as true surrogates less frequently than tumor response. The development of novel, targeted therapies in the upcoming new era of cancer treatment will require biomarkers and surrogate endpoints that are relevant for cytostatic modes of action and applicable for monitoring treatment of early disease. Delivery of these surrogate endpoints will require the concerted, sustained effort of the entire oncology community (Kelloff et al. 2004; Park et al. 2004). Encouragingly, many pharmaceutical investi gators, academic clinicians, and regulatory scientists have begun large scale projects to help develop alternative oncology endpoints robust enough for clinical and regu latory use (Early Detection Research Network) (Reynolds 2003). In clinical studies, biomarkers can support creative clinical development plans that optimize information gained from trials and speed drug development. Tradi tional dose range finding clinical trials in oncology escalated up to the maximum tolerated dose (MTD), which was the intended clinical dose for cytotoxic agents. Through use of biomarkers, however, clinical trials of targeted therapies also can evaluate the minimum efficacious dose (MED) (Booth 2007), as defined by preclinical studies, and deter mine the optimum biological dose (OBD) based on clin ical measurement of a desired biological response (Csiki and Johnson 2006; Rojo et al. 2007). New treatment

regimens based on MED or OBD should have fewer adverse effects in single and combination drug therapies than tra ditional treatment at the MTD. With the growth in num bers of targeted therapies for clinical testing, investigators more frequently employ biomarkers to select patient populations that express or overexpress the target of inter est, as illustrated in the development of trastuzumab (Slamon et al. 2001; Piccart Gebhart et al. 2005). Alterna tively, if not used to actively enrich patient populations, biomarkers allow investigators to stratify patients for pro spective or retrospective evaluation of differential clinical responses and for identification of specific responder sub populations (Ludwig and Weinstein 2005; Hartwell et al. 2006). In addition to demonstrating proof of mechanism at pharmacological and pharmacodynamic levels, clinical investigators can monitor safety risks using biomarkers, which is especially important in oncology where most treatment regimens contain combinations of highly toxic drugs. As described earlier, clinicians and regulators can use highly validated biomarkers, surrogate endpoints of efficacy, as predictors of clinical outcome.

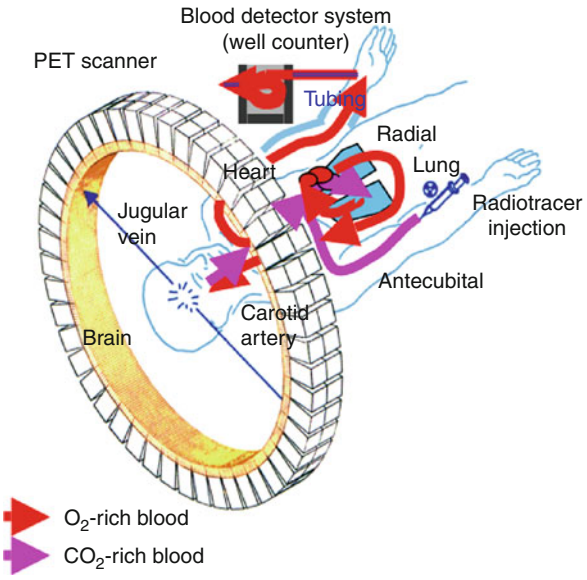

Figure C.11-7
Positron-Emission Tomography (PET)

C.11.4 "In Vivo" Pharmacodynamic Evaluation Using Positron-Emission Tomography (PET)

What is the definition for PET: PET is a sensitive and specific noninvasive technique that uses external detectors to measure the three dimensional (3D) distribution and kinetics of injected compounds that have been labeled with short lived positron emitting radioisotopes (Maziak et al. 2009). Drugs and molecular probes can be radiolabeled with positron emitters such as carbon 11 (physical half life, t1/2=20.4 min), nitrogen 13 (t1/2= 10 min), oxygen 15 (t1/2=2.03 min), fluorine 18 (t1/2= 109.8 min), and iodine 124 (t1/2=4.2 days). The short half life of most positron emitting radionuclides limits the time available for radiosynthesis and formulation and hence the need for an on site cyclotron (Aboagye et al. 2001). Current commercially available clinical scan ners can achieve in plane spatial resolution of the order of 4 8 mm, whereas animal scanners can achieve 0.5 2 mm resolution. Considering the steps involved the application of PET to clinical research, early interaction, and shared risks in technology development between academia and industry (*Fig C.11 7*). After drugs have been shown to act in a predictable fashion, the next step in drug devel opment is to show that they are efficacious (Price 2000). Several probes have been developed to assess efficacy, particularly in the field of oncology wherein alterations in processes downstream of receptor occupancy (e.g., phosphorylation of response elements), for example, could invalidate the relationship between receptor occu pancy and efficacy. Currently, PET methods are being used predominantly as observational endpoints because the level of validation required to use them in making "go no go" decisions (i.e., pivotal decisions whether or not to take the drug to the next phase of development) in the course of drug development has not been achieved for most probes. PET methodology is available for measuring thymidine incorporation into DNA and thus for provid ing an index of proliferation rate, which can be superior to current methods for monitoring tumor response includ ing tumor shrinkage and time to progression (Mankoff et al. 1998). PET methods might be superior because response assessment with anatomical images can be con founded by inflammatory or fibrotic masses. Furthermore, metabolic changes can precede changes in size, an impor tant consideration for monitoring anticancer agents that are cytostatic. The optimal probe for measuring prolifera tion is, however, an issue of current investigation. 2 [^{11}C] thymidine is the PET "gold standard" for measuring pro liferation (Willett et al. 2005). The main limitation of using 2 [^{11}C]thymidine PET is its rapid catabolism ulti mately to ^{11}CO2. A dual scan approach, involving initial scanning with H^{11}CO3 to enable correction of ^{11}CO2 contribution to subsequent 2 [^{11}C]thymidine scans, has been developed and used to determine fractional retention and incorporation rate constants for 2 [^{11}C]thymidine

(Mankoff et al. 1999). This technique has been validated by the demonstration that fractional uptake of 2 [^{11}C] thymidine derived radioactivity correlates with a well known index of cell proliferation, the MIB 1 index. Despite its limitations, 2 [^{11}C]thymidine has been used in several pilot studies to measure response to therapy. For example, in patients with metastatic small cell lung cancer and abdominal sarcoma, the 2 [^{11}C]thymidine flux constant (measured 1 week after chemotherapy) declined by 100% in complete responders and 35% in a partial responder compared with a much smaller decline (15%) in a patients showing progressive disease (Shields et al. 1998). Another strategy for overcoming the rapid catabo lism of 2 [^{11}C]thymidine is the use of less readily metab olized analogues. One of the more promising thymidine analogues for measuring proliferation is 3' deoxy 3' [^{18}F] fluorothymidine (FLT) (Shields 1998). This radiotracer is metabolized to a lesser extent than 2 [^{11}C]thymidine. The retention of FLT is determined not only by the degree of proliferation but also by levels of cell cycle regulated thy midine kinase 1. Studies are ongoing to evaluate the role of this tracer in clinical imaging of proliferation. As a pharmacodynamic endpoint, [^{18}F]FDG has been used in several relatively small studies to monitor the response of tumors to treatment (Brock et al. 2000). These studies include: assessment of the response to temozolomide (in glioma), multidrug chemotherapy (breast and glioma), hormonal treatment, and protracted 5 FU with or with out interferon (colorectal liver metastases). Pharmacody namic studies with [^{18}F]FDG are usually carried out at baseline and soon after the first or second cycle of therapy. The European Organization for Research and Treatment of Cancer PET group (EORTC PET group) has already ten years ago published guidelines for common measurement criteria and for reporting of alterations in FDG PET studies to enable much needed comparison of smaller clinical stud ies and larger scale multicenter trials. Tumor response assessment as defined by the group (progressive, stable, partial, or complete metabolic response) is based on the observation that, on average, a 15 30% reduction in SUV or metabolic rate of glucose utilization can predict response and that this precedes tumor shrinkage and clinical response (Young et al. 1999).

C.11.5 Conclusions

A considerable amount of information is available on the pharmacokinetics of anticancer drugs, but much less is known of their pharmacodynamics, that is, of the rela tionship between therapeutic or toxic response and drug concentration. Drug dosage regimens that are to achieve defined therapeutic objectives can only be designed when both the pharmacokinetic and the pharmacodynamic characteristics of a drug are known. There are a few reports in the literature of relationships in man between toxic response and pharmacokinetic parameters of anticancer drugs, and an even smaller number of reports of relation ships between therapeutic response and pharmacokinetic parameters. It is suggested that the lack of pharmacody namic information is currently limiting the application of pharmacokinetic information to cancer therapy. Ways of improving knowledge of the pharmacodynamics of anti cancer drugs will pass through a number of challenges that need to be tackled before modalities will be widely adopted. The use of biomarkers, and any kind of biomarkers and methodological developments. In addition, there is a need for oncologists, clinical trialists, and industry to be aware of PET, its capabilities, and limitations. It is also essential for the fraternity involved in clinical trials to be convinced that the information provided will be unique, complementary, and unlikely to be gained by other means. This will allow more time and money to be invested and the integration of functional imaging into therapeutic clinical trials, which is likely to benefit the overall development of the compound. The capabilities and limitations of the technology need to be carefully considered before the start of any research, and methodological support that has been validated should be in place in order to process the study and gain meaningful results.

In summary, in addition to its use and value in radio therapy planning and research, functional imaging with PET can be utilized in a variety of applications for the research and therapy of cancer. Its value in the elucidation of pathophysiological processes, development of anticancer drugs, and as a response and prognostic indicator is likely to increase by the day. PET imaging and data interpretation is truly multidisciplinary, requiring cooperation between ani mal biologists, pharmacologists, physicists, PET techni cians, data modelers, radiochemists, and clinicians. Finally, the importance of development and validation of methodology and the need for carefully planned studies prior to the universal adoption of novel radioligands, meth odology, and machines cannot be over emphasized. Func tional PET imaging has tremendous potential and we need to harness it in order to use it successfully.

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Regulations

D.1 Development of Regulations for Submitting Pharmacogenomic Data to Authorities

Klaus Burger

With the advent of genomic methods, expectations soared to exploit them for making better medicines with respect to efficacy, safety, and tolerability. In particular, genomic information should enable the pharmaceutical industry to target specific patient populations that are more likely to respond to the drug therapy, or to avoid individuals who are likely to develop specific adverse events in their clinical studies, thereby "enriching" for appropriate populations. This will reduce the cost of clinical research and accelerate the drug development process. Often neglected in such considerations, drug targets should become identified much more easily with these technologies.

Before discussing the implications of these methods, it is necessary to delimit the topic. There are numerous definitions of pharmacogenomics and pharmacogenetics, however, many in part not consistent which each other, and some not helpful as they confound just the issue they want to define using cloudy nomenclature. This chapter starts the discussion based on definitions proposed by regulatory bodies and authorities, to name primarily the ICH E15 Note for guidance (ICH Topic E15 2007; Other European Pharmacogenomics Guidelines 2009; FDA 2008a; Terminology in Pharmacogenomics 2008) adopted by the European Medicines Agency (EMEA), the US Federal Drug Administration (FDA), and the Japanese Ministry of Health, Labor, and Welfare (MHLW). It defines

pharmacogenomics as: "The study of variations of DNA and RNA *characteristics* as related to drug response," whereas

pharmacogenetics should be a subset of pharmacogenomics being stipulated as "the study of *variations in DNA sequence* as related to drug response."

Important here is the definition of "characteristics" as given in the guidance (e.g., DNA sequence variations, copy number variations, or RNA expression levels). The E15 definitions exclude other "omics" such as proteomics, metabolomics, etc. These definitions are quite similar to those which have been already established by the EMEA's Committee for Proprietary Medicinal Products (CPMP 2002) in its "Position paper on terminology in pharmacogenetics" in 2003; however, the latter also addresses potential uses in its definition of pharmacogenomics (drug design, discovery, clinical development).

Though drug manufacturers have been using genomic methods in their molecular toolbox for some years already, it was not clear how to present these data to the authorities and what risk the disclosure of exploratory data would entail. This was the topic of a workshop (Lesko et al. 2003) the FDA organized jointly with industry in May 2002. In this workshop, basic requirements (technologies, reference populations) and goals of this type of research in different development phases of a drug were highlighted (thereby covering research, preclinical in particular toxicogenomics early and late clinical phases). A concept of "safe harbor" for the submission of genomic data was developed. This term was coined to outline a possible scheme in which exploratory genomic based data generated and submitted under an active investigational new drug (IND) application would be submitted to the FDA but would not undergo formal regulatory review until more would be known about the validity of the technology used and the appropriate interpretation of the data.

In a second workshop (Salerno and Lesko 2004), held in November 2003, the "safe harbor process," now called "voluntary genomic data submission (VGDS)," had been dealt with in greater detail. Again, nonclinical and clinical pharmacology as well as clinical case studies were in focus.

The workshop served as a forum for input into the newly crafted FDA guidance for "pharmacogenomic data submissions (FDA 2005a)." This guidance delineates when and which data in what format has to be submitted to the authority for regulatory action; the guidance is accompanied by an attachment (FDA 2005b) specifying many different scenarios with examples for situations when a VGDS or a regular submission would be appropriate. In addition, the main document clarifies how the data will be used in regulatory decision making. At the same

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time, the authority opened a "Genomics at the FDA (2009)" Web site to provide up to date information on genomics in the regulatory context. In 2007, a companion guide (FDA 2007) was drafted incorporating the experience gained with the numerous VGDS and IND/NDA/biologic license applications (BLA) containing pharmacogenomic data.

In the guidance for pharmacogenomic data submissions, a decision tree helps to classify such data in two ways: either to be submitted mandatorily or voluntarily (Submission of Pharmacogenomic Data Decision Tree 2005). Shortage of space only allows examples to be highlighted. The tree offers instructions on a situation starting with data submitted to an IND/BLA, an NDA, or an approved NDA/BLA. For illustrative purposes, the first situation has been selected (abridged form, for details the source documents are to be consulted).

Data submitted voluntarily will not be used for regulatory decision making by the FDA and is not included in the evaluation of an IND or market application.

Since issues are very much related in Europe, the EMEA and the FDA agreed on a common process of how to handle true VGDS (2006) submissions in a common guidance document to industry, describing the process of setting up briefing meetings with the agencies. Briefing material will be reviewed by the European Pharmacogenomics Working Party (PGWP 2008) and the US Interdisciplinary Pharmacogenomic Review Group (IPRG 2005), not by the official review divisions of the agencies (for details of their respective missions, see original references in the guidance document). For insight into the evolving field of bioethics and regulations of pharmacogenomics in Japan, see Ishiguro et al. (2008), Tamaoki et al. (2004), and citations therein.

Since the first VGDS submission, a considerable amount of experience has been gained. Amur et al. (2008) reported that about 40 clinical and nonclinical VGDS were received by the FDA until 2007 in the areas of oncology, Alzheimer's disease, hypertension, diabetes, depression, obesity, and rheumatoid arthritis and others. The questions addressed with these submissions were equally broad as the indication areas covered and comprised everything from genetic variations in drug response, biomarker qualification, genotyping devices, and toxicology to biostatistics.

With the advancement of science, more consideration needs to be given to the issue of biomarkers in general, and this came even more into focus with the creation of the "critical path initiative."

The critical path initiative launched in April 2004 was intended to "stimulate and facilitate a national effort to modernize the scientific process through which

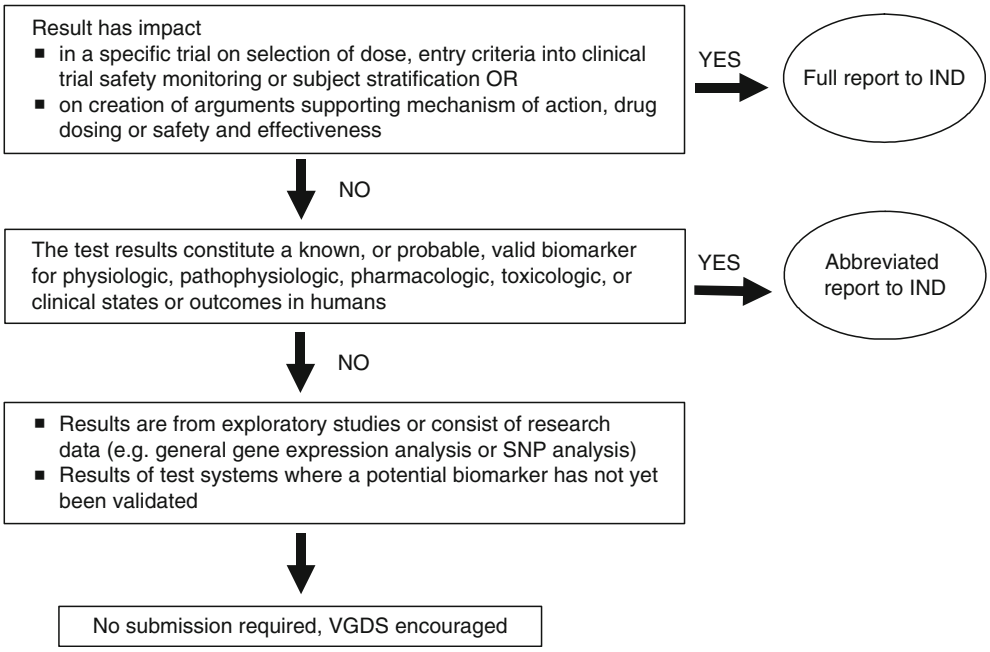

Figure D.1-1

Submission of animal or human PG results to an IND. Adapted from FDA (2005a) http://www.fda.gov/downloads/RegulatoryInformation/Guidances/UCM126957.pdf. Appendix A

a potential human drug, biological product, or medical device is transformed from a discovery or 'proof of concept' into a medical product (FDA 2004)." In face of soaring developing costs and the decline of successful drug registrations, the tools available and the approach to drug development had to be improved. The critical path opportunities lists (FDA 2006) devote nearly an entire section to biomarkers. A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001).

In ICH E15 and the FDA's guidance for industry, only genomic biomarkers were considered, for example, the role of cytochrome variants in drug metabolism or the potential impact of genetic β adrenergic receptor polymorphisms in asthma treatment (see also FDA 2008b for a list of already FDA approved biomarkers). With the critical path and the corresponding European IMI initiatives (Innovative Medicines Initiative 2009), the more or less artificial boundaries between genomic and other biomarkers will disappear. One important result of this new thinking was the establishment (apart from other biomarker consortia) of the Predictive Safety Testing Consortium (PSTC 2006) which includes industry, the FDA, and the EMEA, and is led by the C Path Institute. It is devoted to the identification of safety biomarkers prior to human use of a compound. A specific example of successful research was the identification by industry partners (Merck and Novartis) of the Kidney Injury Group of the consortium of seven biomarkers linked to nephrotoxicity in preclinical settings (EMEA 2008a) now acknowledged by regulators. Meanwhile, EMEA has a guidance for applicants for biomarker qualification under discussion (EMEA 2008b).

Also FDA adapted its schemes to this broader approach. The VGDS program is now promoted into a VXDS scheme to allow all other exploratory data as proteomics, metabolomics, and others to be included (Orr et al. 2007). In fact, the nephrotoxicity biomarker submission was the first one of its kind in the new VXDS process.

Where do we go from here? The rapid pace of science and the increasingly efficient collaborative efforts of agencies, academia, and industry make predictions difficult. Already now, according to an analysis of Frueh et al. (2008), about one fourth of all outpatients (in a cohort of 36.1 million investigated 2006) received one or more drugs that have pharmacogenomic information in the label for that drug. In a similar Japanese investigation (Ishiguro et al. 2008), 16% of the package inserts in 2006 contained pharmacogenomic information. Still only a minority of drugs require mandatory genetic/genomic testing yet; mostly there is not yet sufficient information to guide dosing. However, it is foreseeable that manufacturers will be compelled in special situations to codevelop diagnostic tests ("theranostics," see FDA, 2005c for guidance) to enable physicians to prescribe the drug to the right patients, including also relabeling of existing drugs to adapt to newly identified risks. This is not an easy task, neither for the sponsor nor for the agencies where there are often different bodies which either separately approve the drug or the test. In addition, even if the test is "analytically valid," is it so with respect to "clinical validity" and "clinical utility" (Pendergast 2008)? After establishment of the relationship of a marker with, for example, a genotype, does it relate to the disease and is it of relevance then for therapy?

Already now efficacious treatment of infectious diseases has to employ pharmacogenomic tools; not only the patient's genotype but even more so that of the infectious agent has to be considered (Dorr et al. 2005). Expansion of this approach from viral to other infectious diseases is almost certain.

Toxicogenomics (Khor et al. 2006; National Research Council 2007) might take center stage to guide early administration in man and predict/prevent rare, severe adverse events in later clinical stages and on the market; markers like those identified by the PSTC, even independent of actual drugs, will become extremely important. Proteomic and other non genomic "omics" technologies will gain more importance. The high attrition rates even in phase III of clinical development may make it necessary to use adaptive designs (Freidlin and Simon 2005), including only preselected patients based on experience gained with, for example, certain genomic or nongenomic biomarkers (impacting also the drug label). Adaptive designs may also speed up development through the possibility to submit only a limited dataset on safety and efficacy for registration, which would need to be expanded as a postapproval commitment.

A final but not unimportant issue is the adherence to appropriate privacy rules and proper data protection. This is essentially nothing specific to pharmacogenomics but is contained in the routine set of precautions in doing clinical research. Europe (Directive 95/46/EC of the European Parliament and the Council 1995) has advanced considerably in this respect; however, large areas of the world suffer from special weaknesses in this sensitive field, for example, the modalities of hosting, transfer, and evaluation of data. But also the informational self determination of an individual remains on the table.

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D.2 Pharmacogenomic-Guided Drug Development

Klaus Burger

In an ideal world, a pharmaceutical drug would be highly effective without side effects in patients correctly predicted to be susceptible to the beneficial effects of the respective drug. The drug manufacturer would be able to identify a disease entity worth investing in, because reimbursement would be assured. On the basis of genomic knowledge, molecular diseases and treatment targets would be readily identifiable, and their validation would pose no problem in terms of investment and time due to the availability of appropriate technology. Biomarkers (already validated) should guide an accelerated clinical development process to regulatory approval ensuring no unpleasant surprises concerning safety in the post approval period.

The cornerstone of these dreams possibly coming true one day is pharmacogenomics (PGx). This contribution will center around PGx in development. It goes without saying, however, that the earlier the course is set properly before the development stage (i.e., in discovery and research, in target identification, and lead compound profiling), the smoother the remaining development path will be. An overview is given in *Fig. D.2 1*.

Clinical development comprises phase 1 (first in man studies with limited number of volunteers/patients in oncology and transplantation, to determine drug disposition), phase 2 (proof of concept studies and dose finding in patients), phase 3 (extension into large cohorts of patients and pivotal registration trials which demonstrate efficacy and safety), and phase 4 (post marketing trials to gain experience in more real life situations and as registration commitment).

In this contribution, PGx is regarded as entailing pharmacogenetics and toxicogenomics; in order to avoid artificial exclusion of helpful tools, protein biomarkers (as gene products) will be included in the discussion where needed. PGx embraces analyses of patient populations (e.g., to identify disease genes) but also testing the individual case for either management of clinical studies or setting up rational pharmacotherapy (choice of treatment, dose, toxicities to be avoided). The corresponding research tools as for example genetic and gene expression analyses (SNPs, copy number variations, transcriptome assays, RNA interference, etc.), sequencing and chip technologies, high throughput screening, and bioinformatics are presumed to be known to the reader or accessible through other sources (such as Chen et al. 2009), for definitions see Chap. 39.

D.2.1 Drug Toxicities/Toxicogenomics

In an important survey of multinational pharmaceutical companies, and as a result of a workshop (Olson et al. 2000), it was found that of the compounds with a positive efficacy/safety ratio justifying further development, the type of safety risk is correctly predicted in 70% of the cases based on preclinical in vivo studies. However, the remaining 30% would emerge as unexpected toxicities in man during development. Such human toxicities could be responsible for termination of development (some even late in phase III), limitation of dosage, implementation of drug monitoring, or restriction of the target population and the addition of warning labels.

The important decision step to select the least toxic compound could be moved upstream by toxicogenomics, preferentially to the stage of lead compound selection (not in scope here). In preclinical development, more than 40% of failures to proceed to clinical are due to toxicity, whereas in phase 3, attrition of drugs is driven mainly by lack of efficacy (Suter et al. 2004). Global gene expression analyses should detect changes in gene expression that correlate and help predict toxicities using a wide range of molecular, microarray, and bioinformatic technologies (National Research Council 2007) to capture differential data from humans and animals on exposure to a drug. Due to the high sensitivity of gene expression analyses, in the near future compounds could be quickly classified according to their toxic potential even in first animal experiments using appropriate marker genes. In recent years, a number of databases has been compiled and the creation of a "Human Toxicogenomics Initiative" with a wide scope of tasks and an own database is under way (Committee on Applications of Toxicogenomic Technologies to

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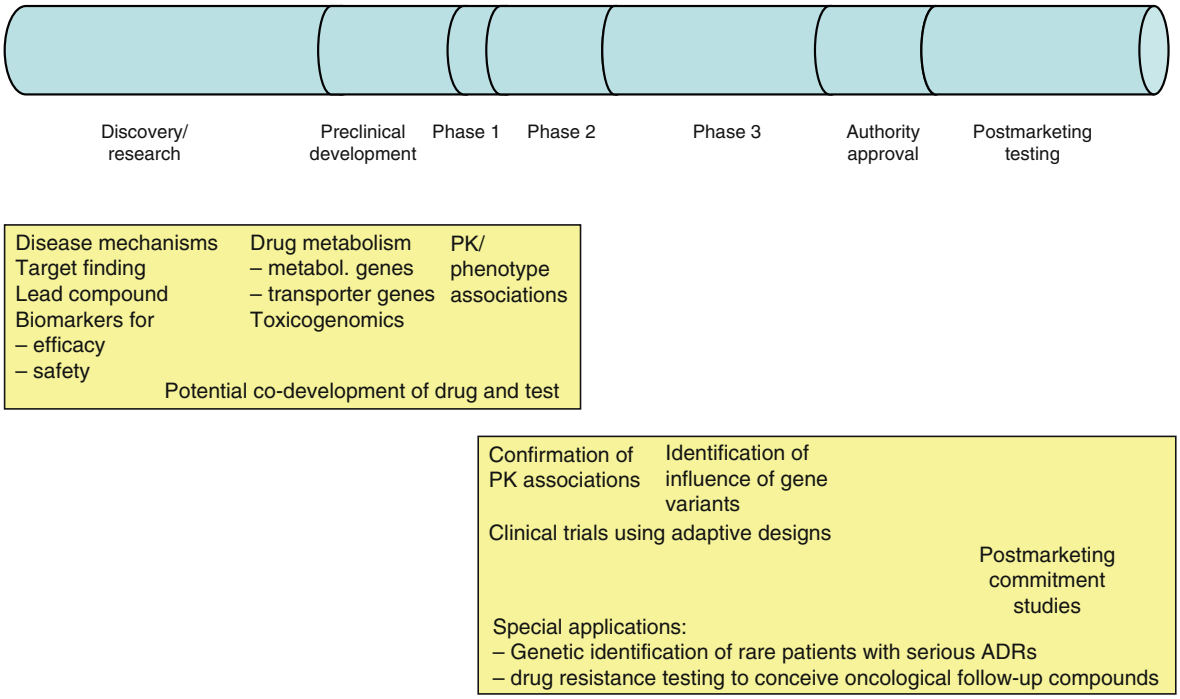

Figure D.2-1
Pharmacogenomics/genetics in drug discovery and development

Predictive Toxicology and Risk Assessment 2007) (for other initiatives and regulatory aspects see Chap. D.1).

PGx analyses are helpful in every step on the route to drug approval. This chapter does not deal with genome wide association and other genetic hypothesis generating studies; clinical development, however, will depend on their results (Bromley et al. 2009). Once it has been decided to proceed to clinical, phases 1 and 2 trials can employ genetic and non genetic biomarkers considered valid by authorities to serve as surrogate endpoints first for toxic ity, second for efficacy. In phases 2 and 3, trials can be "enriched" with more suitable patients.

D.2.2 Phase 1

Pharmacogenetics is an essential tool in phase 1 studies, DNA samples should be taken to analyze absorption, dis tribution, metabolism, elimination (ADME) in dependence on genotype and to gain insight into the PK dose relation ship (for overview about ADME PGx see PhRMA (2008)). In particular, the influence of cytochrome CYP450 gene variants should be clarified early on. However, a number of critical questions should be kept in mind (Grossman 2009), first and foremost: does testing for the relevant ADME polymorphisms in the target population lead to improvement in outcomes, or are testing results useful in medical, personal, or public health decision making?

So called poor metabolizer (PM) genotypes would be endangered if there was only a small safety margin with a certain drug. On the other hand, PMs of a prodrug as for example codeine would not benefit from the intended conversion to the therapeutically active drug (morphine) but rather suffer from codeine side effects. However, at least at present it is unrealistic to check individually the about 80 gene variants known for such a drug which is certainly not lifesaving. This example points to the need to consider exposure (area under the curve; AUC) of patients to the drug as well as to metabolites and precursors. On one end of the scale are PMs, on the other one the "exten sive" and "ultra high" metabolizers for whom the scenario needs to be reverted compared to the PM types. Not only codeine, but also compounds as tamoxifen (potentially life prolonging) and many other drugs (about 20 25%) are metabolized by CYP2D6 (Ingelman Sundberg 2005).

If a drug turns out to be subject to metabolism by one of these genetic "polymorphic" cytochromes (besides CYP2D6 having many variant forms, CYP2C19 and CYP2C9), the likelihood of unexpected individual toxic ities and variable responses based on PK variability is high.

Also, if a drug's PK clearance depends on a single path way, clinical consequences are to be expected if there is pharmacogenetic variability. Similarly, if the drug itself is interfering with such a cytochrome, drug drug interac tion studies are warranted. Pharmacogenetic analyses should be extended to other genes beyond cytochro mes, to transporters (Katz et al. 2008), to multidrug resistance (MDR) genes (important for chemotherapeu tics (Gottesman and Ling 2006), to other metabolism genes (an example being thiopurine S methyltransferase (TPMT), relevant for the metabolism of azathioprine, and 6 mercaptopurine (McLeod and Siva 2002), and to recep tors (e.g., single nucleotide polymorphisms (SNPs) in the β2 adrenergic receptor (Hawkins et al. 2006)). Good genotype PK correlations have been established in quite a number of such genes (Katz et al. 2008).

Similarly as in first in man studies, also multiple dose rising studies need to be accompanied by genotype PK analyses.

If there is no approved test but only genotype phenotype correlations of unknown robustness, the trans lation of such PGx data into specific recommendations for rational drug therapy and even more for drug devel opment is not easy. There are recommendations for the first instance (Kirchheiner et al. 2005), for drug develop ment, on the other hand, the strategies of individual manu facturers vary and become often discernible only in the context of public conferences with key players (McCarthy et al. 2004).

D.2.3 Phase 2 Activities: First Results in Different Indications

The extent of collection of genetic samples depends on the quality and validity of the clinical data to be associa ted with (EMEA/EFPIA 2009). If there are phase 1 results available, phase 2 studies should be able to replicate the genotype PK associations identified earlier. If indicated by previous evidence, PK bridging studies using Cauca sians and Japanese should be performed in order to facil itate development also in Japan (due to ethnic differences in metabolizer types, not an easy enterprise) (ICH 1998); similar approaches will apply to other regions with strong ethnic diversity.

An important result of phase 2 is the definition of dose(s) to be administered in future. Depending on genetic PK variability, in a drug with a narrow safety margin, some patients would need exclusion from therapy unless the dose would be adapted accordingly. Hence, a PGx test would need to be employed throughout further development and also for the post approval phase if the drug could cause appreciable side effects. In such a case, the drug would be codeveloped with its test similarly as is being done for tests identifying responders. Such tests need to comply with the "In Vitro Diagnostic Directive of the European Union" (Directive 98/79/EC) or the "Clinical Laboratory Improve ment Amendment of the United States" (US Food and Drug Administration 2009). The differences in attitudes and procedures between the two regulatory bodies and the outcomes of regulatory approvals with genomic implica tions were discussed in a joint FDA industry workshop (Frueh et al. 2009; see also Hinman et al. 2006; for regu latory guidances see Chap. D.1). Kirk et al. (2008), Weiss et al. (2008), as well as Flockhart et al. (2009) discuss and list predictive pharmacogenetic/genomic tests, some of them being approved already.

Patients can also be deliberately selected by genotype in phase 2 (and phase 3) if there is reliable evidence of efficacy in a certain patient subpopulation. Based on greater confidence in the compound by the sponsor, early studies could use this "enriched" population to allow for a quick "proof of concept." Later studies could then expand to a wider group of patients. Any impact on a future label would only ensue if the wider population did not respond properly or suffer from more adverse reactions than the original one.

PGx analyses in phase 2 explore also the variability of drug target genes. Samples are mostly collected under a broad informed consent for genotyping in order to elucidate the involvement of still unknown genes in later analyses (not specifically hypothesis driven), and offers the option to analyze samples in other disease contexts. This requires a professional operational biobanking setup and high ethical standards to substantiate consideration of patients' rights in front of ethical boards. The shrinking costs of genome wide association analyses will lead to such scenarios in future much more frequently.

First successes of these PGx based strategies in devel opment are notable, in particular in oncology. There, in contrast to other indications, the target organ can (and often should) be sampled, which allows identification of gene expression levels in the tumor (being itself genetically heterogeneous with a high degree of genetic instability). Breast cancer is a field which is rich in PGx assays deter mining cancer stage, prognosis, and treatment options in the individual patients, some of them exploratory, some approved by authorities (Fan et al. 2006; Reis Filho et al. 2006). Novel therapies in many oncological entities have been developed that exploit unique genetic mutations and rearrangements as for example imatinib (targets Bcr Abl), bevacicumab (VEGF inhibitor), cetuximab (EGFR),

trastuzumab (HER 2) (Desany 2004). In the case of imatinib, GIST therapy gene signatures for the low number of nonresponders have been found (Rink et al. 2009).

The so called "Cancer Genome Project" is a very ambitious enterprise. The initiative wants to obtain a comprehensive description of genomic, transcriptomic, and epigenomic changes in 50 different tumor types and/or subtypes that are of clinical and societal impor tance across the globe (International Cancer Genome Pro ject). Each cancer project will involve sourcing and sequencing both tumor and non tumor tissue from some 500 patients (Nicholls 2008). The expectation is that also non oncological investigations will benefit from new technologies and knowledge.

Advances are also remarkable in other indications. Therapies active in the central nervous system need time to be established; often having individualized dosage schedules, they are fraught with adverse effects leading to switching drugs or even drug classes. Investigations into antiepileptic, antipsychotic, and antidepressive therapies are currently identifying first biomarkers to help reduce or avoid toxicities (Patnaik et al. 2008). Beyond pharma cogenetics, PGx allows more insight into pathogenesis and more causal treatment concepts in dementias as Alzheimer's disease (Gupta et al. 2008).

Cardiovascular investigations and first applications have been reviewed by Peireira and Weinshilboum (2009). Good insight into several other indications were presented in a workshop (Armstrong et al. 2009).

D.2.4 Phase 1/2/3 and Adaptive Trial Designs

Regulatory agencies and pharmaceutical industry are now becoming increasingly aware of the benefits of adaptive clinical study designs to make clinical trials more efficient and speed up development. In face of the high attrition rates even in phase 3 *adaptive designs* are a must to be considered. However, they need special justification and should be executed only in areas where it is necessary to cope with difficult experimental situations (EMEA 2007).

Adaptive designs use knowledge from an ongoing study to modify key parameters in the later part of the trial according to predefined rules in order not to compromise integrity and validity of the study (including full control of the type 1 error). There are a number of possible adaptive designs. A first step to an adaptive design is the well known study concept of stopping a trial based on interim data for futility (drug is not effective) or for superiority (e.g., drug proves to be superior to a comparator already at interim analysis). Similarly, sample size could be reestimated based on blinded data to rescue a trial by improving its power (inclusion of more patients). In principle, even test statistics could be changed in adaptive designs, this is discouraged, however (in particular modification of the primary end point), because of low regulatory acceptance.

The earliest possibility to employ adaptive designs is used in phase 1 studies in oncology. There, based upon clear rules, the next dose cohort is selected according to toxicities; a certain algorithm allows to use the next dosage, to enlarge the previous dose cohort or to titrate down because of toxi city (approaches reviewed in Rosenberger and Haines 2002).

Target populations cannot only be opened up as des cribed in an earlier section, but could be narrowed down from a wider population (using e.g., a validated bio marker) to select the proper patient group after interim analysis (enrichment). The use of enriched populations only would in the end lead to a restricted label. In general, what has been selected in mid trial as best treatment in for example phase 2b could be moved into phase 3 to combine it with a comparator in a confirmatory setting. However, authorities tend to challenge rigorously the blinded status of the study after interim analysis. Every effort should be made to ensure integrity and confidentiality of these data in terms of process, team organization, and documenta tion (EMEA 2008; EFPIA 2009).

A root cause of late drug failures is improper dose selection. Traditional designs focus on selecting a target dose out of a (mostly) small number of dosages with fixed dose allocation. Designs of the *adaptive seamless design* type have new features; they aim at combining proof of concept studies/dose finding studies (phase 2) with later trials (phase 3) in a single study: an exploratory (learning) phase is followed by a confirmatory phase guided by the outcome of the first stage. Such a design enables flexible changes in dose allocation ratios; doses can be added or dropped according to information accrued in the trial (see, e.g., *Fig. D.2 2*).

In an adaptive seamless design at the end of the con firmatory phase, all data from the relevant cohorts from both stages of the trial will be combined. This methodol ogy if properly executed should reduce time to approval of a drug and cost to achieve endpoints by using less patients; it offers the opportunity for longer following up of pati ents from the exploratory phase until end of confirmatory phase. However, it should not be forgotten that a single adaptive seamless design study would not be sufficient for approval of a new compound for marketing. Such a trial has to be paralleled by another confirmatory trial to be started immediately after interim analysis. In reality this may shrink the time advantage gained by the first trial

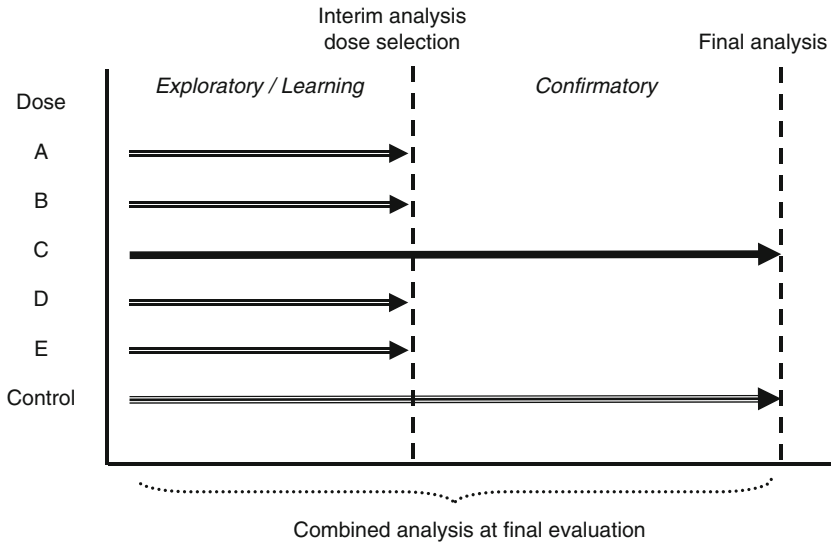

Figure D.2-2
Example of efficient dose finding using an adaptive seamless design

again. There may be other pitfalls, even on the ethical side: educated patient volunteers would prefer to wait to be included only in stage 2 of such trials, creating potential bias between both stages. Further down the road: how should the wording be phrased in an informed consent for such a study? The streamlining power of adaptive designs employing PGx biomarkers or not may not only reside in the methodology but in the teams operating them and the overall preparedness to handle such data without delays (Scott and Baker 2007).

D.2.5 Phase 3: Studies

When approaching submission of the dossier, data should demonstrate that the compound is efficacious, safe, and tolerable in a clearly defined dosage and administration form with an unambiguously positive risk benefit ratio. In today's markets, this is not enough, the drug should compare favorably to competitors in the indication in order to have a good starting position in reimbursement negotiations. Studies in phase 3 should be conducted closer to real life than in phase 2.

If phase 2 results suggest better efficacy or less adverse effects in relevant patient subgroups, selecting these may require codevelopment of a diagnostic test (see above). However, chances for success are low if the drug depends on this test with the result to reach only the safety level of comparators which do not require additional testing. On the other hand, reduction of risks for adverse events by using a PGx test below that of comparators could become a vital factor for market success of a compound (McCarthy et al. 2004).

For many pharmacogenetic polymorphisms, the prevalence is too low to address relevant questions in phase 2. Similarly, PGx enrichment may prove to be necessary only in phase 3. Any PGx selection of patients, however, depends not only on the sensitivity of the test (would it inadvertently exclude many responders?) but would need also consideration of its effects, selection may speed up development and reduce costs; the downside, however, being that no safety data in test negative patients and a reduced chance to detect early on serious adverse events due to smaller study populations. PGx testing is not a panacea for drug development in every respect: there are interesting analyses where testing is for example not cost cutting (Davis et al. 2009). With the rapid development of new methods and fast acquisition of knowledge in mind, general recommendations cannot be given; case by case considerations will be required, even in discussion with authorities.

D.2.6 Phase 4

After approval for marketing, drug exposure of patients usually increases exponentially from a few thousands to possibly millions, and with this the risk for serious unknown and rare adverse drug events to emerge. These events need close monitoring, sometimes in form of post approval

safety studies as consequence of a commitment to author ities in fast approval schemes, always, however, in form of professional drug safety surveillance.

In 1974, long before the era of PGx, clozapin, an atyp ical neuroleptic, was introduced for therapy of therapy refractory schizophrenia. Soon after, it was withdrawn from the Finnish market due to a cumulation of cases of agranulocytosis. The drug remained on the market in some countries as Germany and Switzerland with supervised drug distribution and application. A cornerstone of this procedure has been sampling frequently white blood counts. This simple measurement keeps a pivotal drug available as last therapeutic resort. Today, there is good evidence that clozapine induced agranulocytosis is immune mediated and probably connected to variations in HLA DQB1 (Patnaik et al. 2008) which might become a modern test substrate for future clozapine patients.

The anticoagulant warfarin is a more recent example; its blood levels are influenced largely by CP2C9 and VCORC1. Potentially fatal bleeding by inappropriate war farin concentrations should be avoided. Since many decades the benefits of this drug outweigh the risks. The pharmacogenetic test approved by FDA did not penetrate the market as anticipated; it was considered difficult to interpret and not needed for the patients already on the drug (Roses 2008).

The antiviral compound abacavir may serve as a last (and more positive) example. The drug can cause hyper sensitivity syndromes in a small percentage of patients. Early testing during clinical development did not show reliable correlations of genotype and hypersensitivity. Only after intensified research, retrospective analyses and a prospective clinical trial, was HLA B*5701 identified as a prognostic marker with high sensitivity (>97%) and specificity (>99%) in individual tests of AIDS patients. Contrary to warfarin, where there are conventional clinical "trial and error" methods available, the respective test for abacavir was rapidly taken up by the market since it could identify a patient at risk for hypersensitivity without exposing them to the drug (Roses 2008). The test quickly entered the guidelines and enabled the manufacturer to reach a potential 96% of the market, much more than was conceivable before the prospective trial (Mallal et al. 2008).

D.2.7 Perspective

There is virtually no area in pharmaceutical research and development in which PGx approaches will not be of importance. Due to the rapid advances in technologies, it is difficult to predict which field will progress the most. In future, drug manufacturers will certainly experience even more pressure to contain costs than today and will find themselves squeezed in between the need to create inno vative drugs and satisfy medical need in an ever more aging population and the restrictions imposed by reluc tant governments and payers. The toolbox offered by PGx used in a prudent way should help in this situation, not the least for the sake of patients.

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