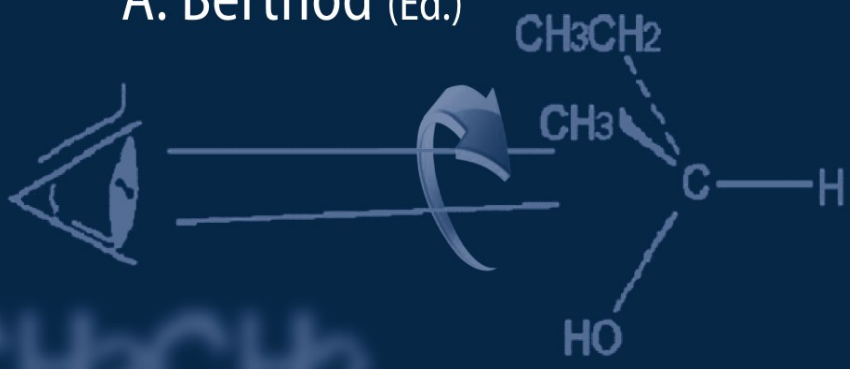
A. Berthod (Ed.)



Chiral Recognition in Separation Methods

Mechanisms and Applications

Springer

Chiral Recognition in Separation Methods

Alain Berthod
Editor

Chiral Recognition in Separation Methods

Mechanisms and Applications

Springer

Editor
Prof. Alain Berthod
University of Lyon
CNRS UMR 5180
69622 Villeurbanne Cédex
France
berthod@univ-lyon1.fr

ISBN 978-3-642-12444-0 e-ISBN 978-3-642-12445-7
DOI 10.1007/978-3-642-12445-7
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2010929210

Cover design: WMXDesign GmbH, Heidelberg

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

What drives a scientist to edit a book on a specific scientific subject such as chiral mechanisms in separation methods? Until December 2005, the journal *Analytical Chemistry* of the American Chemical Society (Washington, DC) had an A-page section that was dedicated to simple and clear presentations of the most recent techniques or the state of the art in a particular field or topic. The "A-page" section was prepared for a broad audience of chemists including industrial professionals, students as well as academics looking for information outside their field of expertise. Daniel W. Armstrong,[1] one of the editors of this journal and a twenty-year+ long friend, invited me to present my view on chiral recognition mechanisms in a simple and clear way in an "A-page" article. In 2006, the "A-page" section was maintained as the first articles at the beginning of each first bi-monthly issue but the pagination was no longer page distinguished from the regular research articles published by the journal. During the time between the invitation and the submission, the A-page section was integrated into the rest of the journal and the article appeared as (2006) Anal Chem (78):2093–2099.

The article was well received. John Dorsey,[2] another very long time friend and colleague, invited me to present it as a lecture in his Dal-Nogare Award session of the 2008 Pittsburg Conference in New Orleans. I presented a talk focusing on the only part of chiral mechanisms that I really know and worked on: chiral recognition mechanism with the macrocyclic glycopeptide chiral selectors. Steffen Pauly, Senior Editor Chemistry for the publisher Springer, heard the talk and asked me to edit a book on the subject. It was so well paid (sigh!) that I could not refuse the offer. . . and now, you have the book in hand.

Author invitations, article redaction time, reviewing and revising process, and text editing took almost 2 years. The book opens with my own general view of chiral mechanisms in separation methods. I was very fortunate in recruiting some of the most distinguished researchers in the field. In many cases, the originators of some of these powerful separation methods agreed to contribute and provide

[1] University of Texas at Arlington
[2] Florida State University, Tallahassee

their unique insight. For instance, Yoshio Okamoto, the discoverer of the powerful carbohydrate-based chiral stationary phases (CSP), and his co-workers prepared a chapter on mechanisms and applications of these CSPs. Cyclodextrins are another class of very useful CSPs. Thomas Beesley, CEO of Astec Inc, recently incorporated in the Supelco-Sigma-Aldrich group, gives his views on cyclodextrin CSPs. Daniel Armstrong introduces the macrocyclic glycopeptide CSPs. In addition, he presents here, with his group, a new class of potentially very powerful CSPs: the cyclofructan CSPs. In capillary electrophoresis (CE) the chiral selector must be added to the mobile phase since there is no real chromatographic stationary phase. Bezhan Chankvetadze of the Tbilisi State University details all possible mechanisms of chiral separations in CE. The sixth chapter written by Brian He of Bristol-Myers Squibb provides the point of view of an expert in chiral separations from the pharmaceutical industry. Next, the macrocyclic glycopeptide CSP properties and interaction mechanisms are presented by Dan Armstrong, people of his group and myself. Tim Ward of Millsaps College, Mississippi, reminds us that vancomycin, one of the macrocyclic glycopeptide selectors, has strong antibiotic properties and proposes, using vancomycin as an example, and that the antibiotic and enantioselective interactions are related. The ninth chapter, presented by Cristina Minguillon of University of Barcelona, deals with countercurrent chromatography and chiral interactions in liquid phases. Eric Peyrin of Grenoble University explains aptamers capabilities in chiral separation and the book ends with a chapter by the Isiah Warner group (Louisiana State University) on another new class of chiral selectors: the chiral ionic liquids.

In drawing this preface to a close, while all authors presented their unique point of view on chiral mechanisms in enantiomeric separations, they would like to impress upon the readers that we are still a very long way from full understanding of the enantiomer–chiral selector interactions leading to chiral separation. For instance, solvents are used. Solvent effects are very important and yet very difficult to predict accurately. The different author approaches should give an idea to the reader on the complexity of the chiral separation problem.

I want to acknowledge and to thank all the authors for the hard work and amount of effort and information that they put in their chapters. We all sincerely wish that this book will be useful to beginners and students as well as to confirmed practitioners in this unique separation field.

Villeurbanne, France Alain Berthod
May 20, 2010

Contents

Contributors

Daniel W. Armstrong Department of Chemistry, University of Texas-Arlington, 700 Planetarium Place, Arlington, TX 76019-0065, USA, sec4dwa@uta.edu

Thomas E. Beesley 148 Waughaw Road, Towaco, NJ 07082, USA, tebeesley@gmail.com

Alain Berthod Laboratoire des Sciences Analytiques, Université de Lyon, CNRS UMR 5180, Bat. CPE, 69622 Villeurbanne, France, berthod@univ-lyon1.fr

David K. Bwambok Department of Chemistry, Louisiana State University, Baton Rouge, LA, 70803, USA, dbwamb1@lsu.edu

Bezhan Chankvetadze Department of Physical and Analytical Chemistry, School of Exact and Natural Sciences, Tbilisi State University, Chavchavadze Ave 3, 0179 Tbilisi, Georgia, bezhan_chankvetadze@yahoo.com

Sayo O. Fakayode Department of Chemistry, Winston-Salem State University, Winston-Salem, NC 27110, USA, fakayodesa@wssu.edu

Aprile Gilmore Department of Chemistry, Millsaps College, 1701 N. State street, Jackson, MS 39210, USA, gilmorea@millsaps.edu

Brian Lingfeng He Bristol-Myers Squibb Research and Development, 1 Squibb drive, New Brunswick, NJ 08903, USA, lingfeng_he@hotmail.com

Tomoyuki Ikai EcoTopia Science Institute, Nagoya University Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan, ikai@chiral.apchem.nagoya-u.ac.jp

Mikhail A. Kuznestov Chemistry Department, BioChemMack S&T, Lomonosov State University, b1/11 Leninskie Gory, Moscow 119992, Russia, mkuznestov@kge.msu.ru

Min Li Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA, minli@lsu.edu

Cristina Minguillón Parc Científic de Barcelona, Baldiri Reixac 10, E 08028 Barcelona, Spain, cminguillon@pcb.ub.es

Yoshio Okamoto College of Material Science and Chemical Engineering, Harbin Engineering University, 145 Nantong St. Harbin, 150001 P. R. China, okamoto@apchem.nagoya-u.ac.jp

Eric Peyrin Département de Pharmacochimie Moléculaire UMR 5063 CNRS, Institut de Chimie Moléculaire de Grenoble FR 2607, Université de Grenoble I, Bât E (C) André Rassat, Domaine Universitaire, 301 avenue de la Chimie, BP 53, 38041 Grenoble Cédex 9, France, eric.peyrin@ujf-grenoble.fr

Hai Xiao Qiu Department of Chemistry, University of Texas-Arlington, 700 Planetarium Place, Arlington, TX 76019-0065, USA, haixiao.qiu@mavs.uta.edu

Núria Rubio Departament de Quimica Organica, Parc Científic de Barcelona, Baldiri Reixac, 10, E 08028 Barcelona, Spain, nrubio@pcb.ub.es

Sergey M. Staroverov Chemistry Department, BioChemMack S&T, Lomonosov State University, b1/11 Leninskie Gory, Moscow 119992, Russia, staroverov@analyt.chem.msu.ru

Ping Sun Department of Chemistry, University of Texas-Arlington, 700 Planetarium Place, Arlington, TX 76019-0065, USA, ping.sun@mavs.uta.edu

Courtney Vowel Department of Chemistry, Millsaps College, 1701 N. State street, Jackson, MS 39210, USA, courtneyv@millsaps.edu

Chunlei Wang Department of Chemistry, University of Texas-Arlington, 700 Planetarium Place, Arlington, TX 76019-0065, USA, chunlei.wang@mavs.uta.edu

Karen Ward Department of Chemistry, Millsaps College, 1701 N. State street, Jackson, MS 39210, USA, wardkdl@juno.com

Timothy J. Ward Department of Chemistry, Millsaps College, 1701 N. State street, Jackson, MS 39210, USA, wardtj@millsaps.edu

Isiah M. Warner Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA, iwarner@lsu.edu

List of Abbreviations

AA	Amino acid
ACN	Acetonitrile
ADP	Adenosine diphosphate
AGP	Acid glycoprotein
AGT	Aminoglutethimide
AMP	Adenosine monophosphate
APCI	Atmospheric pressure chemical ionization
AQC	Amino quinilyl carbamate
ATP	Adenosine triphosphate
ATPS	Aqueous two phase systems
ATR-IR	Attenuated total reflection – infrared
AVI	Avidin
BGE	Background electrolyte
Bis–Tris	Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane acetate buffer
BNDA	Binaphthyl diamine
BSA	Bovine Serum Albumin
C12-Pro	*N*-Dodecyl-L-proline
CB-AC	Acetylated-beta-cyclodextrin
CB-DM	Dimethyl-beta-cyclodextrin
CBH	Cellobiohydrolase
CB-RN	*R*-naphthylethyl carbamate-beta-cyclodextrin
CB-RSP	Hydroxypropylated-beta-cyclodextrin
CBZ	Carboxybenzoxy
CCC	Countercurrent chromatography
CCD	Central composite design – or – charged coupled device
CCS	Charged chiral selector
CD	Cyclodextrin
CE	Capillary Electrophoresis
CEC	Capillary electrochromatography
CF	Cyclofructan
CGE	Capillary gel electrophoresis

CHARM	Charged resolving agent model
CICS	Complexation-induced chemical shift
CIEF	Capillary isoelectric focusing
CIL	Chiral ionic liquid
CIP	Cahn–Ingold–Prelog rule
CMPA	Chiral mobile phase additive
CPC	Centrifugal Partition Chromatography
CS	Chiral selector
CSP	Chiral Stationary Phase
CZE	Capillary zone electrophoresis
DEA	Diethylamine
DFT	Density functional theory
(DHQD)2PHAL	Bis-1,4-(dihydroquinidinyl)phtalazine
DIM	Dimethindene
DM	Dual-mode
DMA	Dimethylacetamide
DMO	Desmethyl meloxifene
DNA	Deoxyribonucleic acid
DNB-(±)-Leu	*N*-(3,5-dinitrobenzoyl)-(±)-leucine
DNB-(±)-Leu-*t*Bu	*N*-(3,5-dinitrobenzoyl)-(±)-leucine-*t*-butylamide
DNS	Dansyl (5-sulfonyl chloride)
DNS-(±)-Nle	Dansyl-(±)-norleucine
DNS-D-Nle	Dansyl-D-norleucine
DNS-L-Nle	Dansyl-L-norleucine
DNZ-(±)-NPG	*N*-(3,5-dinitrobenzyloxycarbonyl)-(±)-neopentylglycine
EDDP	Ethylidene dimethyl diphenyl pyrrolidine
EDTA	Ethylene diamine tetraacetic acid
ee	Enantiomeric excess
EFGF	Electric field gradient focussing
EKC	Electrokinetic chromatography
ELISA	Enzyme-linked immunosorbent assay
EMO	Enantiomer migration order
ENFB	Ethoxynonafluorobutane
EOF	Electroosmotic flow
ESI	Electrospray ionization
FAB	Fast atom bombardment
FCCE	Flow counterbalanced capillary electrophoresis
FDA	Food and drug administration
FLEC	Fluorenyl ethyl chloroformate
GC	Gas chromatography
HILIC	Hydrophilic interaction chromatography
HP-CD	Hydroxypropylated cyclodextrin

HPLC	High Performance Liquid Chromatography
HSA	Human serum albumin
IL	Ionic liquid
IPA	Isopropyl alcohol
IUPAC	International union of pure and applied chemistry
$\boldsymbol{Ka_{R/S}}$	Association constant CS/enantiomer
$\boldsymbol{K_D}$	Distribution ratio
LC	Liquid chromatography
LLE	Liquid–liquid extraction
LSER	Linear solvation energy relationship
MALDI	Matrice-assisted laser desorption ionization
MD	Molecular dynamics
MDM	Multidual mode
MED	Micromachinated electrophoretic device
MEKC	Micellar electrokinetic chromatography
MIBK	Methyl isobutyl ketone
MIP	Molecular imprinted polymer
MLR	Multilinear regression
MM	Molecular mechanic or molecular modeling
MS	Mass spectrometry
MTBE	Methyl *tert*-butyl ether
NARP	Nonaqueous reversed phase
NEC	Naphthyl ethyl carbamoyl
NIR	Near infra-red
NMF	*N*-methyl formamide
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NP	Normal phase
NPLC	Normal-phase liquid chromatography
$\mathbf{NTf_2}$	Bis-trifluoromethyl sulfonylamide anion $[(CF_3SO)_2N]^-$
OVM	Ovomucoid
PBD	Plackett–Burmann design
PIM	Polar ionic mode
POM	Polar organic mode
PEG	Polyethylene glycol
PTC	Phenyl thiocarbamate
PGA	Penicillin G acylase
PCA	Principal component analysis
PLS	Partial least square
QD	Quinidine
QN	Quinine
QSAR	Quantitative structure activity relationship

Rs	Resolution factor
RPLC	Reversed-phase liquid chromatography
R	Ristocetin
RP	Reversed phase
ROESY	Rotating Overhauser exchange spectroscopy
RNA	Ribonucleic acid
RTIL	Room temperature ionic liquid
SCCE	Synchronous cyclic capillary electrophoresis
SDS	Sodium docecyl sulfate
SELEX	Systematic evolution of ligands by exponential enrichment
S_f	Stationary-phase fraction retained in a CCC column
SFC	Supercritical fluid chromatography
SMB	Simulated Moving Bed
SPE	Solid phase extraction
SR	Stereocenter recognition
SULL	Sodium undecanoyl-L-leucine leucinate
SULV	Sodium undecanoyl-L-leucine valinate
***S*-β-CD**	Sulfated β-cyclodextrin
T	Teicoplanin or Absolute temperature in K
TAG	Teicoplanin aglycon
TEAA	Triethylammonium acetate
Tf^-	Triflate anion (CF_3SO^-)
TFA	Trifluoroacetic acid
TFAE	Trifluoroanthryl ethanol
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TOF	Time of flight
TPI	Three-point interaction
UHPLC	Ultra high pressure liquid chromatography
UV-vis	Ultraviolet-visible light
V	Vancomycin
VCD	Vibrational circular dichroism
VP	Verapamil
±-WSA	Racemic mixture of the Whelk-O® selector analogue
XRD	X-ray diffraction
α_{CCC}	Enantioselectivity factor in CCC
α_{HPLC}	Enantioselectivity factor in HPLC
ΔG	Gibbs free energy
ΔH	Enthalpy variation
ΔS	Entropy variation
$18C6H_4$	(+)-(18-crown-6)-tetracarboxylic acid

Chiral Recognition Mechanisms in Enantiomers Separations: A General View

Alain Berthod

Contents

Abstract In 1858, Louis Pasteur, the first to accomplish the separation of two enantiomers wrote: "Most natural organic products, the essential products of life, are asymmetric and possess such asymmetry that they are not superimposable on their image. This establishes perhaps the only well-marked line of demarcation that can at present be drawn between the chemistry of dead matter and the chemistry of living matter." Enantiomers have exactly the same properties in isotropic conditions.

A. Berthod (✉)
Laboratoire des Sciences Analytiques, Université de Lyon, CNRS, Bat. CPE,
69622, Villeurbanne, France
e-mail: berthod@univ-lyon1.fr

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_1,

They behave differently only in anisotropic conditions. Chiral–chiral interactions are needed for enantiomeric separations. The fundamental mechanisms for chiral separations are listed along with the commercially available chiral selectors. Two chemometric examples are commented: one on quantitative structure enantioselectivity relationship and the second one on linear solvation energy relationships. It is shown that the solvents used in the mobile phase may play the most critical role in the chiral mechanism.

1 Introduction

After the thalidomide tragedy (1957–1961), a strict control of the purity of enantiomers used in medicine was inducted. Worldwide, governmental agencies control all active drugs produced by the pharmaceutical industry with a special attention on the enantiomeric purity in case of chiral drugs. With time, less and less new drugs are introduced as racemates. Figure 1 shows the evolution of the numbers of news drugs introduced worldwide as pure enantiomers, achiral molecules, and racemates over the last 20 years. The steady increase of pure enantiomers is associated with the sharp decrease of racemate introduction with only seven racemates introduced over the 2003–2006 4-year period. This figure should be compared to the 246 pure enantiomers and 131 nonchiral drugs introduced over the same period of time [1–3]. It is interesting to note that 99% of the pure enantiomers had a natural or semi-synthetic origin when most of the nonchiral molecules were synthetic drug substances [3]. Such concern on the interaction of enantiomers with the living world is now going beyond the pharmaceutical industry expanding to the food and agriculture industries and wherever animal and vegetable organisms are involved.

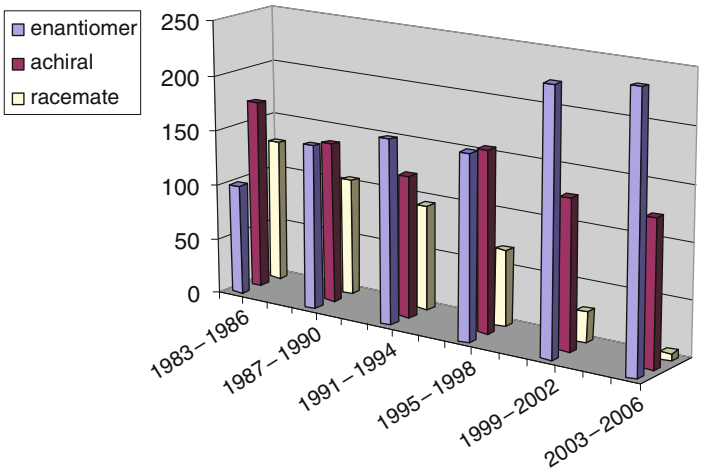


Fig. 1 Time distribution of the number of worldwide newly approved drugs according to chirality character (data from [1–3])

Enantiomer separation and chiral mechanisms go together. The separation of enantiomers is a very difficult task that cannot be achieved without a different pure enantiomer called the selector. This is the first most important point to understand: two enantiomers have exactly the same properties in anistropic, asymmetric, or achiral environments. Some differences in enantiomer behavior can occur only in isotropic or chiral environments. For reasons beyond the scope of this chapter, nature uses single enantiomers of, e.g., amino acids and carbohydrates to build asymmetric living organisms which produce very different interactions with chiral molecules. The metabolic pathway of the (*R*)-thalidomide enantiomer produced the desired sedative effect when that of (*S*)-thalidomide displayed dramatic teratogenic effects in pregnant women. It is because living organisms are asymmetric that chiral separation and pure enantiomers gained such a high significance.

This first chapter will present the world of chiral separation by listing and defining the terms used in the field, giving a brief historical view followed by a general description of enantiomeric interactions and mechanisms involved in enantioseparations. Chromatographic techniques are greatly emphasized due to the background of the author.

2 Nomenclature

2.1 Term Definitions

Absolute configuration	The fully identified spatial arrangement of all stereogenic centers in a chiral molecule.
Achiral molecule	A molecule that does not contain any asymmetric center. Its mirror images are superimposable upon each other.
Asymmetric center	The carbon atom bearing four substituents in a chiral molecule. The tetrahedral sp^3 hybridization of carbon with four different substituents is responsible for more than 95% of the chirality in the living world. Figure 2 shows chiral molecules that do not contain a defined asymmetric center.
Chiral molecule	A molecule with at least one asymmetric center. Its mirror images are not superimposable. The use of the adjective "chiral" is extended to describing involvement with enantiomers, e.g., chiral chromatography, chiral separations.
Diastereoisomers:	Isomers differing by the spatial arrangement of their functional groups not being mirror image of each other. They may contain multiple asymmetric centers. Diastereoisomers may or may not be optically active.

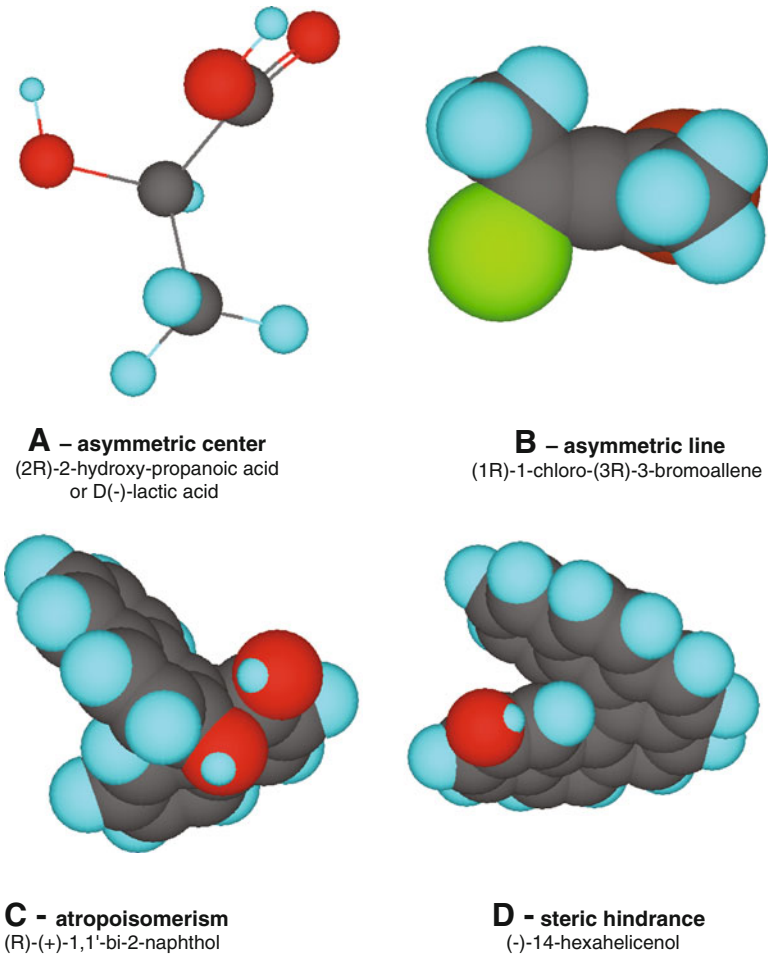


Fig. 2 Examples of chiral molecules with and without asymmetric center. **a** The sp^3 hybridized carbon bearing four different substituents is by far the most common asymmetric center. **b** The C=C=C allene arrangement forms a chiral axis. The 1-chloro-3-bromoallene is chiral. **c** Atropoisomerism occurs when the free rotation around a σ bond is hindered. **d** Steric hindrances create a chiral plane in helicenes

Enantiomer:	One member of a pair of molecules that are mirror images of each other and not superimposable. Enantiomers are optically active.
Enantiomeric excess	The percent excess of an enantiomeric form over the racemate in a mixture of a pure enantiomer and its racemate. Symbol *ee*, it is also termed "optical purity," the specific optical rotation of an enantiomer mixture over the specific rotation of the pure enantiomer. For example, if the *ee* or optical purity of a mixture of

	two enantiomers is 40%, it contains 70% of one enantiomer and 30% of the other. These percentages are seen as 60% of the racemate nonoptically active and 40% "excess" of an optically active enantiomer.
Enantiopure	Quality of a compound that is made of a single isomer not containing its enantiomer according to available analytical methods.
Epimers	Diastereoisomers differing in configuration at one of the two or more asymmetric centers, e.g., sugars. Epimers are optically active.
Meso compound	A diastereoisomer with two or more asymmetric centers and a plane of symmetry within the molecule reducing the number of possible enantiomers. A meso compound is not optically active.
Optical purity	Measure of the enantiomeric excess determined by optical rotation measurement, see "enantiomeric excess".
Racemate	Synonymous of racemic mixture or racemic compound containing exactly the same amount of both enantiomers.
Racemic mixture	A mixture composed of equal amount of enantiomers. This mixture is not optically active.
Specific rotation	The angular rotation $[\alpha]$ observed if a 1 dm length unit tube is used with a compound present at a 1 g/mL unit concentration. $[\alpha]$ is usually expressed in degree $cm^2\ g^{-1}$.
Stereoisomers	Isomers that differ from each other only in the way atoms are oriented in space. There are two types of stereoisomers: enantiomers and diastereoisomers.

2.2 Molecule Nomenclature

The internationally accepted nomenclature for chiral molecule uses the Cahn–Ingold–Prelog (CIP) rules for sp^3 hybridized carbons [4]. The four substituents are sorted by increasing mass of the first atom attached to the asymmetric center. If two atoms are identical (carbons in the case of 2-butanol, Fig. 3), the next heaviest atom one bond further away is considered and so on. Next, the molecule is held by the lightest substituents (-H for 2-butanol in Fig. 3) and the way the three other substituents are arranged in decreasing mass order define the R-enantiomer (Fig. 3 for 2-butanol with the order OH → ethyl → methyl rotating clockwise), R is for the latin word "rectus" right. The mirror image of (*R*)-2-butanol is the S-enantiomer (S is for the latin word "sinister" or left). These rules allow for the absolute configuration of any chiral compounds.

Historically, the first chiral separation of the enantiomers of sodium ammonium tartrate by Louis Pasteur in 1858 was done separating the crystals by hand

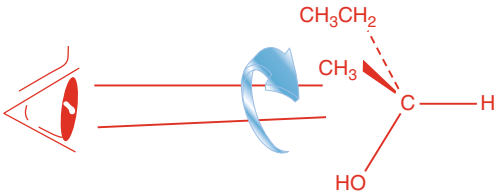


Fig. 3 The Cahn–Ingold–Prelog rules applied to 2-butanol. The decreasing substituents mass order is -OH > -CH_3-CH_2>-CH_3>-H. Seeing the molecule held by the lightest – H atom, the substituent masses decrease rotating clockwise: the R-enantiomer is pictured

using tweezers and a magnifier [5]. In the nineteenth century, the optical activity of the solutions was the only mean to recognize chiral molecules that were sorted in *d*- and *l*-isomers for dextrorotatory or levorotatory the right and left, respectively, optical rotation of the vertically polarized orange sodium light (589 nm). The *d*- and *l*-nomenclature is no more in use today supplanted by the (+) or (–) signs associated with the (R) and (S) CIP notation. Indeed, there is no known relation between the absolute molecular configuration of a compound and its optical rotation. In 1891, Emil Fisher devised a method of representing a three-dimensional molecule on a page. By a lucky guess, he correctly defined the structures of D- and L-glyceraldehyde and consequently of D- and L-tartaric acid [6]. His method was used to name sugars and amino acids for more than 50 years. It is still accepted today for these natural compounds only. Setting glycine apart since it is nonchiral, it must be noted that all amino acids found in proteins are L-amino acids and also have the S-configuration at the exception of cysteine whose –CH_2-SH substituent precedes the carboxylate-COOH in mass making L-cysteine the R-enantiomer.

3 Interaction Between Molecules

3.1 The Bases: The Three-Point Interaction Model

As already said, two enantiomers have exactly the same properties in anisotropic environment. To separate enantiomers, interactions with an isotropic selector are needed. The key step in enantiomer separation and chiral recognition is the formation of labile diastereoisomeric complexes between the enantiomers and the chiral selector. The selector will be able to discriminate between the two enantiomers if there are at least three point of interaction between the chiral selector and one or both of the enantiomers as illustrated by Fig. 4. The left image shows that a chiral molecule can match exactly three sites of the selector. Its mirror image on the right, after all possible rotations, can present a maximum of two groups able to interact with only two sites of the selector. The experimental binding constant of enantiomer (a) will be higher than that of its mirror image (Fig. 4). This difference can be used to separate the two enantiomers. Easson and Stedman were the first to propose in 1933 a minimum of three points of attachment to explain the different physiological

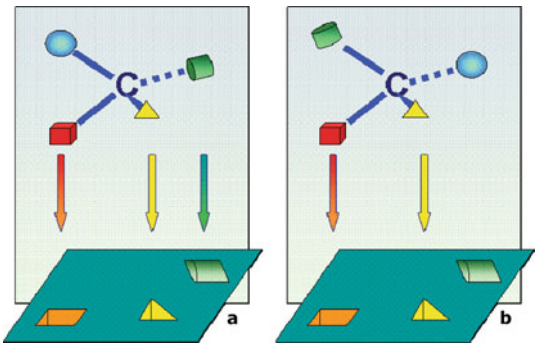


Fig. 4 The three-point interaction model. Enantiomer (**a**) presents three groups that match exactly three sites of the selector when its mirror image, Enantiomer (**b**) can interact with a maximum of two sites of the selector

activities of dissymmetric drugs [7]. Dalgliesh later adapted the model to explain the separation chiral aromatic amino acids that he obtained on paper chromatography, the first use of a cellulose chiral stationary phase! [8].

The "three-point interaction model" was useful in the design of some of the earlier chiral stationary phases (CSP). It is still used to rationalize mechanisms for chiral discrimination. It is very important to use it correctly. Figure 5 shows a fancy

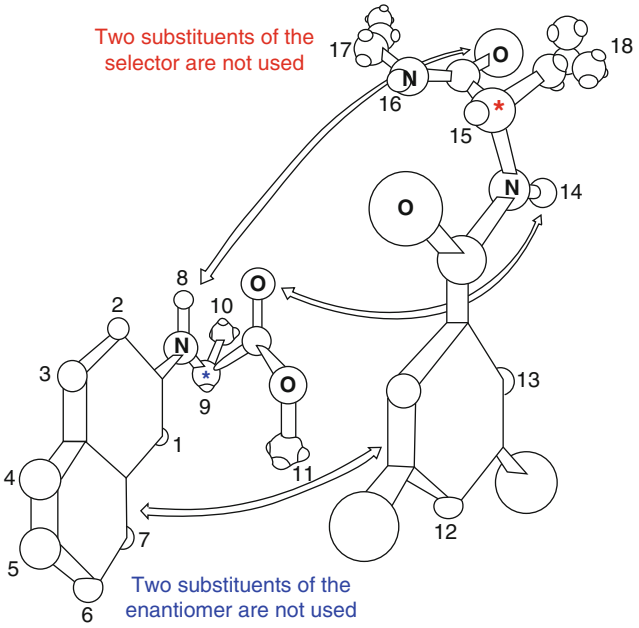


Fig. 5 Incorrect use of the three-point interaction model seen in [9]. Interaction of methyl-*N*-(2-naphthyl)alaninate with the chiral selector *N*-(3,5-dinitrobenzoyl)-(*S*)-leucine *n*-propylamide. Switching Hydrogen 15 and Group 18 on the selector asymmetric center (*) would produce the other enantiomeric form. Switching hydrogen 9 and methyl 10 of the leucine asymmetric center (*) would make the (*R*)-leucine enantiomer. In both cases, the three interactions mentioned would be similarly possible not allowing for any chiral discrimination

molecular modeling that was published by prominent experts in chiral separation in one of the best scientific journal [9]. Sorry to say, this model cannot be right since two of the three proposed interactions occur with the same substituents of the derivatized (L)-leucine chiral molecule. Switching the hydrogen atom 9 for the methyl group 10 (Fig. 5) would produce the (*R*)-leucine enantiomer that could interact with the selector through exactly the same proposed three interactions. This erroneous figure was unfortunately used over and over as an illustration of the three-point interaction model [10–12]. The three interactions must occur between three different substituents of both the chiral molecule and the chiral selector (Fig. 4).

The model is not readily applicable to all cases. The simplification of considering a point of interaction is not appropriate for all enantiomer–selector binding. Steric fits in a cleft or cavity can correspond to more than one interaction. In the original model, all interactions were attractive. From a stereochemical point of view, repulsion is considered as productive an interaction as attraction. For example, two of the interactions can be repulsive if the third interaction is strong enough to promote the formation of at least one of the two possible diastereoisomeric selector–ligand complexes [12]. Also the three-point interaction model can be considered as a geometric model. When the formation of the intermediate diastereoisomer complex involves interaction with a line or a plane or other rigid structures, this interaction can be counted for two or even three. So that this can agree with the idea of the three point of interaction considering that a line is defined by at least two geometrical points or a plane by at least three points [13].

3.2 Intermolecular Forces

All chiral separation methods involve an intermediate diastereoisomeric complex formed between the enantiomers to be separated and a chiral selector. All molecular interactions can play a role in the enantiomer–chiral selector-binding process. Table 1 lists these forces along with their strength, direction, and range.

The strongest interaction is obtained with the *Coulomb force*. The attraction between two electric charges of opposite signs is responsible for the high cohesion of salts. The Coulomb interaction can be attractive as well as repulsive if the two charges have the same sign. The *hydrogen bond* (H-bond) interaction occurs between the positively polarized hydrogen atom of a hydroxyl (or amine) group and the negatively polarized oxygen (or nitrogen) atom of another hydroxyl (or amine) group. H-bonds can be very strong because the negative site can come very close to the hydrogen atom depleted of any remaining repulsive electrons. *Steric hindrances* are due to the intrinsic room needed for an atom or group of atoms. This volume cannot be occupied by another atom or group of atoms. Steric hindrances are repulsive, very strong on very short range.

π–π *interactions* are observed when π-electron molecular assemblies, mainly aromatic rings, interact with each other. Aromatic structures are said to be π-acceptor or π-acid where the ring has electron-rich substituents, mainly $-NO_2$

Table 1 Strength, direction, and working distances of molecular interactions

Type of interaction	Strength	Direction	Working distance
Coulomb or electric	Very strong	Attractive (+/–) or repulsive (same charges)	Medium range ($1/d^2$)
Hydrogen bond	Very strong	Attractive	Long range
Steric hindrance	From weak to very strong	Repulsive	Short range
π–π interaction	Strong	Attractive (donor/acceptor)	Medium range
Ion–dipole	Strong	Attractive	Short range
Dipole–dipole	Intermediate	Attractive	Short range ($1/d^3$)
Dipole-induced dipole	Weak	Attractive	Very short range ($1/d^6$)
London dispersion or van der Waals forces	Very weak	Attractive	Very short range ($1/d^6$)

groups. They are said to be π-donator or π-basic when the π-electron can delocalize such as in a naphthyl group or when electron donating substituents, such as methyl groups, are attached to the aromatic ring. π–π interactions involved in chiral recognition mechanisms are most often attractive with a π-acceptor or π-acid group of the enantiomer interacting with a π-donator or π-basic group of the selector or vice versa. *Ion–dipole*, *dipole–dipole*, and *dipole-induced dipole* interactions act with molecule having a dipole moment. The strongest ion–dipole interaction combines the Coulomb force between the ion and the partial charge of the dipolar molecule. It is always attractive since, by constitution, a permanent dipole structure combines a partial positive charge with an equal partial negative charge. For the same reason, the dipole–dipole interaction is also attractive although weaker than the ion–dipole interaction.

The weakest interaction is that occurring between a permanent dipolar molecule and a dipole induced by the electric field. The *London forces*, part of the van der Waals interactions, are the weakest intermolecular forces. Being the weakest forces does not mean that they have no importance and/or no significant role to play in molecular behavior: these forces are, for example, responsible for the hydrophobic effect that is responsible for a great part of reversed-phase liquid chromatography (RPLC) compound separations and for entropy-driven forces causing oil to separate from water.

4 Assessing Mechanisms

4.1 Rationale of Chiral Recognition Mechanisms

Molecular interactions are responsible for slightly different binding constants between the transient diastereoisomeric complexes formed with the chiral selector and the enantiomers. A full knowledge of the chiral recognition mechanism would

allow predicting which selector will be best to separate the enantiomers of any chiral compounds. The full rationale of chiral recognition is far from being in sight yet although progress is continuous. Chiral recognition mechanisms can be studied most effectively when the exact structure of the chiral selector is known. This is mainly true for the smaller selectors. Most derivatized macromolecules and polymers have little-known structures. However, even with small selectors, too often in liquid chromatography (LC), beautiful molecular modeling studies of chiral molecule—selector association explain a posteriori a particular enantioseparation and have no predictive ability because they do not account for critical solvent effects.

4.2 Methods to Study Mechanisms

Information on chiral recognition mechanisms is mostly obtained by studying differences between binding energies of enantiomers and a chiral selector. Table 2 lists the different methods.

Table 2 Methods for investigating chiral recognition mechanisms

Spectroscopic methods
Circular dichroism and optical rotatory dispersion
NMR
X-ray crystallography
Fluorescence anisotropy
Separation methods
Liquid chromatography
Gas and supercritical fluid chromatography
Capillary electrophoresis
Computer methods
Molecular modeling
Structure properties relationships and handling data

4.2.1 Spectroscopic Methods

Spectroscopic methods can work with the chiral selector associated with the ligand either in solid state or in solution. The chiroptical spectroscopies, circular dichroism, and optical rotatory dispersion, represent an important means for evaluating structural properties of selector–ligand adducts [14]. NMR can specifically investigate ^{1}H proton or ^{13}C carbon atom positions and differentiate one from the other. X-ray crystallography is a powerful technique to investigate the absolute configuration of diastereoisomeric complexes but in the solid state only. Fluorescence anisotropy is a polarization-based technique that is a measure, in solution, of the rotational motion of a fluorescent molecule or a molecule + selector complex [15].

4.2.2 Separation Methods

Separation methods use chiral selectors to separate the enantiomers. Multiple selector–ligand association–dissociation steps occur between the mobile and stationary phase. In chromatography, the selector is most often attached to the stationary phase producing a chiral stationary phase (CSP). The enantiomers are introduced in the mobile phase that is a liquid chromatography (LC), a gas chromatography (GC), or a supercritical fluid chromatography (SFC). They move at slightly different average velocities according to their binding constants with the chiral selector. In capillary electrophoresis (CE) there is not actually a stationary phase: the chiral selector bears a charge, is added to the electrolyte, and moves in the electric field according to its electrophoretic mobility, differentially binding to the two enantiomers. The dissolved chiral selector can be treated as a pseudophase. Alternatively, the chiral analyte may be charged and the selector can be neutral. The migration times of the enantiomers give access to their binding constants. This book focuses on separation methods to obtain insights into chiral recognition mechanisms.

4.2.3 Thermodynamics

Working at different temperatures allows one to perform thermodynamic studies which, in some cases, can provide information on the chiral mechanism. Chromatographic methods give the enantiomer retention factors, k. It is relatively easy to measure the k factors at different temperatures. The slope and intercept of the Van't Hoff plots (Ln k versus $1/T$) contain, respectively, the enthalpy, ΔH, and entropy, ΔS, variations of each enantiomer–selector global (chiral + achiral) interaction.

$$\ln k = -\Delta H/RT + \Delta S/R + \ln \varphi \tag{1}$$

In Eq. (1), R is a perfect gas constant, T is the absolute temperature (°C + 273 in Kelvin) and ϕ is the column phase ratio (ratio of the stationary phase volume over the mobile phase volume).

Comparing the selectivity values α (ratio of the two retention factors k_1 over k_2) for the two enantiomers gives information on the enantioselective part of the interaction [16].

$$\Delta(\Delta G) = -RT \ln \alpha = \Delta(\Delta H) - T\Delta\ (\Delta S) \tag{2}$$

In Eq. (2) $\Delta(\Delta G)$, $\Delta(\Delta H)$, and $\Delta(\Delta S)$ are, respectively, the chiral part of the Gibbs free energy change of the enantiomer–selector phase transfer, the chiral part of the enthalpy and entropy changes occurring with the transfer [16].

The thermodynamic parameters obtained, binding constant , enthalpy, or entropy changes, correspond to the global ligand–chiral selector association. Information concerning the enantioselective separation mechanism can sometimes be inferred

by changing the experimental conditions in a controlled/sequential manner. These changes include the composition of the mobile phase, the pH, the polarity or ionic strength, and substituting and/or derivatizing a chemical group of the analyte and/or the selector.

A statistical thermodynamic study of CSP–enantiomer interaction demonstrated that the possible enantioselectivity factor α was not significantly different when an interaction dominated the two others or when the three interactions were of comparable strength. However, in the former case, ln α should be a linear function of $1/T$, with T, the absolute temperature and a departure from this Van't Hoff behavior would suggest that multiple retention modes compete [17].

Bi-Langmuir adsorption isotherms of enantiomeric pairs and CSPs were determined to gain information on chiral mechanisms. In the few cases fully studied, it was found that the two isomers interacted with type I nonselective sites as well as with type II enantioselective sites [18]. The bi-Langmuir equation is expressed as:

$$q_{R,S} = \frac{q_{\mathrm{I}} b_{\mathrm{I}} C_{R,S}}{1 + b_{\mathrm{I}}} + \frac{q_{\mathrm{II},R,S} b_{\mathrm{II},R,S} C_{R,S}}{1 + b_{\mathrm{II},R,S} C_{R,S}} \tag{3}$$

in which q is the amount of compound at equilibrium per unit of volume of CSP. The subscripts *R, S,* I, and II refer to the R- or S-enantiomers and the type -I or type II adsorption sites. The constants b subscript I and b subscript II with R and S references depend on the site adsorption energies. C is the enantiomer concentration in the mobile phase. The q_{I} contributions and type I b_{I} constants are identical for the two enantiomers making two unknown parameters. There are a total of six unknown parameters in the two q_R and q_{S} in Eq. (3): q_{I} and b_{I} for the nonselective type I sites and $q_{\mathrm{II},R}$ and $q_{\mathrm{II},S}$ and $b_{\mathrm{II},R}$ and $b_{\mathrm{II},S}$ for each R- and S-enantiomers. The six parameters were fully determined for several enantiomeric pairs allowing to obtain the true enantioselectivity factor α as the ratio of the q_{II} b_{II} products for the two enantiomers [19]. In all cases, it was found that the less retained enantiomer interacts with the enantioselective type II sites [18]. For six enantiomeric pairs well separated with enantioselectivity factors over 1.9, the relative chiral contribution to the retention factors of the less retained enantiomers was between 25 and 77% and between 40 and 89% for the most retained enantiomers [18]. The adsorption studies demonstrated also that heterogeneous mass transfer kinetics was the essential explanation for the poor efficiency of protein CSPs. The adsorption results confirmed that the kinetics of adsorption/desorption is much slower on the chiral selective sites than on the nonselective ones [19].

4.2.4 Molecular Modeling and Statistical Analyses

Computer methods use chemical theory to establish chiral recognition mechanisms. Software computes the atom coordinates and calculates the best molecular conformation that minimizes energy between the chiral selector and the ligand.

Beautiful models of chiral molecule–selector association are particularly useful in crystallography and GC. In LC, they may well explain a particular enantioseparation but often have no predictive ability because, so far, models ignored critical solvent effects in a particular interaction.

Another computer approach is to compile a large amount of results and performs quantitative structure retention relationships. This approach classifies experimental results associating conditions, selectors, and enantiomeric pairs successfully separated, not giving great information on the chiral recognition mechanism [20]. However, using the database with probability rule and a statistical approach was proved to have a very good predictive ability [21]. Section 6 of this chapter will detail parts of the author's personal work on associating chemometry and chiral separations.

5 Chiral Selectors in Separation Methods

5.1 Chiral Separations

Enantiomers need an isotropic medium to show different properties. In separation methods, there are three ways to make enantiomers and chiral selectors interact: (1) a chiral derivatization agent can be used to react with the enantiomeric pair turning it into a diastereoisomeric pair that can be separated by classical means; (2) a chiral selector can be added to the mobile phase so that labile diastereoisomers can be formed with the enantiomeric pair during the separation process. Again a classical column will be able to separate the formed diastereoisomers; (3) a chiral selector can be attached to the stationary phase. Labile diastereoisomers can be formed with the chiral stationary phase (CSP) producing different progression of the two enantiomers within the chiral column.

All three methods are used. However, the third method has a significant advantage over the two other methods: a lower than 100% enantiomeric purity of the CSP will not produce erroneous results in chiral analyses. Indeed, a drawback of method 1 is that the derivatization agent used to prepare the diastereoisomers of an enantiomeric pair may be less that 100% pure. If it is only 99% pure, all optical purity analyses done on the diastereoisomeric pair obtained will be systematically biased by 1%. Also, the chemical reaction involved to prepare the diastereoisomers may change the initial optical purity of the enantiomeric pair. Chiral additives to the mobile phases must also have the highest optical purity in order to give accurate results. When a CSP is used to separate enantiomers, e.g., 99% optical purity can be tolerated: the two peaks corresponding to the two enantiomers will be separated by only 99% of the maximum possible resolution factor. However the peak areas will be correct producing accurate optical purity results. The use of CSPs is by far the preferred method in gas and liquid chromatography chiral separations. Chiral mobile phase additives are used in capillary electrophoresis chiral separations.

5.2 Different Classes of Chiral Selectors

The quest for chiral selectors can be arbitrarily separated in two paths: the synthetic route and the natural route. The synthetic route studies the chiral molecule evaluating possible interactions (Table 1) and designs a selector that will interact differently with an enantiomeric form than with its mirror image. The natural route follows Pasteur and uses the fact that the living world is made of countless chiral selectors and produces pure enantiomers. Once a natural chiral selector has been selected, it is tested with its natural chiral target(s) and with many other enantiomers. The observation of the results allows estimating a posteriori possible chiral mechanisms.

Actually, neither of these two classes of selectors is 100% pure: the semi-synthetic class would almost be the actual class since many synthetic selectors are based on a natural molecule and many natural selectors are chemically modified to enhance their initial properties. Table 3 lists most of the selectors used for the separation of enantiomers sorted according to their main origin: synthetic or natural.

Table 3 Chiral selectors and their primary interaction

Appellation	Mechanism	Primary interaction
Synthetic selectors[a]		
Ligand exchange	Diastereoisomeric selector/metal ion/analyte complex	Coulomb or ion-dipole (lone electron pair coordination)
π-complex selectors	Transient 3-point selector/analyte association	π–π interaction
Molecular imprinted polymers	Key and lock association	Selective shape interaction with the imprint
Chiral crown ethers	Inclusion complexation	Ion (primary amino group)-dipole
Polymers	Diastereoisomeric selector/analyte complex	H-bond
Natural selectors[a]		
Proteins	Multiple-binding sites	Variable
Polysaccharides	Insertion in helical structures	H-bond or dipolar or steric
Cyclodextrins	Inclusion complexation	H-bond
Cyclofructoses	Inclusion of NH_2 + multiple-binding sites	Variable
Macrocyclic glycopeptides	Multiple-binding sites	Variable
Cinchona alkaloids	Ion pairing	Coulomb

[a]Most ligand-exchange and π-complex selectors have a natural amino acid core and most natural selectors are artificially derivatized to enhance their performance.

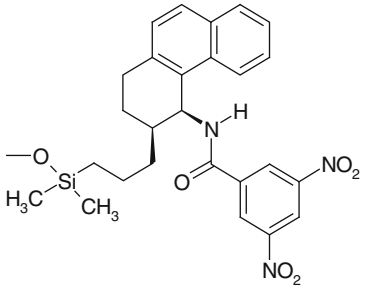
5.2.1 Ligand Exchange Mechanism

The chiral ligand-exchange principle was established in the late 1960s [22]. The basic mechanism involves a metal ion, most often Cu^{2+}, that will be at the core of a complex with the enantiomers and the chiral selectors. To insure an acceptable

chromatographic efficiency, the complex must be kinetically labile forming and dissociating at a high rate. The central metal ion has definite positions in its coordination sphere (six for Cu^{2+}) that can each be occupied by a lone electron pair of organic groups (or water molecules). The chemical functional groups meeting these requirements, a lone electron pair and lability, are the amino, carboxy, hydroxy, amido, and thio derivatives, all bearing at least one lone electron pair on the heteroatom. The chiral selector is an amino acid derivative and other analogous chiral bidentate ligands. Through its amino and carboxylic groups, it occupies two positions of the copper ion coordination sphere. Two positions are occupied by small water molecules leaving two positions for the ligand. The enantiomer analytes must be able to form bidentate chelates. They are α- or β-amino acids, amino alcohols, hydroxyl acids, diamines, amino amides, and dicarboxylic acids. The two interactions described are necessary but not sufficient; the third interaction, required for chiral recognition, is provided by steric- or dipole-type interaction with the selector. Bulky and/or rigid groups in the analyte situated close to the stereogenic center will greatly enhance the chiral recognition as indicated by the good chromatographic enantioselectivity of the separation [22].

5.2.2 Molecular Adjustment for Three-Point Interaction

The π-donator or π-acceptor chiral selectors were introduced in the late 1970s [23]. Later, the (*R*)-*N*-(3,5-dinitrobenzoyl) phenyl glycine selector was specifically designed to have π-bonding capabilities [24]. The π-donator character of the dinitrobenzoyl group of the selector can interact with an added π-acceptor substituent of the enantiomer. Dipole stacking, H-bond, and steric repulsion will provide the two other necessary interactions. The interest of the concept was demonstrated when it was shown that, making the (S) version of the phenyl glycine selector, it was possible to observe the reversal of the elution order of the π-donator substituted enantiomers [25]. Some rigidity in the molecule enhances chiral recognition. At the moment, the most successful π-complex selector, the Whelk-O-1, has two stereogenic centers that are part of a ring and two bonds with two bulky π-electron-rich (acid and basic) substituents.



The synthetic (3R,4S) Whelk-O-1 selector

5.2.3 Key and Lock Recognition with MIPs

Molecular imprinted polymers (MIP) are prepared in solvent solution with the imprint pure enantiomer, a functional monomer (e.g., methacrylic acid), a cross-linker (e.g., ethylene glycol dimethacrylate), and an initiator (e.g., 2,2-azobis-(2-methylpropionitrile)). The mixture is reacted for several hours at elevated temperature. The resultant bulk rigid polymer should be ground in a sieved powder and the template enantiomer will be washed off. Knowing the way the MIP was prepared makes it easy to understand that it will have a strong affinity for the enantiomer that served as template. The interactions are mainly steric and shape recognition associated with other interaction solute depending [26]. The drawback is that MIPs are too specific. They essentially play no role in practical/commercial enantiomeric separations. They are limited by their poor capacity and the lability of the imprint to varying solvent conditions.

5.2.4 Host Crown Ether and Chiral Guest

Chiral crown ether selectors are derivatized forms of polyoxyethylene crown-6 [27]. This crown ether has a cavity that exactly match the size of an ionized primary amine group, $-NH_3^+$. The host–guest ammonium-crown ether interaction, one point of attachment, is the driving force of the enantiomer with this class of chiral selector. The two other necessary interactions are a steric and a hydrophobic one. They will occur between the crown ether substituents and the host substituent. Chiral crown ether can only discriminate chiral molecules with a primary amine group at low pH (where the amine is protonated).

Crown ether type-cyclic oligosaccharides could soon become another class of very efficient chiral selectors. The crown ether cavity could be used as well as the fructose sugar on the ring. Derivatized forms of cycloinulooligosaccharides showed excellent chiral recognition ability for primary amines and a variety of chiral compounds (Armstrong, 2009, personal communication).

5.2.5 Synthetic Polymers

The helical polytriphenylmethyl methacrylate was the first synthetic chiral polymer able to separate a very limited number of enantiomers [28]. Recently a fully synthetic chiral stationary phase based on polymerized diacryloyl derivative of *trans*-1,2-diaminocyclohexane [either (R, R) or (S, S)] bonded to silica gel in the form of a very thin layer was proposed as a new LC CSP [29]. This CSP could not resolve many enantiomeric pairs. However, when it could resolve a racemate, it was shown that the amount that could be loaded was much larger than that on most other CSPs. It means that the number of active sites is large. Hydrogen bonds were found to be pivotal in the chiral recognition mechanism of this CSP. The enantioselectivity was adjusted by the methanol content in the organic mobile phase. Polysodium *N*-undecanoyl-L-leucyl-leucinate (poly-SULL) and −L-leucyl-valinate

(poly-SULV) were dipeptide polymers forming micelles that were found very useful in micellar electrokinetic chromatography with a broad range of applications [30].

5.2.6 Proteins

Proteins were very early introduced as natural chiral selector [31]. This was a highly logical choice since such bio-macromolecules are responsible for the chiral discrimination of drugs and nutrients in the living body. Proteins can discriminate a wide spectrum of charged and neutral molecules. However, they may be difficult to use since small changes in the experimental conditions, pH, ionic strength, added organic solvent, may cancel the enantiorecognition. It is not possible to give a simple mechanism since a single protein may contain several sites acting as chiral selectors. All listed interactions may be involved.

5.2.7 Polysaccharide Selectors

Cellulose, amylase, and chitin are the most abundant optically active natural polymers. They can be readily modified to carbamates or esters through reactions with isocyanates and acid chlorides, respectively [32]. These selectors are very successful and have broad selectivity. They associate individual chiral carbohydrate monomers in a long-range helical secondary structure, also chiral. This association was found to be highly effective for HPLC enantiomer separations. Since the most popular selectors (Chiralcel® OD and Chiralpack® AD in coated forms or Chiralpak® IA and IB in bonded forms) are cellulose and amylose derivatized with 3,5-dimethylphenyl carbamate, a π-donator or π-basic group, it is likely that π–π interactions will be part of the mechanism. However, these chiral polymers offer so many possible interacting sites that many enantiomers are discriminated finding three different points of interaction without possibility to know exactly the mechanism.

5.2.8 Inclusion Complexation

Cyclodextrins (CD) are small cyclic polysaccharides forming a cone-shaped cavity with 6, 7, or 8 glucopyranose units for the α-, β-, or γ-CD, respectively. The interior of the cavity is rather nonpolar with ether groups; the larger and smaller rims of the cavity are lined with polar primary and secondary hydroxyl groups, respectively. Inclusion complexation is the driving interaction in chiral recognition by CDs. Native CDs were proposed in the early 1980s as chiral selectors [33]. Polar secondary interactions with the hydroxyl groups were predominant. Derivation of these hydroxyl groups produced a wide variety of CDs with adjusted polarities and functionalities. Derivatized CDs were able to separate a broad spectrum of enantiomers [34]. For example, naphthyl-ethyl carbamate-substituted CDs associated $\pi-\pi$ interactions, H-bond, and inclusion complexation widening the applicability

of the selector [35]. Since CDs are the main chiral selectors used in enantioseparations done in GC and CE, then, as a class, CDs have the broadest selectivity of any class of selectors in existence today.

It was found that polar enantiomers could be separated with CDs in nonaqueous polar medium (e.g., 99% acetonitrile with 1% methanol). In this situation, inclusion complexation is unlikely, the solvent molecules occupying the CD cavity. The chiral mechanism involves H-bonds with the spatially oriented hydroxyl groups at the rims of the cavity and other interactions with the numerous asymmetric carbons of the glucopyranose units [36]. Polar organic mobile phases were tried with other CSPs and greatly extended their usefulness enhancing the role of H-bond interactions that were screened by water molecules.

5.2.9 Macrocyclic Glycopeptide Selectors

Reading in a biological journal that macrocyclic antibiotics were inhibiting the development of Gram-positive bacteria by blocking the cell wall development by binding to the D-Ala–D-Ala terminal part of an essential protein, Armstrong thought that these molecules should be wonderful chiral selectors for amino acids. He proposed and patented the macrocyclic glycopeptide chiral selectors in 1994 [37]. As expected, these selectors were the best ones in separating native amino acids, the binding constant of the D-form being always significantly stronger thanthe L-form [38]. The critical role of the carboxylic acid group (in the -COO^- ionized form) was demonstrated. The methylation of this group cancels all chiral recognition [39].

It turned out that these selectors could do much more than amino acids through their numerous active parts with many possible mechanisms. The most useful selectors are vancomycin, ristocetin, and, especially, teicoplanin. All three have similarities in their complex structures. They contain one or two charged sites, hydroxyl groups, aromatic rings, polar (e.g., amido) and apolar (e.g., alkyl chain) groups that give them the capability of interaction through all possible forces listed in Table 1 through a mechanism most often difficult to establish [40]. These three selectors show a “complementary separation” effect when used in LC chiral selectors. If a partial separation of a given pair of enantiomer is obtained with one glycopeptide, good chance exists that a full baseline be obtained with one of the two other selectors. From a mechanistic point of view, it means that there are subtle differences in the stereo-binding sites between these related selectors.

5.2.10 Cinchona Alkaloids

The two Cinchona alkaloid selectors will be used to really get into a particular chiral recognition mechanism. Quinine is a natural alkaloid extracted from the bark of the South American Cinchona tree of the *Rubiaceae* family and used as an anti malaria drug (Fig. 6). Its 8 and 9 positions are, respectively, substituted in the S and R configuration. By chance, quinidine is the mirror image form of quinine, also found in the Cinchona bark with the 8R and 9S configuration. These two alkaloid enantiomers

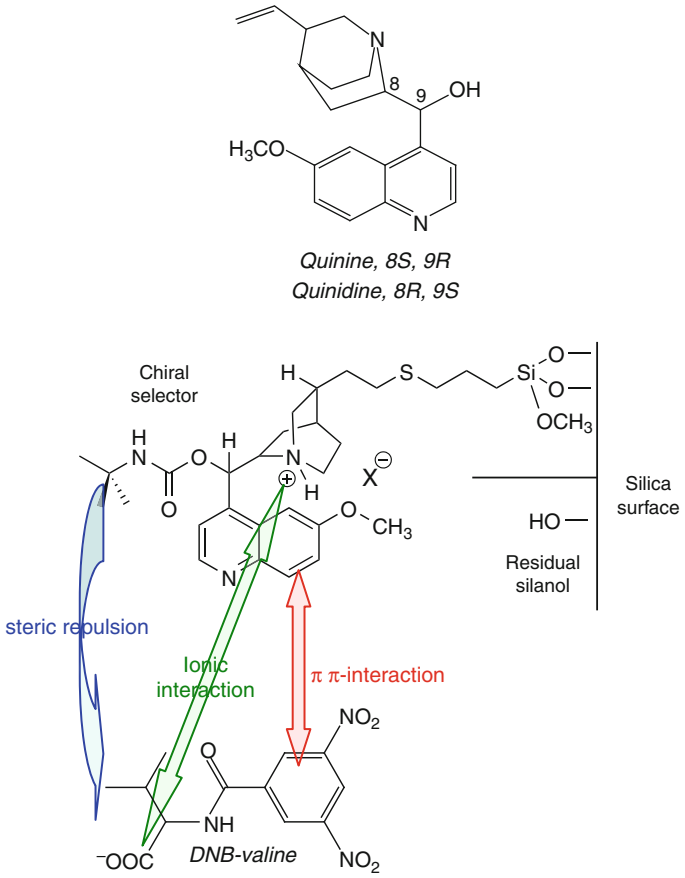


Fig. 6 *Top*: the quinine and quinidine selectors. *Bottom*: Chiral recognition mechanism by a quinine-based chiral stationary phase (*CSP*). The strongest interaction is the ionic docking attraction between charges of opposite signs. DNB-D-valine is more retained by the quinine CSP than its L-enantiomer. DNB-L-valine is more retained by the quinidine CSP. All three interactions occur between three different substituents of both the quinine selector and amino acid selectand. Compare with Fig. 5. Adapted from [41]

can easily be individually separated and derivatized to prepare two useful CSPs with opposite configurations [41].

The active quinine site responsible to the enantiomer separation can interact through (1) an ionic interaction due to the quaternary ammonium, (2) π–π interaction with the quinoline group, and (3) dipole and H-bond or steric hindrance interaction with the carbamoyl substituent. The quinine selector can well separate the enantiomers of *N*-3,5-dinitrobenzoyl (DNB) derivatized amino acids. The docking interaction is the ionic attraction between the negative carboxylate charge of the DNB amino acid and the positive ammonium group of the CSP. Then, the DNB π-acid group can interact with the quinoline π-basic group of the CSP making the

second attractive interaction. The third interaction is a repulsive steric hindrance between the bulky tertiobutyl substituent of the carbamate group on the CSP and the substituent of the amino acid, e.g., phenyl group for phenylalanine, isobutyl group for leucine, or methyl group for alanine (Fig. 6).

In the case of DNB amino acids, the relevance of the mechanism was established by the following results: (i) Methyl esterification of the amino acid carboxylic group cancels all chiral recognition making the docking approach impossible; (ii) A relationship between log α, the enantioselectivity factor, and log k_2, the retention factor of the most retained enantiomer, was found depending on the amino acid side chain size. The retention factors, k_1, of the first eluting DNB amino acids were similar [41]; (iii) The enantioselectivity factors and elution order, respectively, obtained on the quinine and quinidine CSPs are similar and opposite for the same enantiomeric pairs; (iv) Native amino acid enantiomers are not separated (no π–π interaction) [41].

It is because the chiral selector was a relatively simple molecule having naturally its stereoisomer that the chiral recognition mechanism could be fully established in the case of DNB-derivatized amino acid enantiomer separation. Most chiral selectors are very complicated molecules making extremely difficult to predict a priori a chiral recognition mechanism.

6 Chemometry and Chiral Mechanisms

6.1 Quantitative Structure Enantioselectivity Relationship

Chemometric methods are very useful allowing for predictions of chemical properties. Quantitative structure activity relationships (QSAR) are used in many modern chemical softwares to give a very decent calculated value for chemical properties such as molar volume, boiling point, acidity constant, or octanol/water partition coefficient [42]. The molecule is seen as an association of fragments, each of which contributing to the overall property [43].

Almost 20 years ago, I had the chance to work with a meticulous student that kept well-documented records of all successful as well as unsuccessful chiral separations that he was doing [35]. It is very difficult to teach students that failed experiments contain information and that it is important to record them carefully. He tested 121 racemic compounds on the same CSP, the (*S*)-naphthylethyl carbamoyl-substituted β-cyclodextrin (S-NEC-β-CD), with two mobile phases, hexane-isopropyl alcohol (IPA) normal mobile phase and acetonitrile (ACN)-ethanol (EtOH) polar organic mobile phase, writing down all data and saving chromatograms. The S-NEC-β-CD CSP is unfortunately no more available today, supplanted by the more broadly efficient Cyclobond® I 2000 DNP β-CD and Cyclobond® I 2000 SP β-CD marketed by Supelco (Bellefonte, PA). Considering the large amount of data, we thought to use a QSAR approach assuming that each one of the four substituents of the asymmetric center was responsible for an identical contribution to the experimentally

observed enantioselectivity. Equation (2) relates the experimental enantioselectivity factor α to the chiral Gibbs energy $\Delta(\Delta G)$ of the enantiomeric pair–CSP interaction:

$$\alpha = \exp\left(-\frac{\Delta(\Delta G)}{RT}\right) \tag{4}$$

The quantitative structure enantioselectivity approach will consider that the overall $\Delta(\Delta G)$ energy for the interactions of the enantiomeric pair with the CSP in a particular mobile phase is the sum of four independent $\Delta(\Delta G)_i$ contributions coming from the four different substituents attached to the asymmetric carbon:

$$\Delta(\Delta G) = \Delta(\Delta G)_1 + \Delta(\Delta G)_2 + \Delta(\Delta G)_3 + \Delta(\Delta G)_4 \tag{5}$$

The 121 compounds were made with only 81 different substituents arranged differently. For example, 119 compounds had the hydrogen atom as one of the four substituents of their asymmetric center. This observation prompted for the use of the H atom as the energy reference substituent [35]. It must be pointed out that more than 1.6 million different enantiomeric pairs could be made with 81 different substituents. Using the only 121 enantioseparations, the experimental $\Delta(\Delta G)$ values were entered for all resolved enantiomeric pairs and all unsuccessful separations were also entered with a nil value for $\Delta(\Delta G)$. Using a data mining software and selecting the H atom as the energy reference with a $\Delta(\Delta G)_H = 0$ cal/mol contribution, it was possible to deconvolute the 484 (121×4) contributions to obtain the substituents free energy contribution for enantiomeric recognition of the 81 atoms or groups of atoms listed in Table 4.

A positive energy value in Table 4 means that the presence of the substituent in the chiral molecule increases the chiral recognition by the *S*-NEC-β-CD CSP more than the H atom does and vice versa.

Table 4 allows for the computation of the theoretical enantioselectivity factor for any of the possible 1.6 million enantiomeric pairs built with four of the substituents. However, this value is for the *S*-NEC-β-CD CSP with a hexane-IPA mobile phase only. For example, the dinitrobenzoyl derivative of propranolol has a chiral center bearing substituents #3 (H-), #36 (-O-CO-DNB), #77 (-CH_2-O-(1-naphthyl), and #80 (-CH_2-N(*t*-Bu)-CO-DNB. These four substituents contribute, respectively, for 0, 146, 20, and 101 cal/mol making a $\Delta(\Delta G)$ of 267 cal/mol that would produce an enantioselectivity factor α of 1.58 [Eq. (3), with 586 cal/mol for the *RT* product at 22°C or 295 K and $\exp(-267/586) = 1.58$]. The experimental α was only 1.06. Table 4 shows at the bottom that the four substituents do not interact fully independently. Of course, two identical substituents make the chirality disappears, so the $\Delta(\Delta G)$ energy is necessarily nil ($\alpha = 0$). It was found that two DNB derivatives were detrimental for the enantioselectivity factor decreasing the $\Delta(\Delta G)$ energy by 230 cal/mol (Table 4). Both the amine and alcohol group of propranolol were DNB derivatized, so the calculated 267 cal/mol for the four substituents must be decreased by 230 cal/mol giving a final $\Delta(\Delta G)$ value of only 37 cal/mol and the corresponding enantioselectivity factor $\alpha = 1.065$ very close to the experimental value.

Table 4 Substituent-free energy contributions in cal/mol for enantiomeric recognition on (*S*)-naphthylethyl carbamate-substituted β-cyclodextrin chiral stationary phase

	R substituents		Mobile phase	
No.	Formula	Structure	Hexane-IPA	ACN-EtOH
1	Br	-Br	−27	–
2	Cl	-Cl	−40	–
3	H	**Reference substituent**	0	0
4	CHO_2	-COOH	–	7
5	CH_2NO	-NH-CHO	–	0
6	CH_3	-methyl	−74	−34
7	CH_3N_2O	-NH-CO-NH_2	–	27
8	CH_3O	-CH_2-OH	−122	–
9	CH_3O	-O-CH_3	−94	–
10	$C_2H_2O_2$	-COO-CH_3	14	–
11	C_2H_4NO	-NH-CO-CH_3	14	7
12	C_2H_5	-ethyl	−81	–
13	C_2H_5O	-CH_2-O-CH_3		–
14	C_2H_5O	-CHOH-CH_3	−115	–
15	C_2H_5S	-CH_2-S-CH_3	−27	−27
16	C_3H_5	-cyclopropyl	−108	–
17	$C_3H_5O_2$	-COO-CH_2-CH_3	54	–
18	C_3H_7	-*n*-propyl	−81	−34
19	C_3H_7	-isopropyl	−20	−27
20	C_4H_9	-*n*-butyl	−40	−34
21	C_4H_9	-CH_2-CH-$(CH_3)_2$	−47	−47
22	C_4H_9	-CH(CH_3)CH_2CH_3	–	−14
23	C_4H_9	- *t*-butyl	−81	–
24	$C_5H_3N_4O_3$	-NH-DNBPy	–	47
25	$C_5H_9O_2$	-COO-$(CH_2)_3$-CH_3	142	–
26	$C_5H_9O_2$	Solketal	−20@	–
27	$C_5H_{10}NO$	-CO-NH-*t*-Bu	68	–
28	$C_5H_{10}NO_2$	-NH-CO-O-*t*-Bu	0	−27
29	C_5H_{11}	-*n*-pentyl	−81	–
30	C_6H_5	-phenyl (−Φ)	81	81
31	C_6H_5O	-Φ-OH	68	–
32	C_6H_5O	-O-Φ	54	–
34	C_6H_{11}	-cyclohexyl	81	–
35	C_6H_{13}	-*n*-hexyl	−81	–
36	$C_7H_3N_2O_6$	-O-CO-DNB	142	–
37	$C_7H_4NO_4$	-COO-Φ-NO_2	14	–
38	$C_7H_4N_3O_5$	-NH-CO-DNB	230	68
39	$C_7H_4N_3O_5$	-CO-NH-DNB	263	–
40	$C_7H_4N_3O_6$	-O-CO-NH-DNB	182	–
41	C_7H_6NO	-NH-CO-Φ	27	14
42	C_7H_6NO	-CO-NH-Φ	148	–
43	$C_7H_6NO_2$	-NH-CO-O-Φ	27	−27
44	C_7H_6Cl	-CH_2-Φ-Cl	14	–
45	C_7H_7	-CH_2-Φ	0	0
46	C_7H_7O	-CH_2-Φ-OH	94	27
47	C_7H_7O	-CH_2-Φ-OH(meta)	−27	–
48	$C_7H_{12}NO$	-CH-NH-cyclohexyl	155	–
49	C_7H_{15}	-$(CH_2)_6$-CH_3	−54	–

Table 4 (continued)

No.	R substituents		Mobile phase	
	Formula	Structure	Hexane-IPA	ACN-EtOH
50	$C_8H_{14}NO$	-CH-N(CH_3)-cyclohexyl	148	–
51	$C_8H_5N_2O_6$	-CH_2-O-CO-DNB	94	–
52	$C_8H_6N_3O_5$	-CH_2-NH-CO-DNB	20	–
53	$C_8H_6N_3O_5$	-N(CH_3)-CO-DNB	243	–
54	$C_8H_6N_3O_6$	-CH_2-O-CO-NH-DNB	68	–
55	C_8H_7	-1-benzocyclobutene	−122@	–
56	C_8H_9N	-CH-NH-CH_2-Φ	122	–
57	C_8H_9N	-CH-N(CH_3)-Φ	94	–
58	C_8H_9	-CH_2-CH_2-Φ	–	−7
59	C_8H_9O	-CH_2-Φ-O-CH_3	–	0
60	C_9H_7N	-CH_2-(3-indole)	27	14
61	C_9H_7NO	-CH_2-O-(7-indole)	14	–
62	C_9H_9	-(1-indanyl)	–	–
63	$C_9H_{10}NO$	-CH-NH-CH_2-CH_2-Φ	142	–
64	$C_9H_{10}NO$	-CO-N(CH_3)-CH_2-Φ	94	–
65	$C_9H_{17}O_2$	-CO-O-$(CH_2)_7$-CH_3	230	–
66	$C_{10}H_7$	-(1-naphthyl)	108	–
67	$C_{10}H_{11}$	-(1-tetrahydronaphthyl)	−20@	–
68	$C_{10}H_{11}$	-(2-tetrahydronaphthyl)	−108@	–
69	$C_{10}H_{11}O$	-CH_2-O-Φ-CH_2-CH=CH_2	20	–
70	$C_{10}H_{11}O_2$	-CH_2-O-Φ-O-CH_2-CH=CH_2	14	–
71	$C_{10}H_{11}O_2$	-CH_2-O-Φ-CH_2-CH_2-O-CH_3	34	–
72	$C_{11}H_7O_2$	-CO-O-(1-naphthyl)	0	–
73	$C_{11}H_8NO$	-NH-CO-(1-naphthyl)	54	34
74	$C_{11}H_8NO$	-CO-NH-(1-naphthyl)	162	–
75	$C_{11}H_9$	-CH_2-(1-naphthyl)	–	7
76	$C_{11}H_9$	-CH_2-(2-naphthyl)	–	−7
77	$C_{11}H_9O$	-CH_2-O-(1-naphthyl)	20	–
78	$C_{12}H_9$	-(1-acenaphthenyl)	−34@	–
79	$C_{12}H_{13}N_2O_2S$	-NH-DNS	61	–
80	$C_{12}H_{14}N_3O_5$	-CH_2-N(*t*-Bu)-CO-DNB	101	–
81	$C_{13}H_9O$	-CH(Φ)-O-DNB	88	–
Synergistic and antagonistic substituent interactions				
Two identical substituents		No asymmetry	Cancel all values	
30 and 38		Φ and NHCO-DNB	175	–
30 and 39		Φ and CONH-DNB	175	–
38 and 66		NHCO-DNB and naphthyl	486	432
38 and 34		NHCO-DNB and cyclohexyl	−142	–
Two different DNB derivatives		Decrease the energy by	−230	–

Abbreviations: DNB = $C_6H_3N_20_4$ = 1-(3,5-dinitrobenzene); DNBPy = $C_5H_2N_30_4$ = 2-(3,5-dinitropyridine); DNS = $C_{12}H_{12}NO_2S$ = dansyl derivative. All phenyl rings (Φ) are para substituted, unless otherwise indicated. @ means the asymmetric carbon is part of a ring structure. The mobile phase compositions were normal phase, hexane-isopropyl alcohol with between 10 and 30% v/v IPA; polar organic mode, acetonitrile–ethanol 50–50% v/v with 1% acetic acid. Data taken from [35].

From a mechanistic point of view, it is interesting to consider the energetic contribution of a substituent when the mobile phase is changed. For many substituents the contributions in normal-phase and polar organic mode are similar, e.g., $-CH_2$-S-CH_3 #15 −27 cal/mol, isobutyl #21 −47 cal/mol, phenyl #30 +81 cal/mol, -NH-CO-naphthyl #73 +54 cal/mol in normal phase, and +34 cal/mol in polar organic mode (Table 4). For other substituents, significant differences can be seen as for the methyl group #6 with −74 cal/mol in normal phase and only −34 cal/mol in polar organic mode. The −NH-CO-O-Φ substituent #43 has a beneficial contribution of +27 cal/mol in normal phase and a detrimental contribution of −27 cal/mol in polar organic mode. These data definitively show the critical role of the solvent molecules in the chiral mechanism.

The substituent interactions are also interesting to discuss. It was expected that the presence of a π-acid DNB group in the chiral molecule be very beneficial for the chiral recognition by a π-basic naphthyl selector explaining that all DNB-containing substituents have large positive energy values in Table 4. However it makes sense that the presence of two DNB substituents is not beneficial for chiral recognition. With similar π–π interactions occurring with two substituents of the asymmetric center, the two enantiomers interact similarly with the π-basic CSP rendering their recognition more difficult. Conversely, the synergistic contribution observed for a π-acid and a π-basic substituents is coherent. The values of +175 cal/mol for a DNB and phenyl group and +486 for a DNB and naphthyl group (Table 4, bottom) show that the naphthyl group is a better π-basic substituent than the simple phenyl ring. The antagonistic (negative) contribution of the association of a DNB substituent and a cyclohexyl non-π-basic substituent on the same asymmetric center, both substituents having independently a great positive contribution (Table 4), is likely due to steric effects.

6.2 Linear Solvation Energy Relationships

6.2.1 The Chemometric Technique

Linear solvation energy relationships (LSER) are general relationships linking analyte retention factors or partition coefficients to numerical measures of properties of the analyte, of the mobile phase and of the stationary phase [44]. Using LSER an understanding of the types and relative strengths of the chemical interactions that control retention and selectivity in chromatography is possible. The aim of LSER studies is to understand the intermolecular processes controlling retention and doing so will be able to quantitatively predict the experimental conditions to achieve an acceptable separation. In chromatographic studies, a thermodynamic parameter, i.e., the solute retention factor in the form of log k', is used. Log k' is associated with parameters attributed to the solute, called solute descriptors, and with the retentive or chromatographic system (mobile + stationary phases, temperature), called system parameters. Solute descriptors are independent of the chromatographic system. Numerous tables of solute descriptors have been published

[45]. The chromatographic system (mobile + stationary phase, temperature) is calibrated using solutes with known descriptors to establish its system parameters. Once the later are known, the retention factors of any solute whose LSER descriptors are known can be predicted on this particular chromatographic system.

One of the more widely accepted symbolic representation of the LSER model was proposed by Abraham as follows:

$$\log\ k' = c + eE + sS + aA + bB + vV \tag{6}$$

where k' is the solute retention factor $((t_R - t_0)/t_0)$. The capital letters, *E, S, A, B,* and *V*, are the solute descriptors independent of the mobile and/or the stationary phase used. *E* is the solute excess molar refraction modeling the solute polarizability due to *n*- and/or π-electrons in excess of that of a comparable-sized *n*-alkane; *S* is the solute descriptor for the dipolar character and also polarizability of the molecule; *A* and *B* are, respectively, the H-bond solute acidity (H-donor) and basicity (H-acceptor) descriptors; and *V* is the McGowan's characteristic molecular volume calculated using the solute structure [45].

The lowercase letters, *c, e, s, a, b,* and *v,* are the system parameters or constants reflecting the difference in solute interactions between the mobile and stationary phases. The constant *c* is the intercept obtained in the regression calculation; it depends on the experimental system used (nature of the organic modifier, phase ratio) and not on the solute. The system parameter *e* indicates the tendency of the mobile and stationary phases to interact with the solute through π- or *n*-electron pairs; the parameter *s* is related to dipole- or induced dipole-type interactions; the system parameter *a* denotes the difference in hydrogen bond (H-bond) basicity between the phases and the solute since an acidic solute (corresponding *A* descriptor) will interact with a basic stationary phase; the parameter *b* is a measure of the difference in H-bond acidity between phases and solute; and the system parameter *v* corresponds to the difference in cavity formation energy between the mobile and stationary phases [44, 45]. A positive value for a system parameter indicates that the corresponding interaction increases retention; hence, it is more favorable for the stationary phase. A negative sign on a system parameter denotes more favorable interactions in the mobile phase leading to decreased retention.

6.2.2 The LSER Theory and Chiral Separations

In the LSER theory, two enantiomers have identical sets of descriptors, which correctly indicate that enantiomers are not separated by any isotropic stationary phase. They can, however, be separated by a CSP. This allows the relative Dolan–Snyder approach with a reference solute to be used for this special case [46]. In chiral separations, the reference solute will not be ethylbenzene, as recommended by Snyder, but it will be the less retained enantiomer of the enantiomeric pair. It is considered that one enantiomer sees a different CSP domain than the other enantiomer. Both enantiomers form two different transient diastereoisomeric complexes

with the same CSP. Thus, the enantioselectivity factor, α, will be modeled as follows:

$$\log\ k_2 - \log\ k_1 = \log\ \alpha = \Delta eE + \Delta sS + \Delta aA + \Delta bB + \Delta vV \qquad (7)$$

in which k_1 and k_2 are the retention factors of the first and last eluted enantiomers and all the Δ terms correspond to energy changes responsible for the observed enantioselectivity. LSER studies in chiral separation have a heavy workload. It is first necessary to determine the system parameters, *e, s, a, b*, and *v*, of the studied CSP using achiral solutes. Next, the $A-V$ descriptors of a set of enantiomers must be evaluated using the system parameters previously obtained for classical, achiral columns. Only after these two series of experiments are completed, the enantiomers with known $A-V$ descriptors can be separated by the CSP with known $a-v$ system parameters. The experiments will make possible the evaluation of the Δa to Δv parameters and relative $\Delta a/a$ to $\Delta v/v$ values corresponding to the enantioselective interactions.

6.2.3 LSER Study with Macrocyclic Glycopeptide Chiral Stationary Phases

We performed this difficult study using the Supelco Chirobiotic® CSPs based on macrocyclic glycopeptide selectors. The system parameters, *e, s, a, b*, and *v*, of the four different columns, namely ristocetin, vancomycin, teicoplanin, and teicoplanin aglycon, were obtained for six different normal heptane–ethanol mobile phases and ten different acetonitrile or methanol–water reversed mobile phases [47, 48]. The 44 achiral probe solutes contained aldehydes, ketones, amides, halogenated phenols, nitro-substituted benzenes, and nitro-alkanes. About 2,400 chromatograms were needed to obtain the 5 $a-v$ system parameters for the 4 CSP columns and the 16 mobile phases [47].

Thirteen different enantiomers were tentatively used to obtain a significant number of $A-V$ chiral solute descriptors. They were selected because they were well resolved on the four Chirobiotic® columns. The set contained eight ionic or zwitterionic amino acids or amino acid derivatives and five molecular solutes. The 13 enantiomers were separated on 5 well-characterized achiral columns, namely a classical polymeric C18, a diphenyl bonded column, a weak anion exchanger (Primesep D, SiELC), a divinyl benzene bonded column, and a strong anion exchanger (SAX, Alltech). Five different ethanol–water mobile phases were used making more than 300 chromatograms to develop and to analyze.

Unfortunately, the LSER theory was designed for molecular solutes [44, 45], not for charged molecules. The Chirobiotic® CSPs work very often with solutes such as organic acids or bases and, especially, amino acids involving charge–charge interactions. The regression LSER calculations done with all enantiomers bearing a charge returned negative descriptors. This is not possible. A negative polarizability or H-bond acidity or basicity or a negative molecular volume, all have no chemical or physical meaning. Only the five molecular solutes returned the significant $A-V$ descriptors listed in Table 5 [48].

Table 5 LSER solute descriptors of five molecular enantiomeric pairs and the corresponding Eq. (6) enantioselectivity coefficients obtained on the teicoplanin macrocyclic glycopeptide chiral stationary phase

Compound	E	S	A	B	V	r^2	SE	n
5-methyl-5-phenyl-hydantoin	1.416	1.59	0	1.49	1.403	0.945	0.049	22
Bromacil	1.180	1.41	0	1.66	1.631	0.953	0.025	20
Dihydrofurocoumarin	1.034	1.19	0	0.59	1.663	0.945	0.026	23
1,1-dimethyl-3-phenyl-propyl toluyl sulfoxide	1.935	0.40	0	1.67	2.392	0.932	0.037	22
α-naphthalenyl methyl sulfoxide	2.145	1.55	0	0.19	1.448	0.952	0.058	22
Enantioselectivity on teicoplanin[a]	ΔeE	ΔsS	ΔaA	ΔbB	ΔvV	log α	n refractive index	
5-methyl-5-phenyl-hydantoin[a]	0.5	−0.05	0	0.23	−0.42	0.26	1.61	
Bromacil	0.21	−0.01	0	0.08	−0.22	0.06	1.56	
Dihydrofurocoumarin	0.11	0	0	0.11	−0.12	0.10	1.54	
1,1-dimethyl-3-phenyl-propyl toluyl sulfoxide	0.21	0.01	0	0.14	−0.21	0.15	1.60	
α-naphthalenyl methyl sulfoxide	0.45	0	0	0.36	−0.61	0.20	1.72	
5-methyl-5-phenyl-hydantoin[b]	0.12	0.21	0	−0.10	0.24	0.47	1.61	

E: descriptor related to interaction with the stationary phase through polarizable bonds; S: descriptor related to dipole- or induced dipole-type interactions; A: descriptor related to the solute acidity; B: descriptor measuring the solute basicity; V: descriptor linked to the size of the solute (McGovan volume); r^2: regression coefficient; SE: regression standard error; n: number of experiments in the regression or number of data points.

[a]Mobile phase ethanol/buffer 75/25% v/v pH 4 with 10 mM trietylamine + acetic acid; n: solute refractive index calculated using the SPARC software (http://ibmlc2.chem.uga.edu/sparc).

[b]Normal mobile phase heptane/ethanol 75/25% v/v.

Data from [48].

Now that the *e, s, a, b*, and *v* system parameters are known for the four Chirobiotic® columns and the *A, B, E, S*, and *V* solute descriptors are obtained for five enantiomeric compounds, it is possible to calculate the retention factor k' of the five enantiomeric compounds using Eq. (5) and compare it with the experimental two retention factors obtained for the two enantiomers on the CSP. Figure 7 illustrates the results: three situations were observed. (i) The retention factors predicted by LSER corresponded to the first eluting enantiomer (e.g., dihydrofurocoumarin, Fig. 7a); (ii) the LSER-predicted retention factors corresponded to the last eluting enantiomer (e.g., 5-methyl-5-phenyl hydantoin in the normal-phase mode, Fig. 7b); and (iii) the LSER-predicted retention factors did not correspond to a particular enantiomer for all mobile phase compositions (e.g., 5-methyl-5-phenyl hydantoin in the RPLC mode and bromacil, Fig. 7c and d). From a mechanistic point of view, it can be speculated that in case i, the chiral selector has overall attractive enantioselective interactions with the second enantiomer more retained than the LSER prediction; in case ii, the chiral selector has overall repulsive enantioselective interactions with the first enantiomer less retained than the LSER prediction; and in case iii, the chiral selector has enantioselective interactions with both enantiomers.

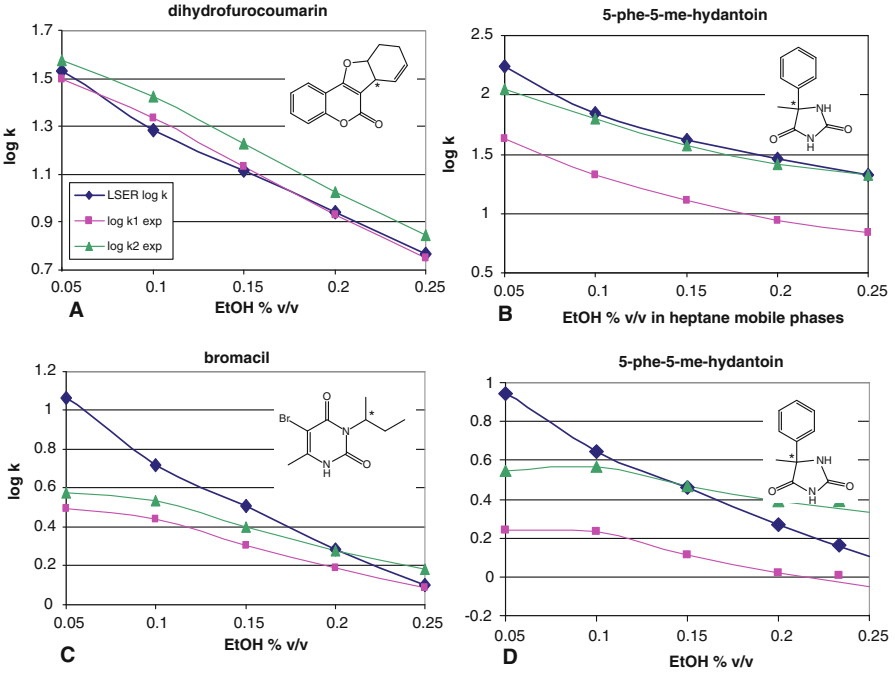


Fig. 7 Enantiomer retention factors obtained on the Chirobiotic® T column (teicoplanin selector) plotted versus the organic modifier content in the mobile phase. **a**, **c,** and **d**: RPLC mode with ethanol/pH 4 buffer mobile phases; **b**: normal-phase mode with ethanol/heptane mobile phases. *Diamonds* and *thick line*: LSER calculated retention factors using system parameters [48] and Table 5 solute descriptors; *squares*: experimental retention factors of the first eluting enantiomer; *triangles*: experimental retention factors of the last eluting enantiomer

The enantioselectivity factors α were obtained as the ratio k'_2/k'_1. From an LSER point of view, it appears that the enantiomers were effectively seeing two different stationary phases. The $a_1 - v_1$ and $a_2 - v_2$ system parameters correspond to these two hypothetical stationary phases. The parameters were determined for the two enantiomers using, respectively, the log k'_1 and log k'_2 experimental results and Eq. (6). Actually, in case (i) only the log k'_2 were used to obtain $a_2 - v_2$ and in case (ii) only the log k'_1 were used to obtain $a_1 - v_1$ system parameters. The $a-v$ parameters of references [47, 48] were used as the other parameters. Next the five ΔxX terms of Eq. (2) were obtained as $(x_2 - x_1)X$, x being an a to v parameter and X is the corresponding A to V solute descriptor. Since the solute A coefficients are all nil, the ΔaA terms of Eq. (2) also have a zero value. Table 5 (bottom) lists the enantioselective terms obtained with excellent regression coefficients ($r^2 > 0.990$). It should be noted that only five solutes were used for the LSER study in the reversed-phase mode and, in the normal-phase mode, only one solute, 5-phenyl-5-methyl hydantoin, could be separated in enough different mobile phases to perform a statistically significant LSER regression.

Before commenting on the results, it is worthwhile to point out that this LSER study done on the enantioselectivity factors does not respect the Vitha and Carr recommendation recently set out [44]. First, the solute set is not large enough since we had to drop the eight amino acid derivatives and all five studied solutes have the same nil A descriptor. Second, the 10 mobile phases tested are somewhat correlated but it is not possible to use any mobile phase composition since the separation of the two enantiomers is required. Last, the regression is very good because it is done with a too low data set. We still proposed this study because we followed exactly the Vitha and Carr statement saying that one of the main reason for performing LSER analyses is to obtain a better understanding of the system being studied [44]. It will be important to validate our results with a larger set of both enantiomeric pairs and CSPs.

For the five enantiomers studied in the reversed-phase mode, two terms dominated: the ΔeE and the ΔvV terms, the first being positive and the second being negative (Table 5, bottom). They almost cancel each other. The ΔeE term encodes interactions through polarizable n and π electrons. The e coefficient has a minor importance in overall solute retention [47]. It has a major effect on enantioselectivity. The negative enantioselective contribution of the ΔvV term is likely an indication of steric repulsion. Since these two terms almost cancel each other for our five test solutes, it means that the dipolar, ΔsS, and especially H-bonding, ΔbB, terms will be mainly responsible for the experimentally observed enantioselectivity factor.

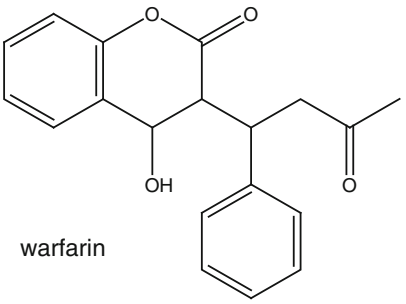
The last line of Table 5 shows that the normal-phase enantiorecognition mechanism of 5-methyl-5-phenyl hydantoin is completely different from that observed in reversed-phase mode. Especially, the ΔvV term is positive meaning that steric interactions contribute to enantiorecognition, a trend opposite to what was obtained in reversed-phase mode. The magnitude of the ΔsS term for dipolar interactions is also significant and completely different from the reversed-phase mode (Table 5, bottom).

It is striking to find that the main conclusion obtain by the QSAR and LSER chemometric studies presented is that the solvent used in the mobile phase have

a critical role in the chiral recognition mechanism. Interactions between a given chiral selector and a particular enantiomer can be completely changed by the solvent molecules. H-bond interactions, critical in the polar organic mode, can be completely screened by water molecules in the reversed-phase mode.

7 Conclusion

It is a challenge to separate the enantiomers of 3-methyl hexane when a chiral selector unable to separate the enantiomers of warfarin can be considered as useless. Indeed, the four substituents of 3-methyl hexane are a H atom, a methyl, an ethyl, and a propyl groups having very similar properties. They interact similarly making the molecule shape (steric interactions) the only way to separate its enantiomers by gas chromatography and cyclodextrin selectors [49]. The four very different substituents of warfarin varying in size and polarity make the two enantiomers easy to recognize by almost all listed chiral selectors. The two principal points that must be made by this introductory chapter are (1) chiral implies chiral. No enantiomer separation can be done in isotropic media. Chiral selectors and chiral stationary phases are needed to separate enantiomers. (2) In liquid chromatography, the mobile phase plays a critical role in any chiral interaction. The solvent molecules are too often forgotten when making chiral recognition models.



warfarin

References

1. Thayer AM (2008) Interaction yields. Chem Eng News 86(31):12–20
2. Caner H, Groner E, Levy L (2004) Trends in the development of chiral drugs. Drug Discov Today 9:105–110
3. Millership JS, Fitzpatrick A (1993) Commonly used chiral drugs: a survey. Chirality 5: 573–576
4. Cahn RS, Ingold C, Prelog V (1966) Specification of molecular chirality. Angew Chem Int Ed 5:385–415
5. Pasteur L (1858) Transformation of the two kinds of tartaric acid into racemic acid. Discovery of inactive tartaric acid. New method of separating racemic acid into the two tartaric acids, right and left. C R Acad Sci 46:615–618

6. Kunz H (2002) Emil Fischer—unequalled classicist, master of organic chemistry research, and inspired trailblazer of biological chemistry. Angew Chem Int Ed 41:4439–4451
7. Easson LH, Stedman E (1933) Studies on the relationship between chemical constitution and physiological action. V. Molecular dissymmetry and physiological activity. Biochem J 27:1257–1266
8. Dagliesh CE (1952) Optical resolution of aromatic amino acids on paper chromatograms. J Chem Soc 137:3940–3952
9. Pirkle WH, Pochapsky TC (1987) Chiral molecular recognition in small bimolecular systems: a spectroscopic investigation into the nature of diastereomeric complexes. J Am Chem Soc 109:5975–5982
10. Taylor DR (1993) Chiral separations by gas chromatography. In: Baugh PJ (ed) Gas chromatography, a practical approach, Ch 8. IRL Press, New York, NY, p 252
11. Bargmann-Leyder N, Caude M (2001) Séparation chirale par chromatographie en phase liquide, en phase supercritique et gazeuse. In: Techniques pour l′ingénieur, article P1470, Paris
12. Davankov VA (1997) The nature of chiral recognition: is it a three-point interaction? Chirality 9:99–102
13. Berthod A (2006) Chiral recognition mechanisms. Anal Chem 78:2093–2099
14. Nafie LA (1997) Infrared and Raman vibrational optical activity: theoretical and experimental aspects. Annu Rev Phys Chem 48:357–386
15. McCaroll ME, Billiot FH, Warner IM (2001) Fluorescence anisotropy as a measure of chiral recognition. J Am Chem Soc 123:3173–3174
16. Berthod A, He L, Beesley TE (2004) Temperature and enantioseparation by macrocyclic glycopeptide chiral stationary phases. J Chromatogr A 1060:205–214
17. Boehm RE, Martire DE, Armstrong DW (1988) Theoretical considerations concerning the separation of enantiomeric solutes by liquid chromatography. Anal Chem 60:522–528
18. Fornsted T, Sajonz P, Guiochon G (1998) A closer study of chiral recognition mechanisms. Chirality 10:375–381
19. Fornsted T, Zhong G, Bensetiti Z, Guiochon G (1996) Experimental and theoretical study of the adsorption behavior and mass transfer kinetics of propranolol enantiomers on cellulose protein as the selector. Anal Chem 68:2370–2378
20. Roussel C, Piras P (2009) Chirbase: a molecular database for liquid chromatography. http://chirbase.u-3mrs.fr . Accessed February 3, 2010
21. Kafri R, Lancet D (2004) Probability rule for chiral recognition. Chirality 16:369–378
22. Davankov VA (2000) 30 years of chiral ligand exchange. Enantiomer 5:209–223
23. Mikes F, Boschart G, Gil-Av E (1976) Resolution of optical isomers by high-performance liquid chromatography, using coated and bonded chiral charge-transfer complexing agents as stationary phases. J Chromatogr 122:205–221
24. Pirkle WH, Finn JM, Schreiner JL, Hamper BCJ (1981) A widely useful chiral stationary phase for the high-performance liquid chromatography separation of enantiomers. J Am Chem Soc 103:3964–3966
25. Welch CJ (1994) Evolution of chiral stationary phase design in the Pirkle laboratories. J Chromatogr A 666:3–26
26. Lu Y, Li CX, Zhang H, Liu XH (2003) Study on the mechanism of chiral recognition with molecularly imprinted polymers. Anal Chim Acta 489:33–43
27. Kaplan L, Sousa LR, Hoffman DH, Cram DJ (1974) Total optical resolution of amino esters by designed host-guest relations in molecular complexation. J Am Chem Soc 96: 7100–7101
28. Yuki H, Okamoto Y, Okamoto I (1980) Resolution of racemic compounds by optically active poly(triphenylmethyl methacrylate). J Am Chem Soc 102:6356–6358
29. Cavazzini A, Dondi F, Marmai S, Minghini E, Villani C, Rompietti R, Gasparrini F (2005) Adsorption equilibria of benzodiazepines on a hybrid polymeric chiral stationary phase. Anal Chem 77:3113–3122

30. Shamsi SA, Valle BC, Billiot F, Warner IM (2003) Polysodium N-Undecanoyl-L-leucylvalinate: a versatile chiral selector for micellar electrokinetic chromatography. Anal Chem 75:379–387
31. Schill G, Wainer IW, Barkan SA (1986) Chiral separations of cationic and anionic drugs on an α1-acid glycoprotein-bonded stationary phase (Enantio-Pac). II. Influence of mobile phase additives and pH on chiral resolution and retention. J Chromatogr 365:73–88
32. Shibata T, Mori K, Okamoto Y (1989) Polysaccharides phases. In: Krstulovic AM (ed) Chiral separations by HPLC. Ellis Horwood, New York, pp 336–398
33. Armstrong DW, DeMond W (1984) Cyclodextrin bonded phases for the liquid chromatographic separation of optical, geometrical, and structural isomers. J Chromatogr Sci 22:411–415
34. Han SM, Han YI, Armstrong DW (1988) Structural factors affecting chiral recognition and separation on β-cyclodextrin bonded phases. J Chromatogr 441:376–381
35. Berthod A, Chang SC, Armstrong DW (1992) Empirical procedure that uses molecular structure to predict enantioselectivity of chiral stationary phases. Anal Chem 64:395–404
36. Chang SC, Reid GL, Chen S, Armstrong DW (1993) Evaluation of a new polar-organic high-performance liquid chromatographic mobile phase for cyclodextrin-bonded chiral stationary phases. Trends Analyt Chem 12:144–153
37. Armstrong DW, Tang Y, Chen S, Zhou Y, Bagwill C, Cheng JR (1994) Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography. Anal Chem 66:1473–1484
38. Berthod A, Liu Y, Bagwill C, Armstrong DW (1996) Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase. J Chromatogr A 731:123–137
39. Cavazzini A, Nadalini G, Dondi F, Gasparrini F, Ciogli A, Villani C (2004) Study of mechanisms of chiral discrimination of amino acids and their derivatives on a teicoplanin-based chiral stationary phase. J Chromatogr A 1031:143–158
40. Xiao TL, Armstrong DW (2004) Macrocyclic antibiotics, Chapter 4 . In: Gübitz G, Schmid MG (eds) Chiral separations, methods and protocols. Humana Press, Totowa, NJ, pp 113–171
41. Lämmerhofer M, Lindner W (1996) Quinine and quinidine derivatives as chiral selectors. I. Brush type chiral stationary phases for high-performance liquid chromatography based on cinchonan carbamates and their application as chiral anion exchangers. J Chromatogr A 741:33–48
42. Leo A, Hansch C, Elkins D (1971) Partition coefficients and their uses. Chem Rev 71:525–616
43. Ghose AK, Viswanadhan VN, Wendoloski JJ (1998) Prediction of hydrophobic (lipophilic) properties of small organic molecules using fragmental methods: An analysis of AlogP and ClogP methods. J Phys Chem A 102:3762–3772
44. Vitha M, Carr PW (2006) The chemical interpretation and practice of linear solvation energy relationships in chromatography. J Chromatogr A 1126:143–194
45. Abraham MH, Ibrahim A, Zissimos AM (2004) Determination of sets of solute descriptors from chromatographic measurements. J Chromatogr A 1037:29–47
46. Wislon NS, Dolan JW, Snyder LR, Carr PW, Sander LC (2002) Column selectivity in reversed-phase liquid chromatography: III. The physico-chemical basis of selectivity. J Chromatogr A 961:217–236
47. Berthod A, Mitchell CR, Armstrong DW (2007) Could linear solvation energy relationships give insights into chiral recognition mechanisms? 1-π-π and charge interaction in the reversed versus the normal phase mode. J Chromatogr A 1166:61–69
48. Mitchell CR, Armstrong DW, Berthod A (2007) Could linear solvation energy relationships give insights into chiral recognition mechanisms? 2-Characterization of macrocyclic glycopeptide stationary phases. J Chromatogr A 1166:70–78
49. Kreidler D, Czesla H, Schurig V (2008) A mixed stationary phase containing two versatile cyclodextrin-based selectors for the simultaneous gas chromatographic enantioseparation of racemic alkanes and racemic α-amino acid derivatives. J Chromatogr B 875:208–216

Preparation and Chiral Recognition of Polysaccharide-Based Selectors

Tomoyuki Ikai and Yoshio Okamoto

Contents

Abstract Among more than one hundred commercially available CSPs, those based on the phenylcarbamates of polysaccharides including cellulose and amylose have been recognized as the most powerful for the resolution of a wide range of racemates, and nearly 90% of chiral compounds can be resolved at the analytical level using the polysaccharide-based CSPs. Although the qualitative understanding of the chiral recognition mechanism of polysaccharide-based CSPs is rather difficult in contrast to the small molecule-based CSPs, several attempts have made for comprehension of the chromatographic behavior on the polysaccharide-based CSPs. In this chapter, after describing the development of the polysaccharide-based CSPs with high recognition ability, special emphasis is placed on the mechanistic study of the polysaccharide-based CSPs on the basis of spectroscopic and computational methods.

Y. Okamoto (✉)
Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan
e-mail: okamoto@apchem.nagoya-u.ac.jp

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_2, © Springer-Verlag Berlin Heidelberg 2010

1 Introduction

The development of chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC) with high recognition ability, wide applicability, and high loading capacity has attracted a lot of attention, and the number of commercially available CSPs has surpassed one hundred. These CSPs for HPLC have been prepared using both chiral small molecules and polymers with chiral recognition abilities.

In 1971, Davankov et al. achieved the first baseline separation of enantiomers using a small molecule-based CSP consisting of L-proline [1]. Since then, a wide range of chiral small compounds, which include amino acids, cyclodextrins, macrocyclic glycopeptides, cinchona alkaloids, crown ethers, π-basic or π-acidic aromatic compounds, etc., have been used as CSPs [2−6]. On the other hand, the polymer-based CSPs are further divided into two categories, i.e., synthetic and natural chiral polymers [7, 8]. Typical examples of the synthetic polymers are molecularly imprinted polymer gels, poly(meth)acrylamides, polymethacrylates, polymaleimides, and polyamides, and those of the natural polymers include polysaccharide derivatives and proteins.

Figure 1 shows the distribution of the CSPs for HPLC used for the determination of enantiomeric excess (*ee*) that was reported in the *Journal of the American Chemical Society* in 2005 (a) and 2007 (b) [2, 9]. These statistics show that more than 90% of the *ee* determinations by chiral HPLC are carried out by the polysaccharide-based CSPs.

However, the chiral recognition mechanism of the polysaccharide-based CSPs at a molecular level has not yet been completely clarified. In contrast to the small molecule-based CSPs, the understanding of the chiral recognition mechanism of polymer-based CSPs is usually difficult. This is because a variety of interaction sites with different affinities for enantiomers exist in chiral polymer chains, and the

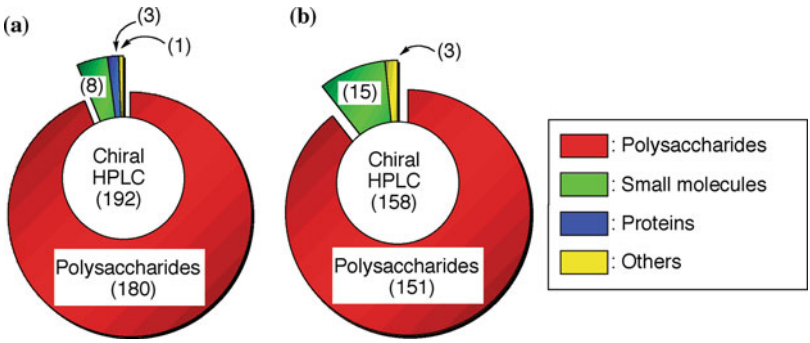


Fig. 1 Distribution of CSPs for HPLC used for the determination of enantiomeric excess reported in Journal of the American Chemical Society in (**a**) 2005 and (**b**) 2007. The values in parentheses represent the number of the counted papers. Reprinted by permission from the Royal Society of Chemistry [2] and International Union of Pure and Applied Chemistry [9]

determination of their precise structures both in the solid and in the solution states is not easy.

In this chapter, we will describe the development and chiral recognition mechanism of polysaccharide-based CSPs capable of the efficient separation of enantiomers. First, the development of the polysaccharide-based CSPs with a high-recognition ability is briefly described, and then special emphasis will be placed on the mechanistic study of the polysaccharide-based CSPs on the basis of spectroscopic and computational investigations.

2 Preparation and Chiral Recognition of Polysaccharide Chiral Selectors

2.1 Polysaccharide Esters

Polysaccharides, such as cellulose **1** and amylose **2**, are the most abundant natural polymer resources on the earth and are optically active (Fig. 2). Although the native polysaccharides themselves can discriminate enantiomers and resolve several racemic compounds by liquid chromatography [10–12], their recognition abilities are not sufficiently adequate to be practically used as CSPs. More useful CSPs can be obtained through modification of the polysaccharides. In 1973, the first practical CSP derived from polysaccharides was reported by Hesse and Hagel [13]. They found that the microcrystalline cellulose triacetate, CTA-1 (**3** in Fig. 3), which was synthesized by the heterogeneous acetylation of the native microcrystalline cellulose, showed a useful recognition ability during liquid chromatography. The crystalline structure of CTA-1 is expected to maintain that of the native cellulose and the chiral recognition ability seems to be derived from its crystalline structure. Therefore, once CTA-I is dissolved in a solvent, its recognition ability is completely changed from that of CTA-I. For example, the opposite elution order of

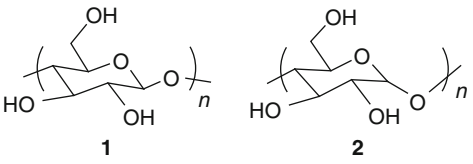


Fig. 2 Structures of cellulose (**1**) and amylose (**2**)

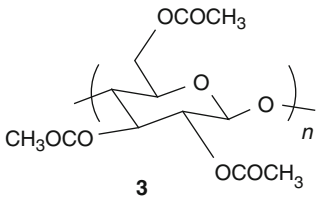


Fig. 3 Structure of cellulose triacetate **3** (CTA-1)

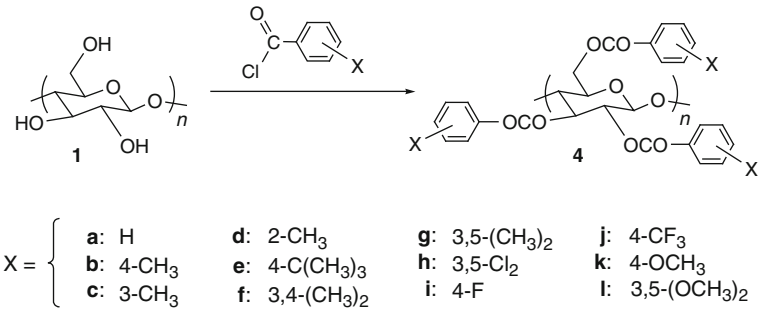


Fig. 4 Synthesis and structure of cellulose benzoates **4**

the enantiomers of Tröger base was observed using two kinds of cellulose triacetate-based CSPs [14]. The difference in the recognition seems to be derived from the change in its structure.

Since the microcrystalline cellulose triacetate CTA-1 was recognized as a practical CSP in the 1970s, various kinds of cellulose esters have been prepared to evaluate their recognition abilities as CSPs for HPLC [14–16]. Among them, the cellulose benzoates (**4** in Fig. 4), which are easily obtained by reacting cellulose with the corresponding benzoyl chlorides, show high-recognition abilities when they are coated on silica gel. The effect of the substituents on the phenyl group, which include alkyl, halogen, trifluoromethyl, and methoxy groups, has been systematically studied [16]. The benzoate derivatives with electron-donating substituents, such as an alkyl group, have a tendency to show a higher recognition ability than those with electron-withdrawing substituents, such as a halogen and trifluoromethyl. The most likely reason for this observation is that the electron density of the carbonyl groups of the cellulose derivatives is significantly influenced through an inductive effect of the substituents on the phenyl groups. However, the electron-donating methoxy group does not work to increase the recognition ability due to the high polarity of the substituent itself. Among these cellulose benzoates, 4-methylbenzoate **4b** exhibits an especially high chiral recognition ability and has been used for the resolutions of a broad range of chiral compounds [17, 18]. In contrast to the cellulose derivatives **4**, the amylose benzoates (**5** in Fig. 5) show almost no recognition abilities as CSPs. This may be due to the lower conformational stability of the amylose derivatives, which causes many conformational isomers to be formed.

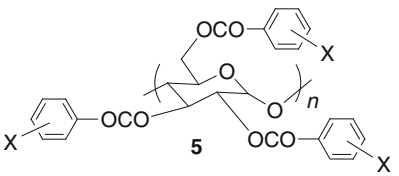


Fig. 5 Structure of amylose benzoates **5**

2.2 Polysaccharide Phenylcarbamates

Cellulose (**1**) and amylose (**2**) are readily converted to various phenylcarbamate derivatives (**6** and **7**) by reacting them with the corresponding phenyl isocyanates (Fig. 6) [19, 20]. The recognition abilities of these derivatives can be significantly changed, depending on the substituents on the phenyl groups as well as the benzoate derivatives. The resolution results of 10 racemates **8–17** (Fig. 7) on the nine *para*-substituted phenylcarbamates of cellulose are given in Table 1, in which the substituents on the phenyl group are arranged in the order of their increasing electron-donating powers from left to right [20]. In addition, the retention times of acetone and the first isomer of alcohol **16** eluted on the *para*-substituted CSPs are plotted versus the Hammett parameter σ of the substituents (Fig. 8).

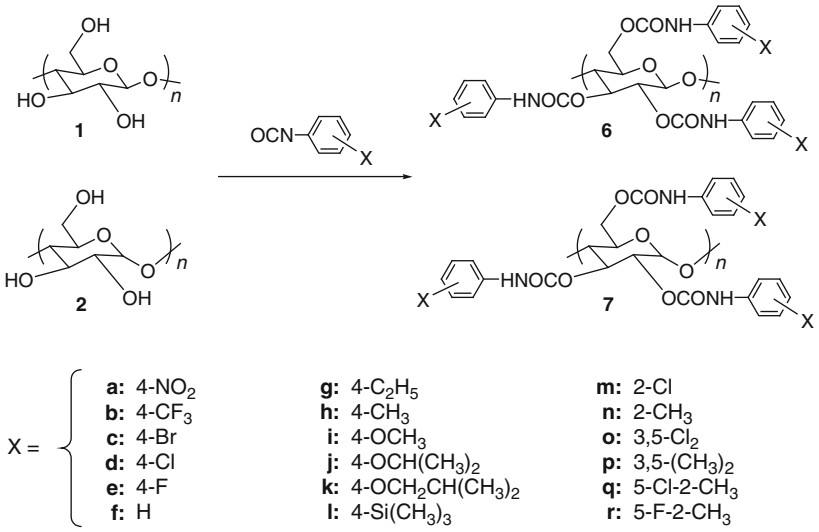


Fig. 6 Synthesis and structures of phenylcarbamate derivatives of cellulose **6** and amylose **7**

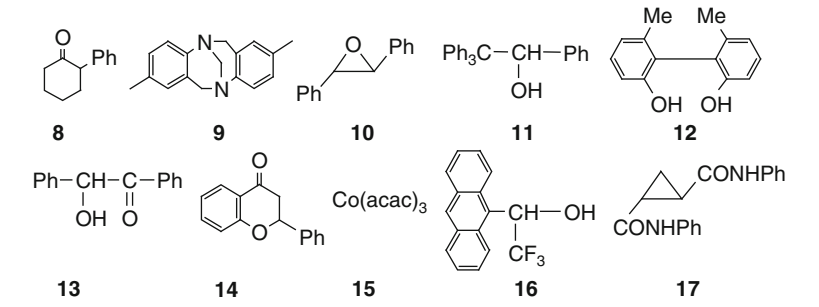
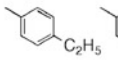
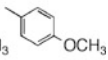


Fig. 7 Structures of racemates **8–17**

Table 1. Separation factors (α) on the *para*-substituted phenylcarbamates of cellulose

Racemates	 6a	 6b	 6c	 6d	 6e	 6f	 6g	 6h	 6i
8	~1 (–)	1.18 (–)	1.17 (–)	1.16 (–)	1.12 (–)	1.17 (–)	1.19 (–)	1.20 (–)	1.13 (–)
9	~1 (–)	1.23 (+)	1.19 (+)	1.16 (+)	1.14 (+)	1.37 (+)	1.11 (+)	1.48 (+)	~1 (+)
10	1.33 (+)	1.61 (+)	1.70 (+)	1.68 (+)	1.38 (+)	1.46 (+)	1.55 (+)	1.55 (+)	1.34 (+)
11	1.00	1.48 (+)	1.95 (+)	1.95 (+)	1.64 (+)	1.22 (+)	1.59 (+)	1.37 (+)	1.00
12	~1 (+)	2.04 (–)	1.21 (–)	1.20 (–)	1.17 (–)	1.65 (–)	1.33 (–)	1.30 (–)	1.15 (–)
13	1.00	1.10 (–)	1.13 (–)	1.20 (–)	1.14 (–)	~1 (+)	1.14 (–)	1.12 (–)	~1 (+)
14	1.00	1.14 (+)	1.13 (+)	1.12 (+)	1.13 (+)	1.10 (+)	1.22 (–)	1.16 (+)	~1 (+)
15	~1 (+)	2.06 (+)	1.79 (+)	1.46 (+)	1.53 (+)	1.24 (+)	1.76 (+)	1.75 (+)	~1 (+)
16	~1 (+)	1.30 (–)	1.29 (–)	1.29 (–)	1.26 (–)	1.45 (–)	1.57 (–)	1.52 (–)	1.35 (–)
17	~1 (+)	1.22 (–)	1.17 (–)	1.44 (–)	~1 (–)	1.45 (–)	2.12 (–)	1.35 (–)	1.00

Column: 25×0.46 cm (i.d.). Flow rate: 0.5 ml/min. Eluent: hexane–2-propanol (90:10). The signs in parentheses represent the optical rotation of the first-eluted enantiomer.

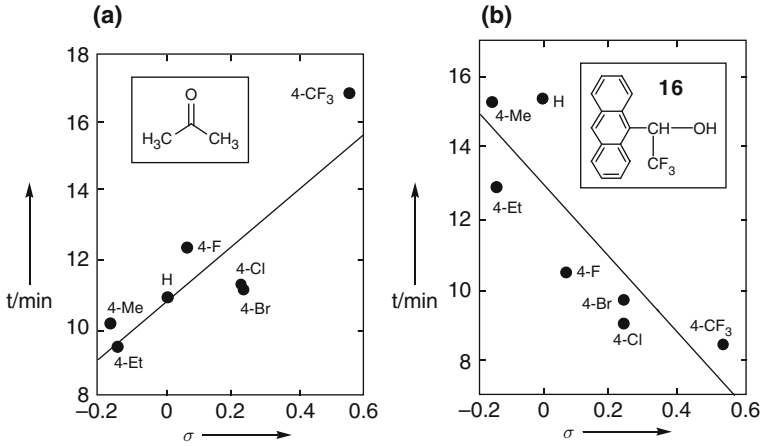


Fig. 8 Plots of retention times t of (**a**) acetone and (**b**) the first enantiomer of **16** to elute on cellulose phenylcarbamate derivatives against the Hammett parameter σ of the substituents. Reprinted by permission from Elsevier [20]

Compared to the non-substituted cellulose derivative **6f**, the phenylcarbamates bearing electron-withdrawing substituents, such as halogens, or electron-donating substituents, such as alkyl groups, exhibit better chiral recognitions. These substituents appear to affect the polarity of the carbamate group via an inductive effect and alter the interaction mode between the cellulose derivatives and the racemates.

When the electron-withdrawing groups are substituted on the phenyl groups, the acidity of the NH proton of the carbamate groups increases. Therefore, the retention time of acetone on the CSPs with the electron-withdrawing groups is increased, because acetone is mainly adsorbed on the derivatives through a hydrogen-bonding interaction with the NH groups. On the contrary, as the electron-donating power of the substituents on the phenyl group becomes more intensive, the electron density at the carbonyl oxygen of the carbamate groups must be increased, and the racemate

16 is more strongly adsorbed on the derivatives through hydrogen bonding with the carbonyl groups. On the other hand, the derivatives bearing fairly polar substituents on the phenyl groups, such as the nitro (**6a**) or methoxy (**6i**) groups, exhibit a rather low recognition ability. Because the nitro or methoxy group is located far from a chiral glucose unit, these polar groups themselves are expected to cause a non-enantioselective interaction with the racemates. Therefore, the introductions of bulky alkoxy groups, such as isopropoxy (**6j**) or isobutoxy (**6k**) groups, instead of the methoxy group can improve the recognition ability by preventing the non-enantioselective interactions at the ether oxygen atom [21].

The chiral recognition on the cellulose phenylcarbamates is also influenced by the position of the substituents on the phenyl group. When a halogen or methyl group is introduced at an *ortho* position (**6m**, **6n**), the recognition ability significantly decreases compared to the non-substituted cellulose derivative (**6f**) [22]. Most cellulose phenylcarbamates with a high-recognition ability form a lyotropic liquid crystalline phase in a highly concentrated solution [20]. This indicates that the phenylcarbamates as the CSPs are presumably arranged in a regular fashion. Such an ordered structure seems to be important for efficient chiral recognition on polymer-based CSPs. However, the *ortho*-substituted derivatives (**6m**, **6n**) do not show such a liquid crystallinity. This means that the *ortho*-substituted derivatives may not possess a regular higher-order structure.

For amylose phenylcarbamates **7** (Fig. 6), the introduction of chloro or methyl groups on the phenyl groups also has a meaningful effect on their recognition ability [23–25]. In contrast to the cellulose derivatives, however, amylose derivatives with substituents at the *ortho* position, such as the 5-chloro-2-methyl- (**7q**) and 5-fluoro-2-methylphenylcarbamates (**7r**), exhibit a relatively high-recognition ability [24, 25]. The difference in the substituent effect on their chiral recognition may be derived from the difference in their higher order structures.

3 Structural Analysis of Phenylcarbamate Derivatives of Cellulose and Amylose

The intimate structural analysis of the polysaccharide derivatives is mandatory in order to clarify the chiral recognition mechanism. Figure 9 shows the stable structures of **6f** and **6p**, which were optimized by molecular-mechanics calculations starting from the proposed X-ray crystal structure of **6f** [26]. These derivatives have similar left-handed 3/2-helical conformations, and the glucose residues are regularly arranged along the helical axis. A chiral helical groove with polar carbamate groups exists parallel to the main chain. The polar carbamate groups are preferably located inside, and hydrophobic aromatic groups are placed outside the polymer chain so that polar racemates may predominately interact with the carbamate residues through hydrogen-bonding and dipole–dipole interactions. These interactions seem to be significant for efficient chiral recognition, especially in normal-phase HPLC using nonpolar eluents. This speculation is supported by NMR studies as described below. Besides these polar interactions, π–π interactions between the phenyl groups

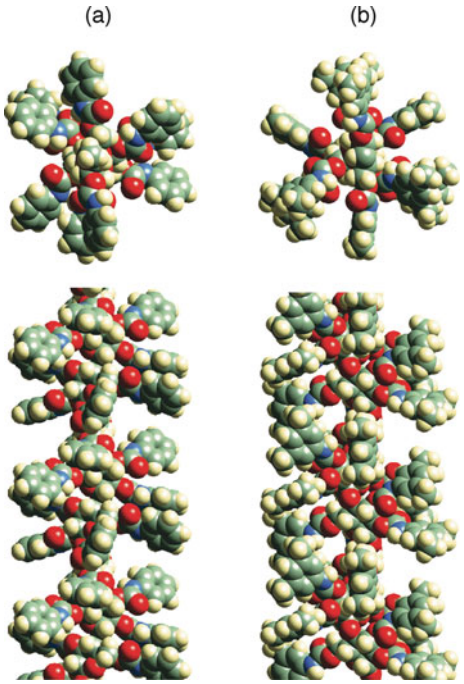


Fig. 9 Optimized structures of (a) phenylcarbamate **6f** and (**b**) 3,5-dimethylphenylcarbamate **6p** of cellulose. Perpendicular (*top*) and along (*bottom*) to the helix axis. Reprinted by permission from The Chemical Society of Japan [26]

of the phenylcarbamates and an aromatic group of a racemate may also play an important role in their recognitions, because several aromatic compounds without any polar groups can also be resolved particularly in reversed-phase mode HPLC with polar eluents.

Meanwhile, the structures of the amylose phenylcarbamate derivatives have not yet been determined by X-ray studies. Wang et al. investigated the structure of **7p** by solid-state NMR and pointed out that **7p** forms a helical structure with less than six-folds in the solid state [27]. Recently, we investigated the structure of **7p**, which had a low degree of polymerization and was soluble in chloroform, by the combination technique involving 2D NMR and computer modeling [28]. Figure 10 shows the optimized structure of **7p** with a left-handed 4/3 helix as the most probable one. Similar to the cellulose derivatives, the polar carbamate groups are located inside the polymer chain and the aromatic groups are on the outside.

4 Chiral Recognition Mechanism

4.1 NMR Studies

NMR spectroscopy is well known to be one of the most powerful tools for the elucidation of the chiral recognition mechanism on a molecular level. Most carbamate

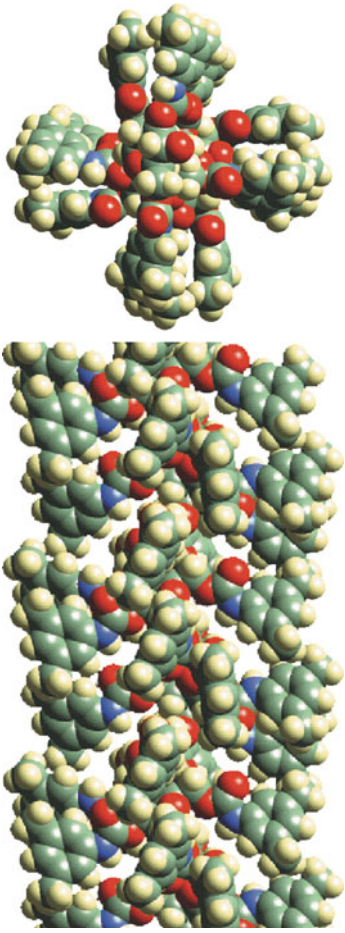


Fig. 10 Optimized structures of amylose 3,5-dimethylphenylcarbamate **7p**. Perpendicular (*top*) and along (*bottom*) to the helix axis. Reprinted by permission from American Chemical Society [28]

derivatives with high-recognition abilities are soluble only in polar organic solvents, such as tetrahydrofuran (THF), acetone, pyridine, dimethyl sulfoxide, etc. In these solvents, the polysaccharide derivatives cannot have a sufficient interaction with enantiomers for efficient recognition due to the stronger interaction between the polysaccharide derivatives and the solvent molecules. Therefore, it is difficult to reveal the chiral recognition mechanism on polysaccharide-based selectors by NMR spectroscopy. In the past 15 years, however, it was found that several carbamate derivatives, such as 4-trimethylsilylphenyl- (**6l**) [29–31], 3,5-dichlorophenyl- (**6o**) [30], 5-fluoro-2-methylphenyl-(**6r**) [32], and cyclohexylcarbamate [33], are soluble in chloroform and can discriminate enantiomers by NMR spectroscopy as well as by HPLC.

Figure 11 shows the 500 MHz ^{1}H NMR spectra of *rac-trans*-stilbene oxide **10** with and without cellulose 4-trimethylsilylphenylcarbamate **6l** in $CDCl_3$[29–31]. The methine proton resonance of the enantiomer **10** was apparently separated into

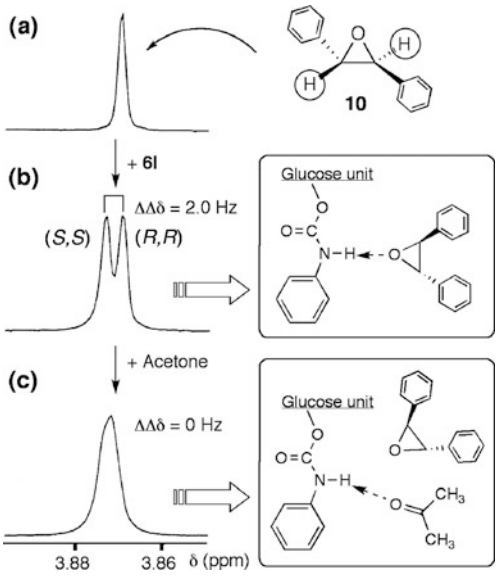


Fig. 11 ^{1}H NMR spectra of *rac*-**10** in the presence of **6l** in $CDCl_3$ (1.0 ml). **6l**: (**a**) 0 and (**b** and **c**) 20 mg, acetone: (**a** and **b**) 0 and (**c**) 65 μL. Reprinted by permission from The Chemical Society of Japan [29]

two sets of peaks in the presence of **6l**, and only the resonance of the (*S,S*)-isomer is downfield shifted. The chemical shift difference ($\Delta\Delta\delta$) of these two peaks increased with a decrease in temperature and with an increase in the amount of **6l**. This indicates that **6l** can recognize the enantiomers even in solution. Because the most important adsorption site for chiral recognition on the phenylcarbamate derivatives must be the polar carbamate groups, the oxygen atom of the oxirane ring in **10** may interact with the NH proton of the carbamate group through a hydrogen bond. Therefore, the addition of acetone, which is a hydrogen-bonding acceptor and attenuates the interaction between **10** and **6l** by hydrogen bonding with the NH proton, causes the splitting of the methine proton resonance to disappear. Many other racemates can be also recognized on **6l** in $CDCl_3$[30].

The cellulose derivative **6r** can also discriminate the enantiomers of **18** by ^{1}H NMR spectroscopy [32]. Figure 12 shows the ^{1}H NMR spectra of *rac*-**18** in the absence (a) and presence (b) of **6r** in $CDCl_3$. Each signal for the hydroxyl and naphthyl (H4 and H6) protons of **18** is clearly split into two sets of peaks due to the enantiomers. The signals for the hydroxy protons of (*S*)-**18** are more downfield shifted with peak broadening than that for (*R*)-**18**, while the signals for the H4 and H6 protons of (*S*)-**18** are upfield shifted with broadening. This means that (*S*)-**18** has a stronger interaction with **6r**. The downfield shift of the hydroxy protons is presumably due to hydrogen bonding between the carbamate group of the cellulose derivative and the hydroxy group of (*S*)-**18**, and the upfield shifts of the aromatic protons may be ascribed to the π-stacking or shielding effect of an adjacent aromatic ring of **6r**. During the HPLC separation of *rac*-**18** on **6r**, the (*R*)-isomer is first eluted followed by the (*S*)-isomer ($\alpha = 4.23$) (Fig. 13). This HPLC elution order agrees with the large shifts in the (*S*)-isomer observed in the ^{1}H NMR spectrum.

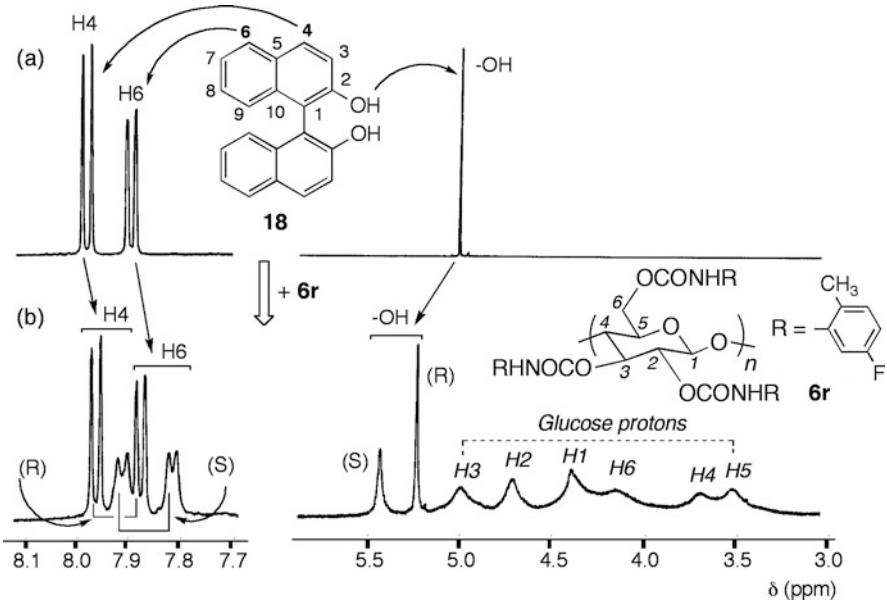


Fig. 12 ^{1}H NMR spectra of selected region of *rac*-1,1′-bi-2-naphthol **18** in the absence (**a**) and presence (**b**) of **6r** in $CDCl_3$. Reprinted by permission from American Chemical Society [32]

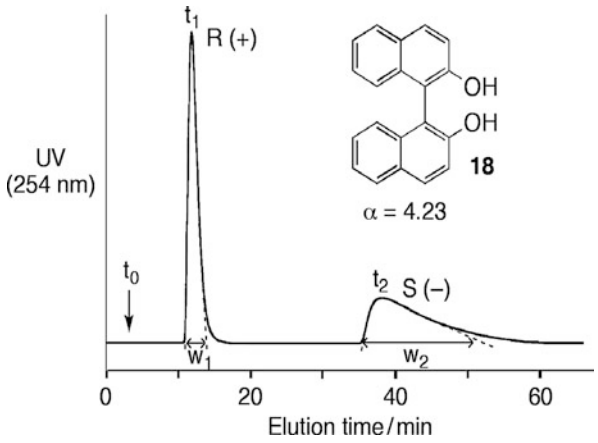


Fig. 13 Chiral separation of *rac*-**18**. CSP: cellulose 5-fluoro-2-methylphenylcarbamate **6r**. Column: 25 × 0.46 (i.d.) cm. Eluent: hexane/2-propanol (90/10). Flow rate: 1.0 ml/min. Reprinted by permission from American Chemical Society [32]

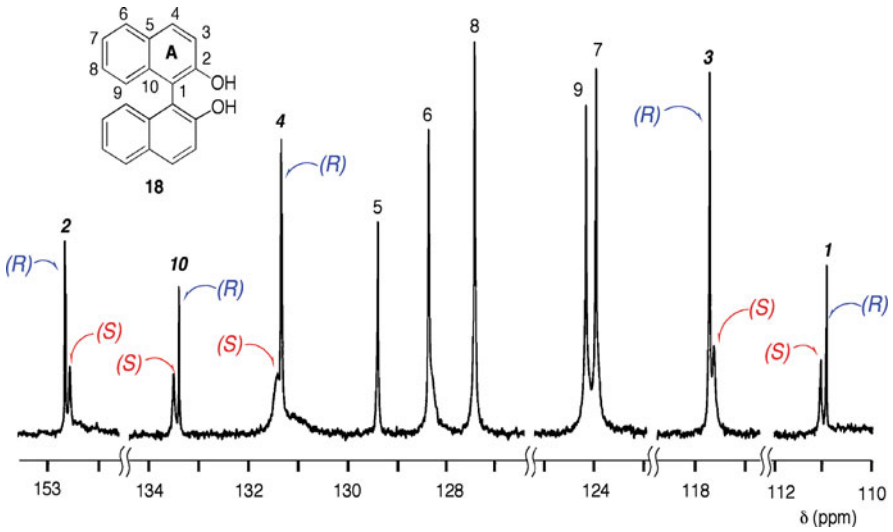


Fig. 14 ^{13}C NMR spectrum of *rac*-**18** in the presence of **6r** in $CDCl_3$. Reprinted by permission from American Chemical Society [32]

The chiral recognition can also be observed by ^{13}C NMR spectroscopy. Figure 14 shows the recognition of the enantiomers **18** by ^{13}C NMR spectroscopy [32]. The resonances of the C1–C4 and C10 carbons of **18**, which are located near the hydroxy groups, are separated into enantiomers in the presence of **6r**, and the carbon resonances of (*S*)-**18** clearly become broader than that of (*R*)-**18** as well as the observation in the ^{1}H NMR spectrum. This indicates that ring A of (*S*)-**18** may be favorably located in the chiral groove of **6r**. Measurements of the relaxation time also support this speculation.

In addition, the ^{1}H NMR titrations of **6r** with (*S*)- and (*R*)-**18** and a Job plot of the continuous changes in the chemical shifts for the complex **6r**–(*S*)-**18** were conducted in order to investigate the binding sites of **6r** and the stoichiometry of the complexation [32]. The Job plot denotes that the maximum complex formation occurs at around 0.5 mol fraction of the glucose unit of **6r**. This represents that each glucose unit of **6r** may have the same binding affinity to (*S*)-**18** probably due to the regular structure of **6r** even in a solution state. During the titrations, the H2 proton resonance of a glucose unit is dramatically upfield shifted as the concentration of (*S*)-**18** increases, while the other glucose proton resonances only move slightly. This upfield shift of the H2 proton resonance implies that the H2 proton may be located above the naphthyl ring of (*S*)-**18**.

More valuable information on the binding geometry and dynamics between the polysaccharide derivatives and the enantiomers can be obtained from the intermolecular nuclear Overhauser effects (NOE). Figure 15 shows the NOE spectroscopy (NOESY) spectra of **6r**-(*S*)-**18** (a) and **6r**-(*R*)-**18** (b) in the region related to the methyl protons on the phenyl group of **6r** and the aromatic protons of **18** [32]. Clear intermolecular NOE cross-peaks shown by the arrows could be observed

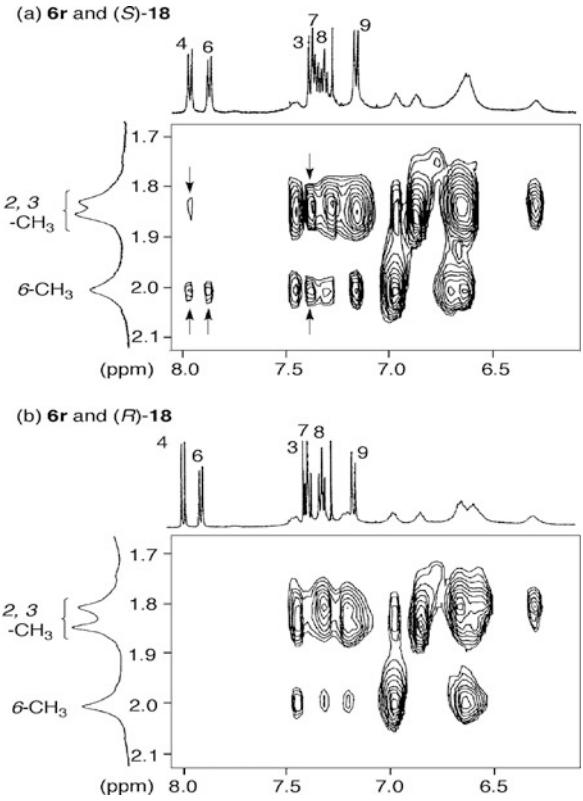


Fig. 15 500 MHz expanded NOESY spectra at a mixing time of 300 ms of the mixtures of (**a**) **6r** and (*S*)-**18** and (**b**) **6r** and (*R*)-**18** in the region between the aromatic protons (**6r** and **18**) and the methyl protons on the phenyl groups of **6r** in $CDCl_3$ at 30°C. Reprinted by permission from American Chemical Society [32]

between the methyl proton of **6r** and the aromatic protons H4, H6, and H7 of (*S*)-**18** (Fig. 15a). On the other hand, the mixture of **6r** and (*R*)-**18** exhibited no intermolecular NOE cross-peaks (Fig. 15b), probably due to a weaker interaction. These results indicate that (*S*)-**18** more strongly binds or interacts with **6r** than (*R*)-**18**, and the naphthyl protons of (*S*)-**18** are closely located to the glucose proton of **6r** within less than 5 Å. These observations correspond to the results of the HPLC and 1D NMR experiments. Based on the HPLC and NMR data combined with the structural data for the cellulose phenylcarbamate (**6f**) determined by X-ray analysis, a computational structure has been proposed for the **6r**–(*S*)-**18** complex (Fig. 16) [32]. This calculation model shows that two hydroxy protons of (*S*)-**18** interact with the carbonyl oxygens of the carbamate groups of **6r** through hydrogen bonding.

Recently, Wirth et al. explained the enantioselective interaction between amylose 3,5-dimethylphenylcarbamate **7p** and the *O-tert*-butyltyrosine allyl ester **19** (Fig. 17) using the intermolecular NOEs [34]. The major differences in the NOESY cross-peaks between the D- and L-isomers are observed in the aromatic region of **19**. Compared to the D-**19**, the greater number and stronger intensity of the NOESY cross-peaks were observed between the aromatic protons of L-**19** and the glucose protons of **7p** including the Ha-H2, Ha-H3, Ha-H5, Hb-H2, Hb-H3, and Hb-H5

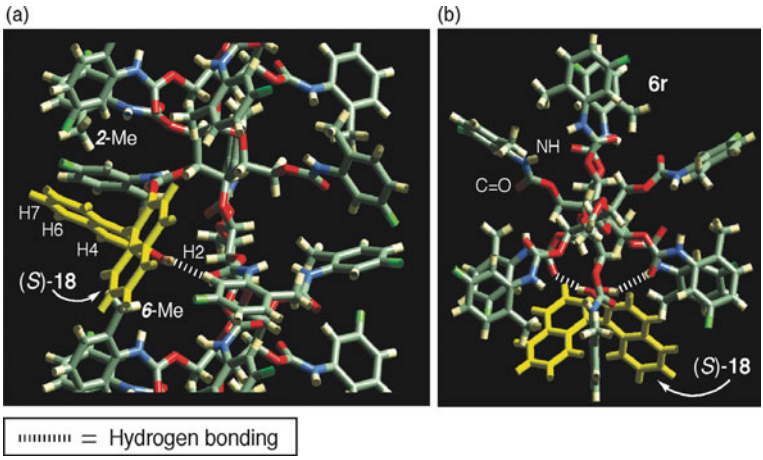


Fig. 16 Calculated structure of the complex **6r**–(*S*)-**18**. (**a**) View along the helix axis and (**b**) perpendicular to the helix axis. Reprinted by permission from American Chemical Society [32]

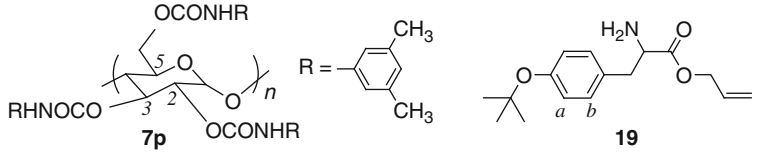


Fig. 17 Structures of amylose 3,5-dimethylphenylcarbamate **7p** and *O-tert*-butyltyrosine allyl ester **19**

pairs. This result indicates that the L-**19** exists in closer proximity to the chiral groove in **7p** than the D-**19**. This structural relationship allows a stronger interaction between the L-**19** and **7p**. The mechanistic study by NMR is well amenable to the HPLC separation results.

4.2 Computational Methods

The chiral recognition mechanism of small molecule-based CSPs has been extensively investigated from theoretical viewpoints, especially by Lipkowitz et al. [35–37]. The interaction energies between the CSPs and enantiomers were calculated by molecular-mechanics (MM), molecular-dynamics (MD), and quantum-mechanical calculations, and the rational interaction models between the CSPs and racemates have been proposed. Until now, several attempts have also been carried out for the qualitative understanding of the chromatographic behavior of the polysaccharide-based CSPs.

The interaction energy calculations between the phenylcarbamate **6f** or 3, 5-dimethylphenylcarbamate **6p** of cellulose and *trans*-stilbene oxide **10** or benzoin **13** were performed using various force fields [26, 38]. During a chromatographic resolution, **10** can be completely separated on both **6f** and **6p** ($\alpha = 1.46$) and ($\alpha = 1.68$). However, their elution orders are opposite; the (*R*,*R*)-isomer is first eluted on **6f**, and the (*S*,*S*)-isomer on **6p**. Meanwhile, **6p** can efficiently separate **13** ($\alpha = 1.58$), while **6f** cannot ($\alpha = \sim 1$).

The applied calculations were roughly divided into the following two methods, which differ in these enantiomer generation methods. In one method, enantiomers were individually generated around the carbonyl oxygen and the NH proton of the carbamate group of **6f** and **6p** and rotated at 15° intervals for the *x*, *y*, and *z* axes. The interaction energy calculation was performed for each carbonyl oxygen and NH proton at the 2-, 3-, and 6-positions of the glucose units with all possible combinations of the rotation angles of the enantiomers. The calculation results are evaluated with the lowest interaction energy and the distribution of the interaction energy. In another method, enantiomers with a particular orientation were randomly generated by the Monte Carlo method on the surface of **6f** and **6p**, and then the interaction energy was estimated step by step through the MM calculation between the molecules [39]. In both calculations, the nonamers of **6f** and **6p** were used as chiral selectors, and the enantiomers were generated around the middle part of their structures in order to avoid the influence of the end groups.

Both calculation results well agreed with the chromatographic results. The averaged or lowest interaction energy between **6f** and (*S,S*)-**10** was lower than that between **6f** and (*R,R*)-**10**, while an opposite enantiomer preference was found in the **6p**–**10** system. In the case of **13**, almost no difference in the interaction energies with **6f** was observed for the enantiomers.

The interaction energy difference between the enantiomers was clearly recognized only when the enantiomers were generated in a chiral groove of **6f** and **6p**. This result indicates that the polar carbamate groups of these phenylcarbamate derivatives may be the most important chiral recognition site for polar racemates.

Figure 18 shows a graphical view of the interaction mode between **6f** and (*S,S*)-**10**, which has the lowest interaction energy obtained by the second calculation method [26]. (*S,S*)-**10** is interned inside **6f** through hydrogen bonding between the NH proton of the carbamate group of **6f** and the ether oxygen atom of (*S,S*)-**10**. In addition, each phenyl group of **10** may interact with the phenyl groups of **6f** through π–π interactions. Although the actual reason for the opposite enantioselectivity of **6f** and **6p** toward **10** is unknown, the different arrangement of aromatic groups in **6f** and **6p** is expected to be responsible for the reversed enantioselectivity.

Aboul-Enein et al. [40] and Grinberg et al. [41] also attempted molecular modeling to provide some insights into the chiral recognition mechanism of cellulose benzoate **4a** and cellulose 4-methylbenzoate **4b**, respectively. The molecular modeling of **4b** and (*R*)- and (*S*)-**20** (Fig. 19) suggests that hydrogen bonding is a primary factor for the separation, and the calculated energy values obtained from the molecular modeling are consistent with the chromatographic results [41].

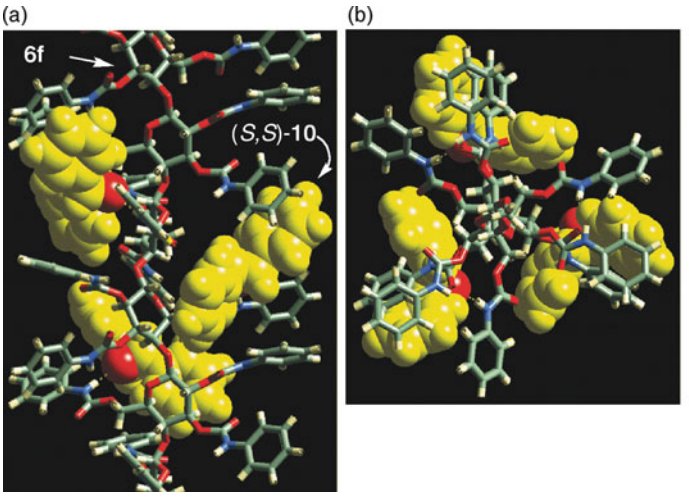
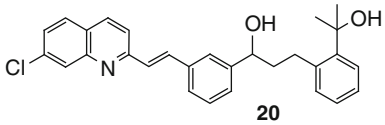


Fig. 18 Calculated structure of the complex **6f**–(*S,S*)-**10** formed through hydrogen bondings. (**a**) View along the helix axis and (**b**) perpendicular to the helix axis. Reprinted by permission from The Chemical Society of Japan [26]

Fig. 19 Structure of racemate **20**

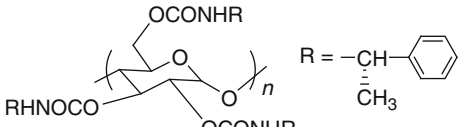


20

4.3 Other Studies for Chiral Recognition Mechanism

The difference in the enantioselective adsorption sites among the cellulose 3, 5-dimethylphenylcarbamate **6p**, amylose 3,5-dimethylphenylcarbamate **7p**, and amylose (*S*)-1-phenylethylcarbamate **21** (Fig. 20) was investigated by Franses et al. using attenuated total reflection infrared (ATR-IR) spectroscopy, X-ray diffraction (XRD), ^{13}C cross-polarization/magic-angle spinning (CP/MAS) and MAS solid-state NMR, and density functional theory (DFT) modeling [42]. The ATR-IR results show that the strengths of the hydrogen bond of the carbamate groups in these polymers are significantly different. Compared to **7p**, **6p** has a weaker intramolecular

Fig. 20 Structure of amylose (*S*)-1-phenylethylcarbamate **21**



21

hydrogen bonding mainly due to the differences in the backbones. Therefore, the chiral groove of **6p** is expected to be slightly larger than that of **7p**. For **21**, on the other hand, the hydrogen-bonding interaction of the carbonyl groups becomes stronger than **7p** due to the additional interactions with the benzyl proton in the same side chains. The XRD results suggest that the packing arrangements are different among the three derivatives, resulting in different nanostructures of the chiral grooves. Based on the CP/MAS NMR, it is inferred that the conformations of the backbone glycoside linkages are similar in **7p** and **21**, but different in **6p** and **7p**. DFT simulations predict that the **6p** and **7p** side chains have a planar conformation, while the side chain of **21** is nonplanar, and possibly has multiple conformations. They concluded that the carbonyl, NH, and phenyl groups of these polymers are expected to be oriented in different ways and that their carbamate groups show quite different interaction energies in their hydrogen bonding. This may be the major factor affecting the selectivity of the chiral analytes.

In order to clarify the role of the eluents in the chiral separation on polysaccharide derivatives, Franses et al. systematically investigated the interaction between **7p** and various organic solvents used as eluents by the above analytical method [43–45]. The polar solvents, such as methanol, ethanol, 2-propanol, or acetonitrile, change in the hydrogen-bonding states of the carbonyl and NH groups in **7p**, and seem to cause changes in the polymer crystallinity and side-chain mobility. On the other hand, the polymer structure remains essentially unchanged upon absorption of the nonpolar hexane. Wang et al. also studied the effects of the eluent on the structure and chiral recognition of **7p** by solid-state NMR [27, 46, 47]. These results indicated that the branched alcohols, such as 2-propanol and *t*-butyl alcohol, caused more twisting of the glucose units on the helical structure than the linear alcohols, such as ethanol, 1-propanol, and 1-butanol. These structural differences in **7p** may cause a change in the recognition ability in various eluents including different types of alcohol modifiers.

Recently, the conformational changes of **7p** in the presence of polar solvents were investigated using solid-state vibrational circular dichroism (VCD) spectroscopy by Grinberg et al. [48]. The VCD results revealed that the conformations of **7p** are drastically changed depending on the concentration of the alcohols, such as ethanol and 2-propanol, in the polymer film. These conformation changes seem to affect the chiral recognition.

5 Conclusions

In this chapter, the development and chiral recognition mechanism of polysaccharide-based CSPs for the efficient chromatographic separation of enantiomers have been outlined. The recognition abilities of native polysaccharides are not sufficient for use as CSPs, but their abilities can be substantially improved by the proper modifications of their structures. At present, more than 10 kinds of polysaccharide-based CSPs are commercially available and practically used around the world as

CSPs for liquid chromatography due to their high-recognition ability and high loading capacity for a the wide range of racemates. Until now, the chiral recognition mechanism of the polysaccharide derivatives has been clarified to some extent by X-ray analysis, spectroscopic analysis, and computational methods. Further understanding of the chiral recognition mechanism on a molecular level will help with the prediction of the separability of enantiomers and the development of more efficient CSPs based on the polysaccharide derivatives.

References

1. Rogozhin SV, Davankov VA (1971) Ligand chromatography on asymmetric complex-forming sorbents as a new method for resolution of racemates. J Chem Soc Chem Commun 490–493
2. Okamoto Y, Ikai T (2008) Chiral HPLC for efficient resolution of enantiomers. Chem Soc Rev 37:2593–2608
3. Subramanian G (ed) (2007) Chiral separation techniques: a practical approach, 3rd completely revised and updated edn. Wiley-VCH, Weinheim
4. Taylor DR, Maher K (1992) Chiral separations by high-performance liquid chromatography. J Chromatogr Sci 30:67–85
5. Pirkle WH, Pochapsky TC (1989) Considerations of chiral recognition relevant to the liquid-chromatographic separation of enantiomers. Chem Rev 89:347–362
6. Armstrong DW (1987) Optical isomer separation by liquid-chromatography. Anal Chem 59:84A–91A
7. Yamamoto C, Okamoto Y (2004) Optically active polymers for chiral separation. Bull Chem Soc Jpn 77:227–257
8. Nakano T (2001) Optically active synthetic polymers as chiral stationary phases in HPLC. J Chromatogr A 906:205–225
9. Chen XM, Yamamoto C, Okamoto Y (2007) Polysaccharide derivatives as useful chiral stationary phases in high-performance liquid chromatography. Pure Appl Chem 79:1561–1573
10. Kotake M, Sakan T, Nakamura N, Senoh S (1951) Resolution into optical isomers of some amino acids by paper chromatography. J Am Chem Soc 73:2973–2974
11. Dalgliesh C (1952) The optical resolution of aromatic amino-acids on paper chromatograms. J Chem Soc 3940–3942
12. Hess H, Burger G, Musso H (1978) Complete enantiomer separation by chromatography on potato starch. Angew Chem Int Ed 17:612–614
13. Hesse G, Hagel R (1973) A complete separation of a racemic mixture by elution chromatography on cellulose triacetate. Chromatographia 6:277–280
14. Okamoto Y, Kawashima M, Yamamoto K, Hatada K (1984) Useful chiral packing materials for high-performance liquid chromatographic resolution cellulose triacetate and tribenzoate coated on macroporous silica gel. Chem Lett 13:739–742
15. Ichida A, Shibata T, Okamoto Y, Yuki Y, Namikoshi H, Toda Y (1984) Resolution of enantiomers by HPLC on cellulose derivatives. Chromatographia 19:280–284
16. Okamoto Y, Aburatani R, Hatada K (1987) Chromatographic chiral resolution XIV- cellulose tribenzoate derivatives as chiral stationary phases for high-performance liquid chromatography. J Chromatogr 389:95–102
17. Okamoto Y, Kaida Y (1994) Resolution by high-performance liquid chromatography using polysaccharide carbamates and benzoates as chiral stationary phases. J Chromatogr A 666:403–419
18. Yashima E, Yamamoto C, Okamoto Y (1998) Polysaccharide-based chiral LC columns. Synlett 344–360

19. Okamoto Y, Kawashima M, Hatada K (1984) Useful chiral packing materials for high-performance liquid chromatographic resolution of enantiomers: phenylcarbamates of polysaccharides coated on silica gel. J Am Chem Soc 106:5357–5359
20. Okamoto Y, Kawashima M, Hatada K (1986) Controlled chiral recognition of cellulose triphenylcarbamate derivatives supported on silica gel. J Chromatogr 363:173–186
21. Okamoto Y, Ohashi T, Kaida Y, Yashima E (1993) Resolution of enantiomers by HPLC on tris(4-alkoxyphenylcarbamate)s of cellulose and amylase. Chirality 5:616–621
22. Chankvetadze B, Yashima E, Okamoto Y (1994) Chloromethylphenylcarbamate derivatives of cellulose as chiral stationary phases for high-performance liquid chromatography. J Chromatogr A 670:39–49
23. Okamoto Y, Aburatani R, Fukumoto T, Hatada K (1987) Useful chiral stationary phases for HPLC amylose tris(3,5-dimethylphenylcarbamate) and tris(3,5- dichlorophenylcarbamate) supported on silica gel. Chem Lett 16:1857–1860
24. Chankvetadze B, Yashima E, Okamoto Y (1995) Dimethyl-, dichloro- and chloromethylphenylcarbamates of amylose as chiral stationary phases for HPLC. J Chromatogr A 694:101–109
25. Yashima E, Yamamoto C, Okamoto Y (1995) Enantioseparation on fluoro- methylphenylcarbamates of cellulose and amylose as chiral stationary phases for HPLC. Polym J 27: 856–861
26. Yamamoto C, Yashima E, Okamoto Y (1999) Computational studies on chiral discrimination mechanism of phenylcarbamate derivatives of cellulose. Bull Chem Soc Jpn 72:1815–1825
27. Wenslow RM, Wang T (2001) Solid-state NMR characterization of amylose tris (3,5-dimethylphenylcarbamate) chiral stationary-phase structure as a function of mobile-phase composition. Anal Chem 73:4190–4195
28. Yamamoto C, Yashima E, Okamoto Y (2002) Structural analysis of amylose tris (3,5-dimethylphenylcarbamate) by NMR relevant to its chiral recognition mechanism in HPLC. J Am Chem Soc 124:12583–12589
29. Yashima E, Yamada M, Okamoto Y (1994) An NMR study of chiral recognition relevant to the liquid chromatographic separation of enantiomers by a cellulose derivative. Chem Lett 23:579–582
30. Yashima E, Yamada M, Yamamoto C, Nakashima M, Okamoto Y (1997) Chromatographic enantioseparation and chiral discrimination in NMR by trisphenylcarbamate derivatives of cellulose, amylose, oligosaccharides, and cyclodextrins. Enantiomer 2:225–240
31. Okamoto Y, Yashima E, Yamamoto C (1997) NMR studies of chiral discrimination by phenylcarbamate derivatives of cellulose. Macromol Symp 120:127–137
32. Yashima E, Yamamoto C, Okamoto Y (1996) NMR studies of chiral discrimination relevant to the liquid chromatographic enantioseparation by a cellulose phenylcarbamate derivative. J Am Chem Soc 118:4036–4048
33. Kubota T, Yamamoto C, Okamoto Y (2002) Chromatographic enantioseparation by cycloalkylcarbamate derivatives of cellulose and amylase. Chirality 14:372–376
34. Ye YK, Bai S, Vyas S, Wirth MJ (2007) NMR and computational studies of chiral discrimination by amylose tris(3,5-dimethylphenylcarbamate). J Phys Chem B 111:1189–1198
35. Lipkowitz KB (1994) Modeling Enantiodifferentiation in chiral chromatography. In: Subramanian G (ed) A Practical Approach to Chiral Separations by Liquid Chromatography. Wiley-VCH, New York
36. Lipkowitz KB (1995) Theoretical studies of type II–V chiral stationary phases. J Chromatogr A 694:15–37
37. Lipkowitz KB (2001) Atomistic modeling of enantioselection in chromatography. J Chromatogr A 906:417–442
38. Yashima E, Yamada M, Kaida Y, Okamoto Y (1995) Computational studies on chiral discrimination mechanism of cellulose trisphenylcarbamate. J Chromatogr A 694:347–354
39. Theodorou DN, Suter UW (1985) Detailed molecular structure of a vinyl polymer glass. Macromolecules 18:1467–1478

40. Aboul-Enein HY, Ali I, Laguerre M, Felix G (2002) Molecular modeling of enantiomeric resolution of methylphenidate on cellulose tris benzoate chiral stationary phase. J Liq Chromatogr Relat Technol 25:2739–2748
41. O'Brien T, Crocker L, Thompson R, Thompson K, Toma PH, Conlon DA, Feibush B, Moeder C, Bicker G, Grinberg N (1997) Mechanistic aspects of chiral discrimination on modified cellulose. Anal Chem 69:1999–2007
42. Kasat RB, Wang NHL, Franses EI (2007) Effects of backbone and side chain on the molecular environments of chiral cavities in polysaccharide-based biopolymers. Biomacromolecules 8:1676–1685
43. Kasat RB, Zvinevich Y, Hillhouse HW, Thomson KT, Wang NHL, Franses EI (2006) Direct probing of sorbent-solvent interactions for amylose tris(3,5- dimethylphenylcarbamate) using infrared spectroscopy, X-ray diffraction, solid-state NMR, and DFT modeling. J Phys Chem B 110:14114–14122
44. Kasat RB, Chin CY, Thomson KT, Franses EI, Wang NHL (2006) Interpretation of chromatographic retentions of simple solutes with an amylose-based sorbent using infrared spectroscopy and DFT modeling. Adsorption 12:405–416
45. Kasat RB, Wang NHL, Franses EI (2008) Experimental probing and modeling of key sorbent-solute interactions of norephedrine enantiomers with polysaccharide-based chiral stationary phases. J Chromatogr A 1190:110–119
46. Wang T, Wenslow RM (2003) Effects of alcohol mobile-phase modifiers on the structure and chiral selectivity of amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase. J Chromatogr A 1015:99–110
47. Helmy R, Wang T (2005) Selectivity of amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase as a function of its structure altered by changing concentration of ethanol or 2-propanol mobile-phase modifier. J Sep Sci 28:189–192
48. Ma SL, Shen S, Lee H, Yee N, Senanayake C, Nafie LA, Grinberg N (2008) Vibrational circular dichroism of amylose carbamate: structure and solvent-induced conformational changes. Tetrahedron Asymmetry 19:2111–2114

Description and Evaluation of Chiral Interactive Sites on Bonded Cyclodextrin Stationary Phases for Liquid Chromatography

Thomas E. Beesley

Contents

Abstract Development of chiral separations has been essential to the drug discovery and development process. The solubility requirements for a number of methods and/or the mobile phase requirements for application of certain detection systems have opened up many opportunities for cyclodextrin-based CSPs for liquid

T.E. Beesley (✉)
148 Waughaw Road, Towaco, NJ 07082, USA
e-mail: tebeesley@gmail.com

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_3, © Springer-Verlag Berlin Heidelberg 2010

chromatography. Even though a few chiral stationary phases cover a wide area of enantioselectivity, they do not meet the entire needs of the industry. Cyclodextrin phases offer some unique mechanisms and opportunities to resolve chiral separation problems especially in the aqueous reversed-phase and non-aqueous polar organic modes. This chapter addresses the need to understand the chiral stationary phase structure, the mechanisms at work, and the role mobile phase composition plays in driving those mechanisms to produce enantioselectivity. In addition, the development of certain derivatives has played an essential part in expanding that basic role for certain chiral separations. What these derivatives contribute in concert with the basic structure is a critical part of the understanding to the effective use of these phases. During this study it was determined that the role of steric hindrance has been vastly underestimated, both to the extent that it has occurred and to its effectiveness for obtaining enantioselectivity. References to the entire 20-year history of the cyclodextrin phase development and application literature up to this current date have been reviewed and incorporated.

1 Introduction

Cyclodextrins (CD) are crystalline, homogenous toroidal structures of different molecular size produced by the partial degradation of starch and enzymatic coupling of cleaved glucose units. Three of the most widely characterized cyclodextrins employed in chemical separations are referred to as α-cyclodextrin (six glucose units), β-cyclodextrin (seven glucose units), and γ-cyclodextrin (eight glucose units). The ubiquitous nature of cyclodextrins comes from a long history of exploring a unique property of these cyclic glucose structures referred to as inclusion complexation. This concept refers to the ability of apolar molecules to be selectivity included into the CD cavity and held there by a variety of forces. The cavity of the cyclodextrin, formed from the glucoside oxygens and methylene groups of the glucose units, gives it this apolar character. The rigidity of this structure has been confirmed from X-ray crystallographic data studied in a variety of aqueous and organic solvents. In addition, the crystallographic data help generate computer models that when based on minimum free energy calculations provided evidence for preferred inclusion complex formation. The hydroxyl groups of the glucose units form the hydrophilic surface of the cyclodextrin making it water soluble. These simple facts led to the use of cyclodextrins to solubilize hydrophobic molecules into aqueous solutions through this simple inclusion process.

Given the physical dimensions of the formed cavities, it is easy to see the possibility for separations based on the space occupied of various molecular types and their relative polarity. The ability of the glucoside oxygens, that make up the CD cavity, to create dipole–dipole and London dispersion forces, combined with the high hydrogen bonding surface gives these structures unique abilities to discriminate structural and functional differences. A versatile method for measuring the partition

coefficients of volatile analytes with an aqueous pseudophase of cyclodextrin using headspace gas chromatography has been reported [1]. These simple characteristics alone have made cyclodextrins the basis of studies for mimicking enzymes, catalysis, molecule stabilization, chemical reactions, molecular recognition models, and a whole host of chemical separation techniques including liquid–liquid extraction (LLE), capillary electrophoresis (CE), capillary electrochromatography (CEC), thin layer chromatography (TLC), HPLC, supercritical fluid chromatography (SFC), solid phase extraction (SPE), and capillary GC.

The role of cyclodextrins as chromatographic agents for chiral separations has a history dating back to late 1970s and early 1980s when it was first demonstrated as a separation tool constructed as a polymer or bonded to a polymer. The early systems were very inefficient but the separation capability that was demonstrated was indeed a dramatic demonstration of its potential. It was not until 1983 when Armstrong bonded the first β-cyclodextrin to a silica base that the true separation potential of these structures was realized in liquid chromatography. This was also the beginning of the explosion of the chiral recognition capabilities of these structures. For a 20-year review up to 2002, of bonded cyclodextrins as chiral stationary phases, see *Methods in Molecular Biology*, Humana Press, a chapter authored by Mitchell and Armstrong that covers the complete technology and literature from a historical perspective [2].

The purpose and direction of this treatise is to more closely investigate and understand the potential interactions of the various structural parts of these elegant toroidal structures with a variety of analytes, both chiral and achiral that will lead to better understanding of the mechanisms at work and what set of chromatographic conditions are required to promote enantioselectivity. We will also indicate the influence of linkage chemistry to the performance of the bonded cyclodextrin. From this knowledge base, it is then possible to expand this study to demonstrate the rationale for the variety of derivatives that have been created and how a particular derivative works to further enhance the application of cyclodextrin technology for liquid chromatography chiral separations. Finally, from the current literature, we will categorize published applications under the various derivative headings to further demonstrate the potential interactive mechanisms at work under diverse mobile phase conditions.

2 Cyclodextrin Structure

The cyclic structures formed in the fermentation process of cyclodextrin production orient the chiral hydroxyl groups of the glucose units on the outside of the toroid making the external surface hydrophilic and therefore the CD is water soluble. The 2- and 3-position hydroxyls form the mouth of the larger opening of the toroid with the 2-position facing inward to the cavity and arrange in a right-handed twist, while the 3-position hydroxyls face outward rotated in the reverse direction as seen in Fig. 1.

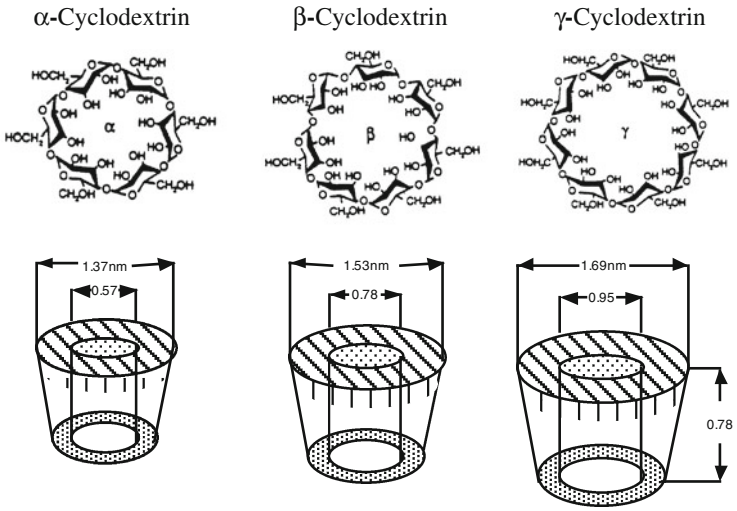


Fig. 1 Structure and dimensions of the three most common cyclodextrin molecules

The restricted conformational freedom and orientation of these secondary hydroxyls is largely responsible for the chiral recognition capabilities of these native structures. It is these functional groups that will also be used to extend the chiral recognition capabilities through derivatization. At the other end of this toroidal basket, the 6-position hydroxyl separated by a methyl group that has reduced chiral ability is typically used to link the cyclodextrin to a solid surface, most often silica gel. As primary hydroxyls, they are most easily activated for derivatization or coupling. The internal cavity, with a high electron density from the glycoside oxygens, is believed to be responsible for the specific orientation of aromatic rings to allow for appropriate hydrogen bonding of the sterically fixed hydroxyl groups and the chiral functional group of an analyte. This latter fact was confirmed chromatographically evaluating the difference in chiral recognition between a saturated ring and an aromatic ring with the same chiral substituents. A saturated ring is included by the weaker London dispersion forces and lacks a specific orientation. In addition, the strength of the inclusion can further enhance the hydrogen bonding differences as can be seen in the separation of ortho-, meta-, and para-xylene as differentiated from ortho-, meta-, and para-cresols in Fig. 2.

Certain functional groups that are electron sharing on the aromatic ring can further enhance selectivity as seen in Fig. 3 with the nitroanilines. The amine function of the nitroanilines, hydrogen bonds to the surface hydroxyls, while the nitro groups π–π bond to the glycoside oxygens of the CD. This concept of inclusion complexation to differentiate aromatic structures based on space filling of the cavity combined with a hydrogen bonding group makes this a unique analytical tool. The utility of this methodology was demonstrated for the achiral analysis of organic acids, a class of drug discovery compounds, in which case it was difficult

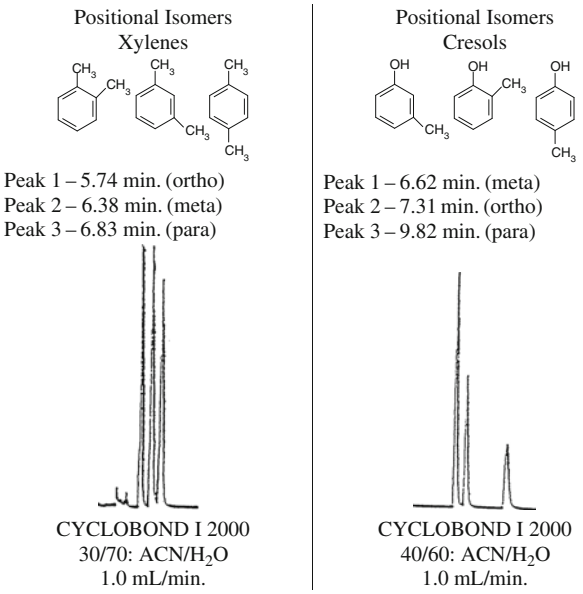
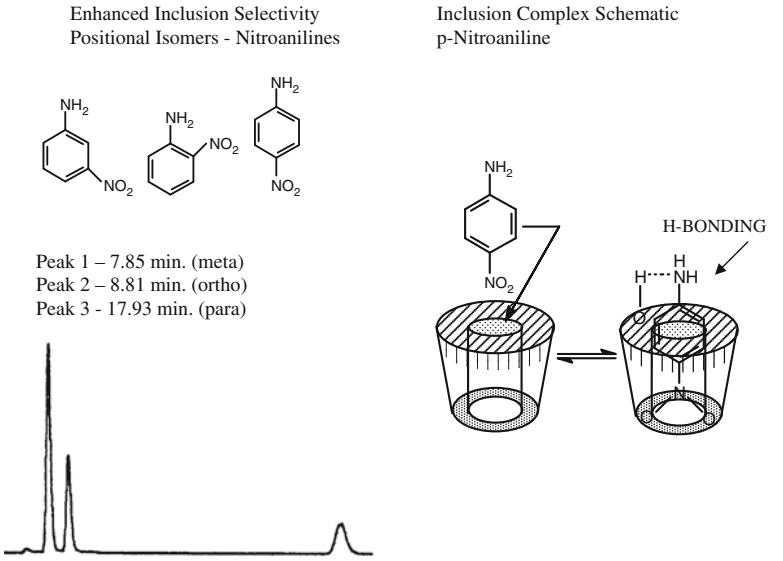


Fig. 2 Examples of inclusion selectivity on Cyclobond® I 2000
Note: The more bulky a molecular structure, the lower the retention



Conditions: column CYCLOBOND I 2000, 25 cm 4.6 mm id, mobile phase: 40/60: MeOH/H_2O; 1.0 mL/min.

Fig. 3 Inclusion selectivity: the case of nitroanilines

to separate all components by typical reversed-phase chromatography [3]. Speed was also a very big factor here where the CD phase outperformed the conventional RP phase.

3 Native Bonded Cyclodextrins

Early work, attempting to create a useful stationary phase, polymerized the cyclodextrins utilizing epichlorohydrin. Control of the polymerization was erratic and typically led to restricted access to the cavity, limiting inclusion complexation, a crucial function. In Armstrong's first successful bonding of the cyclodextrin the primary hydroxyls were activated with sodium hydroxide at high pH. A glycidoxypropylsilane was bonded to a suitable silica and the sodium-activated cyclodextrin opened up the epoxide group resulting in a covalent ether linkage. Other stable chemistries have been used as coupling with isocyanates but these chemistries that added hydrogen donor and acceptor sites to the linkage arm resulted in interference with the interaction of certain analytes with the cyclodextrin cavity. For the purpose of this study we will concentrate on the epoxide linkage as it offers the least interference with the analyte–cyclodextrin interactions. Early studies also indicated that the length of the linkage was of importance to the function of the cyclodextrin in a variety of mobile phases. In addition, early work studying the utility of the α-, β- and γ-cyclodextrin indicated that the β-CD had the broadest applicability seemingly a condition of the unpaired glucose unit (seven units) more than the actual cavity size. The α-CD has some degree of flexibility and accommodates larger molecules than suggested by the actual cavity dimensions but its chiral recognition properties are diminished when compared to the β-cyclodextrin. The limits of utility of the α-CD were best demonstrated for the separation of beta-substituted tryptophans and other tryptophan analogs [4]. Other unusual amino acids have also been successfully separated on the α-CD in the reversed-phase mode as long an aromatic ring was present [5].

The development of mobile phase conditions for these phases led to a number of interesting discoveries as to the functional areas of the cyclodextrin and the controls necessary for chiral recognition. It is important to remember: *Chiral recognition requires the formation of a diastereomeric complex between a functional group on or near the stereogenic center of the analyte and a chiral functional group of the chiral stationary phase. For optimum results these forces must be balanced against the non-chiral forces of retention.* These forces can be attractive as with hydrogen bonding or repulsive as with steric bulk. The utility of the hydrogen bonding groups on the surface of the CD was best evaluated with a study of the separation and characterization of underivatized oligosaccharides [6]. Components of oligosaccharide mixtures up to 11 glucose units long were baseline resolved on a Cyclobond® I 2000 (CB-I, native β-CD) column down to 50 pg with simple acetonitrile/water and ESI-MS detection. This hydrogen bonding mechanism for

separation is quite different from the hydrophobic mechanisms used in C18 separations leading to very different selectivity. In fact, utilizing a non-aqueous polar organic mobile phase for this type of achiral separation, the elution order of a series of catechins is directly linked to the number of hydroxyl groups in the molecule and can be accomplished 10 times faster than on a standard C18 [7]. To provide enantioselectivity for these types of separations, the inclusion mode must be employed in combination with hydrogen bonding; therefore, the reversed-phase mode is used.

In taking advantage of the inclusion complex phenomena for chiral separations, the first mobile phase type studied was reversed phase that used simple alcohol/water mixtures to achieve separation of dansyl amino acids [8]. Acetonitrile for these phases appeared too strong a solvent and caused displacement of compounds easily from the cavity so methanol became the primary organic modifier. The naphthyl group of Dansyl, a 1-dimethyl amino naphthalene-5-sulfonyl derivative of N-terminal amino acids fits nicely into the cyclodextrin cavity. This is now considered as the first recognized step in chiral recognition process, i.e., proper fit of the aromatic portion of the analyte into the CD cavity. If the cavity is too large for the analyte of interest, selectivity is reduced but it is important to remember, both the aromatic ring size and the presence of electron sharing functional groups influence the strength of the inclusion complex. If the cavity size is too restrictive, again selectivity is reduced. In addition, for chiral recognition of the Dansyl amino acids to occur the carboxyl group of the amino acid needs to hydrogen bond with the 2-position hydroxyl group of the CD. If the carboxyl group is methylated, chiral recognition ceases. For this type of separation, simple mixtures of methanol/water are sufficient to separate any Dansyl amino acid. In these cases, methanol controls both the hydrogen bonding and therefore the diastereomeric complex formation and the inclusion function with the CD stationary phase.

If we now look at warfarin, again a naphthyl group is part of the basic structure; we will find no chiral recognition in the reversed-phase mode. Molecular modeling indicates that the chiral hydroxyl group of the warfarin in this case is out of reach of the van der Waals radii of the 2-position hydroxyl group of the cyclodextrin. What then is the solution? There are actually two possible solutions. First, increase the reach of the 2-position hydroxyl through derivatization but an easier solution was to have the analyte interact with the surface and not be fully included. This is accomplished by using acetonitrile in high concentration that saturates the cyclodextrin cavity and rejects the analyte but the analyte can hydrogen bond utilizing both the 2- and 3-position hydroxyls on the surface to satisfy the rule for multiple points of interaction for chiral recognition to occur. This, however, would require a minimum of two potential hydrogen bonding groups. Warfarin does in fact have carbonyl functions in addition to the hydroxyl group capable of interacting with the 3-position hydroxyl of the CD. This non-aqueous new mobile phase design has been referred to as the *polar organic mode*. It uses both acetic acid and triethylamine to control the interaction of the hydroxyl and the carbonyl function. Methanol and acetonitrile in combination can also help control the hydrogen bonding and minimize the

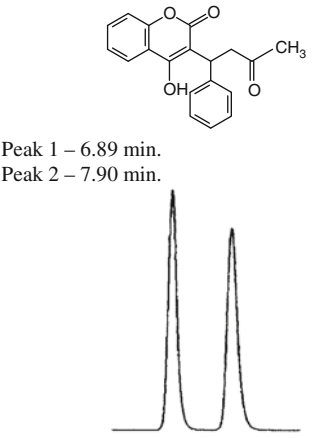


Fig. 4 Separation of warfarin in polar organic mode

amount of acid and base that is used as well as the strength of the cavity interaction. See Fig. 4 for the chiral separation of warfarin in this new mode. A recent publication referenced HILIC, hydrophilic interaction chromatography as a possible explanation for the polar organic mode [9]. This author acknowledges there are cases of a direct similarity to HILIC but not in all cases, and not in my experience, the majority of cases.

From the above description, the following rules can be set, describing possible mechanisms for potential chiral interaction in either aqueous (reversed-phase mode) or non-aqueous (polar organic mode) mobile phase conditions applicable to all CD phases.

Rule 1: A chiral analyte must have at least one aromatic group and it must properly fit into the CD cavity in order to set up hydrogen bonding with the sterically fixed chiral hydroxyl groups of the CD. Certain functional groups on the included aromatic ring enhance this inclusion interaction: halogens, nitrate, sulfate, phosphate, and phenols at low pH. The glucoside oxygens of the CD cavity orient the molecule for appropriate hydrogen bonding to the 2- or 3-position hydroxyls.

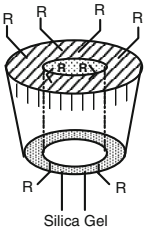
Rule 2: The preferred hydrogen bonding groups: carboxyls, amides, carbonyls, hydroxyls, and amines must be close enough to the included aromatic ring to hydrogen bond to the 2- or 3-position hydroxyl of the CD.

Rule 3: If two or more hydrogen bonding groups are in the extended arm of the chiral analyte, the polar organic mode is a better option than reversed phase because of the faster kinetics of surface interaction and the anhydrous nature of the mobile phase.

For the native bonded cyclodextrins, two mobile phases are responsible for driving the mechanisms for chiral recognition, the aqueous reversed-phase mode (RP), and the non-aqueous polar organic mode (POM). From a review of the applications literature the ratio for RP/POM uses is close to 50/50.

4 Derivatized Bonded Cyclodextrins

Over the last several years, since the introduction of bonded cyclodextrins, certain derivatives have been developed to extend the potential for chiral interaction as shown in Fig. 5. Since the secondary hydroxyls are sterically fixed the distance of a hydrogen bonding group to the included aromatic ring is limited to the influence of a few carbon lengths which can be further influenced by the orientation of the aromatic ring in the cavity by virtue of attached functional groups. The



R =	CYCLOBOND I *2000* SUFFIX	CB-I
—CH_3	DM (2,3-di-O-methyl)	CB-DM
—$COCH_3$	AC (acetylated)	CB-AC
—CH_2*$CHCH_3$ (OH)	SP or RSP/HP-RSP (S or racemic RS hydroxypropyl ether)	CB-RSP CB-HP-RSP
—$CONHCH$*(CH_3)– naphthyl	RN or SN (R or S naphthylethyl carbamate)	CB-RN CB-SN
—$CONH$– (3,5-CH_3, CH_3)	DMP (3,5-dimethylphenyl carbamate)	CB-DMP
–O– (2,6-NO_2, NO_2; 4-OCF_3)	DNP 2,6-dinitro-4-trifluoro methylphenylether	CB-DNP

Fig. 5 Summary of derivatives of Cyclobond® I 2000
*Additional stereogenic center. The CSP's above are products of Sigma-Aldrich Supelco, Bellefonte, PA.

polar organic mode extends that distance, especially using the larger γ-CD cavity, because of the greater number of sterically fixed hydroxyls on the surface of the gamma toroid. It is also possible to add additional mechanisms to further enhance the potential for chiral interaction. We will categorize these derivatives into three groups: *Group 1*: those derivatives enhancing steric effects; *Group 2*: those derivatives extending hydrogen donor and acceptor sites; *Group 3*: those derivatives creating a multimodal CD phase with the addition of π-acidic or π-basic structures.

4.1 Enhanced Steric Effects: Bonded 2, 3-Dimethyl β-Cyclodextrin (CB-DM)

Methylation of the 2- and 3-position hydroxyl groups of the CD blocks the capability for chiral hydrogen bonding and offers instead the potential for weak dipole interactions as well as enhanced steric effects. This phase has proven useful for the separation of single ring structures with bulky side arms as demonstrated for fluoxetine [10] as well as with fused ring structures essentially all in the reversed-phase mode to take advantage of the restricted cavity access of the two spacially different enantiomers. To demonstrate the effectiveness of this mechanism, a series of new chiral fused polycycles have been reported separated by this technique [11]. In a number of cases reported in the literature, in addition to the CB-DM as reported in this latter publication, the CB-RSP has been shown to be a complimentary phase for this technique. The CB-RSP, a hydroxypropylated derivative of CD, offers not only the steric effects of derivatizing the 2-position hydroxyls of the CD that face inward to the cavity but also add an extended more polar chiral hydroxyl group. For a more complete description of CB-RSP series, see Sect. 4.2. In these cases, utilizing CB-RSP, fused ring structures are separated based on steric bulk *and* functional group interaction. Figure 6 demonstrates an example of this application and comparison.

A series of new chiral furan derivatives has also been published demonstrating the utility of both the CB-DM and CB-RSP to separate based on steric bulk in simple methanol/water mixtures [12]. This technique of steric repulsion as a mechanism was also demonstrated for the separation of 16 racemic dihydrofuroflavones with a similar complementary effect for the CB-DM and CB-RSP [13]. Another example of this useful combination of steric bulk and hydrogen bonding mechanism for the CB-RSP can be found in the publication cited for the separation of 12 chiral dihydrobenzofurans [14].

For the CB-DM, reversed phase is the mobile phase of choice, employing methanol predominantly as the organic modifier in water. The choice of modifier is dictated by the strength of the inclusion complex. It has been noted that methyl *tert*-butyl ether (MTBE) greatly improves peak efficiency, reduces retention, and enhances resolution on this phase (Fig. 7). The role of the MTBE is to further restrict the cavity access as it associates with the surface methoxy groups through a weak dipole interaction. It is important to note that in this case only methanol can be used as a modifier as acetonitrile will disassociate the MTBE from the CSP.

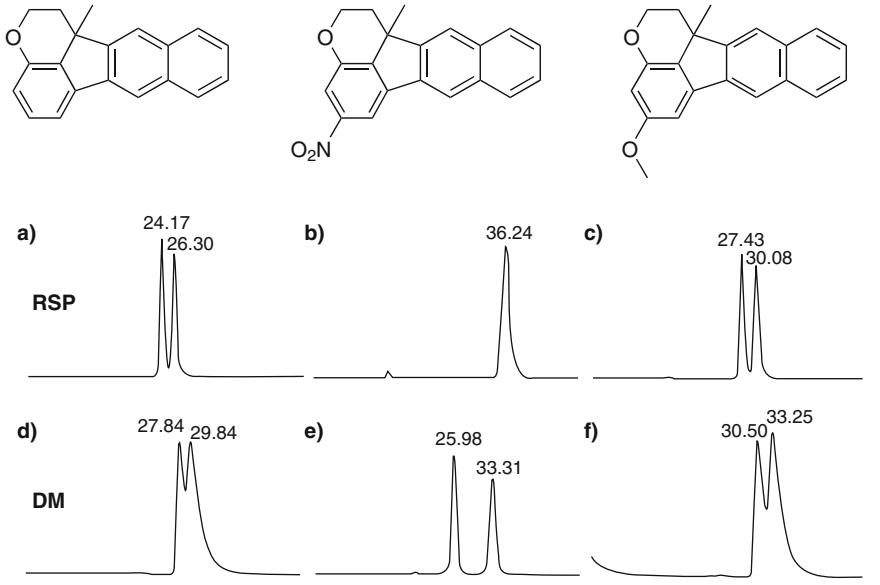


Fig. 6 Complimentary chiral stationary phases: Cyclobond® I 2000 RSP vs DMRSP: mobile phase: CH_3OH/H_2O; 50/50 (**a**), (**b**), (**c**) DM: mobile phase: CH_3OH/H_2O; 50/50 (**d**); 35/65 (**e**), (**f**). Ref. [11], p. 2755

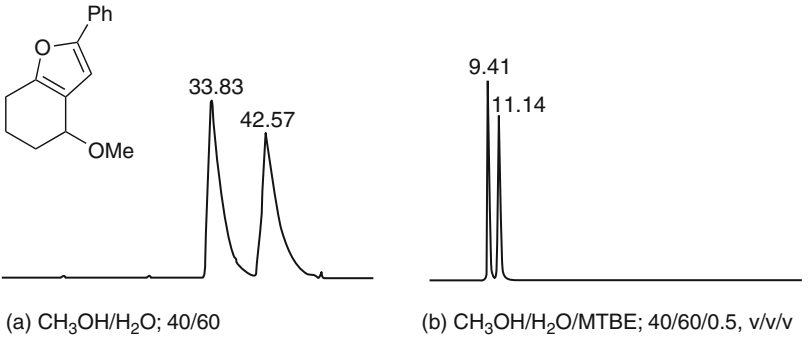


Fig. 7 Effect on a furan derivative peak efficiency of 0.5% MTBE added to the mobile phase (**a**) CH_3OH/H_2O; 40/60, (**b**) CH_3OH/H_2O/MTBE; 40/60/0.5, v/v/v
Column: Cyclobond® I 2000 DM, 25 cm, 4.6 mm id. Ref. [12], p. 118

In addition to the CB-DM, the literature has cited the β-hydroxypropylated cyclodextrin (in the racemic form of hydroxypropyl) (CB-RSP) as the best complementary phase for this type of mechanism where steric bulk as well as hydrogen bonding can play a role in enantioseparation for any fused ring structure. The third most cited phase in this area is the β-acetylated cyclodextrin (CB-AC). More detailed description of these later two phases is given below.

4.2 Extended Hydrogen Bonding Groups: Bonded β-Acetylated (CB-AC) and Bonded β-Hydroxypropylated Cyclodextrins: (CB-SP), (CB-RSP), and (CB-HP-RSP)

A number of functional groups have been reacted with the sterically fixed hydroxyls of the CD to extend the potential for diastereometric complexation. One of the earliest has been the acetylation of these CD hydroxyl groups. The acetyl interacts most favorably with chiral amines, both primary and secondary and chiral hydroxyl groups that are within three carbons of an included aromatic group. Since inclusion and hydrogen bonding are the main driving forces, reversed-phase conditions are the most favorable. For reasons of the distance between carbonyls in the 2- and 3-position, little favorable interactions have occurred in the polar organic mode.

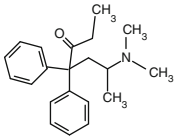
One of the most successful derivatives in a number of separation modes has been the β-hydroxypropylated version derived by the reaction of both chiral and racemic propylene oxide with the native cyclodextrin. Substitution level has been a critical issue in the preparation of this derivative. Full derivatization of all 21 hydroxyl groups of the β-CD has led to less overall selectivity in the same manner as too little. Advanced Separation Technologies, Inc. (Astec) after much experimentation determined that a substitution level of 7 was ideal for both the bonded β-CSP for HPLC and CEC and as an additive for CE. A suitable profile of derivatization is controlled by temperature, rate of addition, and concentration. In studying the chiral *R* form of the propylene oxide it was observed that a strong internal hydrogen bond occurred and little selectivity resulted. The *S* version did show excellent selectivity but the improvement in peak efficiency and resolution over the racemic propylene oxide was insufficient to warrant the cost differential. As a result of these observations the racemic mixture (CB-RSP) was chosen for economic reasons.

As in a number of HPLC phases, the type and length of linkage of these ligands to a silica base lead to changes in overall accessibility and performance. Modifications of these parameters to the first introduced product CB-RSP did lead to a higher performing product the CB-HP-RSP, the HP standing for high performance, to distinguish it from the former CSP with exactly the same chiral ligand. Literature earlier than 2005 citing the CB-RSP should reexamine the applications on the CB-HP-RSP. As in all changes of this type there are still racemates that work better on the original version and in this case it was the t-BOC amino acids. Inclusion complexation is still the preferred mechanism requiring the reversed-phase mode. The polar organic mode is not as effective here as it was for the bonded native β-CD.

This may have more to do with the internal hydrogen bonding from this derivative which is more flexible than the sterically fixed hydroxyls of the native CD. Reversed phase is the driving force with this phase with acetonitrile dominating as the organic modifier. Buffer is often used to control the degree of ionization given the plethora of hydrogen bonding groups and the strength of the additional chiral hydroxyl added through derivatization. See Fig. 8 for a comparison of the CB-RSP versus CB-HP-RSP for the separation of methadone.

In a publication demonstrating the separation of the methadone enantiomers from the major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), the CB-RSP was satisfactory but the CB-HP-RSP would have cut the analysis time in half with little or no change in mobile phase composition [15]. An evaluation of the CB-RSP against other CB derivatives for the separation of a series of 20 chiral isochromenes again demonstrates the utility of this phase in the reversed-phase mode, combining both steric and hydrogen bonding mechanisms [16]. This is a common theme with this type of CSP, as can be reviewed in the analysis of (+)-catechin in fruits, wine, and cocoa products that was conducted using the CB-RSP as seen in Fig. 9, in a reversed-phase mode [17].

Multiple chiral centers can also be resolved on this phase utilizing this combination of H-bonding and steric hindrance as seen in Fig. 10. Direct enantiomeric



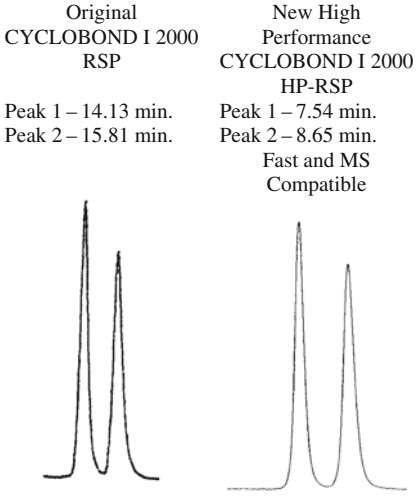


Fig. 8 Separation of methadone. Ref. [34, *Cyclobond* Handbook, 7th edn.,], p. 14

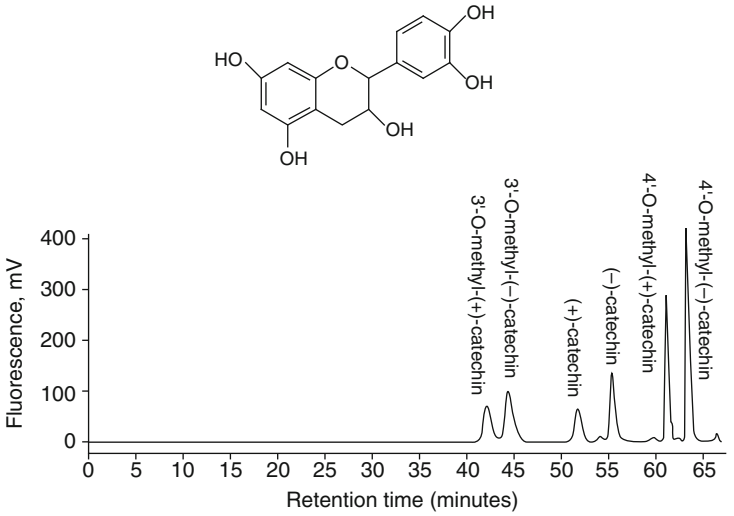
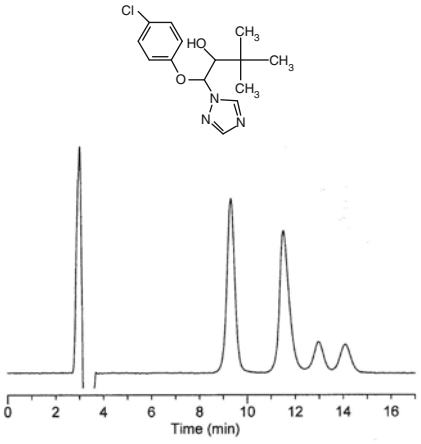


Fig. 9 Separation of catechins Cyclobond® I 2000 RSP 25 cm × 4.6 mm i.d. Gradient: 50 mM $Na_2H_2PO_4$, pH 3 + 80 ACN in 30 mM $Na_2H_2PO_4$. Ref. [17], p. 1030

Fig. 10 Separation of triadimenol enantiomers on Cyclobond® I 2000 HP-RSP 70/30; 20 mM ammonium acetate, pH 4.0/acetonitrile flow @ 1.0 mL/min; detection UV at 220 nm; injection 10μL sample triadimenol (α-tert-butyl-β-(4-chlorophenoxy)-1H-1,2,4-triazole-1-ethanol), 1 mg/mL in mobile phase. Ref. Supelco Application Library



separation and determination of enantiomeric purity of methoxytetrahydronaphthalene derivatives and melatonin ligands have been reported using the CB-RSP in the polar organic mode [18]. Mixtures of acetonitrile and methanol control selectivity and retention indicating that steric bulk played a major role in this separation. The fact that the *cis* conformation (more bulky) was more retained confirms that assumption.

Mechanism for the CB-AC involves both inclusion complexation and hydrogen bonding to the carbonyl of the acetyl derivative. The most successful mobile phase, therefore, is reversed-phase mode, using methanol in this case as the primary organic modifier typically in combination with a buffer in the pH range of 4.0–6.0, as this is the most stable pH range for the acetyl derivative. The best buffers have been triethylamine acetate and ammonium acetate in concentrations of 0.1% w/v.

Highest selectivity among this group of similar ligands is as follows: CB-HP-RSP>CB-RSP

The mechanism largely at work as with the CB-AC is inclusion complexation and hydrogen bonding. From the literature, the reversed-phase composition predominantly utilized acetonitrile as the organic modifier with buffer in the pH range 3.5–7.0. If the amount of hydrogen bonding is very high, then substituting methanol as the organic modifier is appropriate.

4.3 Multimodal Cyclodextrin Phases: Bonded R- or S-Naphthylethyl Carbamate Derivatives (CB-RN/SN); 3,5-Dimethyl Phenylcarbamate (π-bases; CB-DMP) and 3,5-Dinitrophenylcarbamate (π-Acid; CB-DNP) Derivatives

Multimodal is a term that refers to CSPs that have the necessary mechanisms to operate successfully in a variety of mobile phase types, both aqueous and non-aqueous in a range of polarities making them very flexible CSPs. To extend this range of polarities in applications on CD phases into typical normal phase solvents for chiral separations, the derivative must have either π-acidic or π-basic structures in order to anchor the analyte with an opposing electron donating or electron withdrawing structure in hydrocarbon solvents like hexane or heptane. Several π-basic structures have been developed including 3,5-dimethyl phenylcarbamate (CB-DMP); *S* and *R* napthylethylcarbamate (CB-RN and CB-SN) and phenyl carbamate. The β-CD cavity proved to be the best size for this derivative although there are a few examples when the γ cavity improved resolution. These types of derivatives can now allow for separations in reversed-phase, typical normal phase, and the previously mentioned non-aqueous polar organic mode. A number of publications have cited the utility of these phases for chiral separations and one in particular for the separation of ruthenium complexes characterizes the difference between the CB-RN and CB-SN for separations in the reversed-phase mode [19]. The CB-RN demonstrated higher selectivity indicating that in addition to the π-stacking interaction provided by the aromatic group of the CSP, the special structure may change the shape of the cavity providing again a steric contribution not afforded by the *S* form.

These derivatives have demonstrated selectivity in a variety of mobile phase conditions. They offer some unique capabilities, especially in typical normal phase solvents. In a study of a variety of chiral pterocarpans, all enantiomers could be

baseline resolved in a reversed-phase mode, utilizing the CB-RSP and CB-AC but the CB-DMP showed the broadest selectivity in normal phase [20]. The influence of alcohol polarity on the separation of a number of 1- and 2-naphthol analogs was also demonstrated on the CB-DMP [21]. A detailed study was conducted of a variety of racemates containing amines as amino acids, amino alcohols, etc., that were chemically derivatized with a variety of electrophilic tagging reagents to elucidate the chiral recognition sites on a teicoplanin-bonded CSP and on *R*-naphthylethylcarbamate-β-cyclodextrin [22].

Aromatic racemates derivatized with 3,5- DNB yielded better resolutions than non-aromatic racemates in the polar organic mode. This was just the opposite of the teicoplanin CSP indicating that π-bonding is the primary interaction leading to resolution when using the polar organic mode on the CB-RN phase. Further evidence of the utility of the π-basic phases in the polar organic mode was presented for the HPLC enantioseparation of bicalutamide and its related compounds [23].

The π-acidic derivative has been a long sought product but the π-acid structure was the most difficult to stabilize and keep from bleeding. The use of an ether linkage to the CD finally stabilized the product. A thorough review of a number of potential bonding chemistries and their application evaluation for this new π-acidic phase has been published [24]. Optimization of the synthesis for the most promising of the studied derivatives followed [25]. Both the π-acid (CB-DNP) and the π-base (CB-DMP) were evaluated for the resolution of *N*-3,5- dinitrobenzoyl form and *N*-3,5- dimethyl-benzoyl form of unusual β-amino acids meant to create the π-acid and π-base alternate form [26]. The β-amino acids studied were saturated or unsaturated alicyclic β-3-homo-amino acids and bicyclic amino acids. The results were unexpected as the CB-DMP resolved many of the amino acids in the reversed-phase mode while the best results for the CB-DNP were in the polar organic mode. In addition, the CB-DNP was able to resolve all the diastereoisomers (two chiral centers, four peaks) which has not been possible with any other CSP. It appears that the added dimension of steric bulk in the polar ionic mode for the π-electron-deficient CB-DNP increased stereoselectivity. The difference between the ether linkage of the DNP as opposed to the carbamate linkage of the DMP made this possible.

A further evaluation of the CB-DNP was reported by Gahm of Amgen using supercritical fluid chromatography (SFC) [27]. He concluded that inclusion complexation was the primary factor in retention and enantioselectivity. Better enantioseparation, as well as peak shape together with the reversal of elution order, makes CD-based CSPs unique and complimentary to the more commonly used π-basic amylose and cellulose columns.

The CB-DMP has the ability to operate in a typical normal phase solvent but the majority of mobile phases found successful in the literature for this phase have been the polar organic mode followed closely by the reversed-phase mode. The same has been true for the CB-DNP. The number of successful enantioseparation in a typical normal phase conditions has been reported but limited. It appears again that the utility of steric hindrance places a very substantial and successful role for these new derivatives.

5 Additional Application Studies

A number of papers have been published evaluating both the native and all the derivatized bonded cyclodextrins. A publication on the separation of 12 chiral bicyclic or tricyclic β-lactams was typical of the outcomes [28]. Both the CB-DMP and CB-DM resolved these structures in the reversed-phase mode in either ACN/H_2O or MeOH/H_2O. The amount of organic modifier was typically very low, 1% or less. Since this chapter preceded the introduction of the CB-DNP and the fact these structures appear to be resolved based on size, the DNP would have been a good choice. See Fig. 11 for a summary of the performance of the different CD-based CSPs for this type of β-lactam separation. In a subsequent publication, the CD-based CSPs were evaluated against the macrocyclic glycopeptides for the separation of these same β-lactams as well as additional cyclic β-amino acids [29]. The CB-DMP held up well in this study, again in the reversed-phase mode indicating the mechanism of inclusion complexation and steric bulk, plays a very large role in chiral discrimination with these phases.

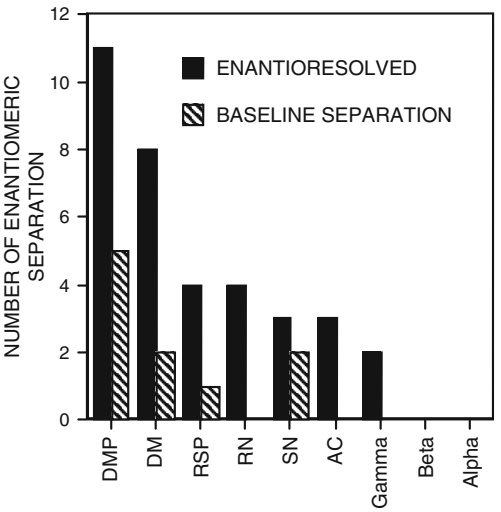


Fig. 11 Separation of 12 chiral β-lactams on CD-CSPs. The cyclodextrin derivative codes are DMP: 3,5-dimethylphenyl carbamate; DM: 2,3-di-O-methyl; RSP: racemic hydroxypropyl; RN: *R*-naphthylethyl carbamete; SN: *S*-naphthylethyl carbamate; AC: acetylated. Ref. [28], p. 1854

5.1 Chiral Liquid Chromatography-Tandem Mass Spectrometric Methods

Mass spectrometric methods have been the life blood of drug discovery and the development process. A number of publications have cited the use of current CD-CSPs for the characterization of drug metabolism and pharmacokinetic profiles of

stereoisomers. Both modes of operation, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), have been used for either the polar organic mode or the reversed-phase mode [30].

5.2 Enantiomeric Impurities in Chiral, Synthons, Catalysts, and Auxiliaries

Chiral synthons, catalysts, and auxiliaries are used in chiral synthesis. Their enantiomeric purity must be very high and known. This knowledge is critical since a 98% pure catalyst will intrinsically synthesize 2% of the wrong enantiomer. Similarly, if an auxiliary reagent is only 98% pure, 2% of the wrong or undesired enantiomer will be produced lowering the enantiomeric purity of the final product. The control of impurities in chiral reagents started in 1998. Two articles were produced in 1998 [31] and 1999 [32]. In the third phase of this continuing study, 84 chiral compounds used in chiral synthesis that were not assayed in the previous studies were evaluated by a variety of chromatographic techniques [33]. Most of the assays for these smaller compounds could easily be developed by chiral GC due to the low volatility of these small compounds with their polar groups derivatized with nonchiral reagents. Few compounds had to rely on HPLC and the two chiral phases most successful for these analyses were the acetylated and dimethylated derivatized CD-CSP referred as CB-AC and the CB-DM, both used in the reversed-phase mode [33].

6 Important Operating Conditions for Cyclodextrin Phases

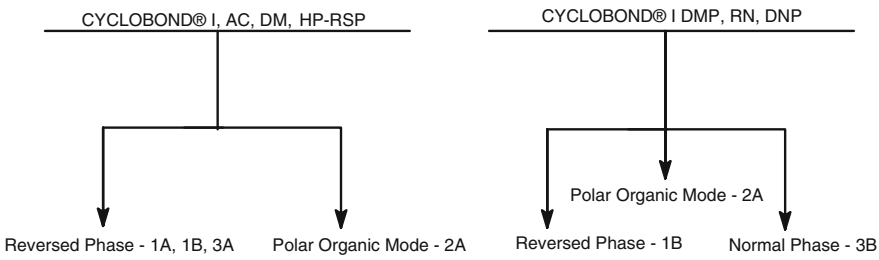
The flow chart shown in Fig. 12 presents a potential method development scheme for these cyclodextrin CSPs. It has been developed over the years from a screening of many applications and is believed to be the best statistical approach to the successful use of these phases. The following outline addresses the key issues in developing and optimizing a separation:

A. Polar organic mode requires:

   – at least two functional groups, one on or near the stereogenic center.

B. Reversed phase requires:

   – an includable group; aromatic or fused ring structure to be present.
   – a hydrogen bonding group outside the inclusion group that is within three carbons.
   – an analyte with suitable solubility.



Starting Mobile Phase Compositions:

1A	Reversed phase 30/70; ACN/20mM NH_4OAc, pH 6.0
1B	Reversed phase 30/70; ACN/20mM NH_4OAc, pH 4.0
2A	Polar organic mode 95/5/0.3/0.2;ACN/MeOH/HOAc/TEA
3A	Reversed phase 20/80; MeOH/20mM NH_4OAc, pH 4.0
3B	Normal phase 30/70; EtOH/Heptane
4	Rinse EtOH

Fig. 12 Cyclobond® method development screen

C. Normal phase requires:

- a π-acidic group to anchor to the π-base CSP or the converse.
- at least one hydrogen bonding group on or near the anchor site.
- adequate analyte solubility.

Details to this outline can be found in the 7th edition of the *Cyclobond* handbook from Supelco, Bellefonte, PA [34]. Unlike conventional chromatography, the inclusion phenomena create some unusual reactions to certain operating conditions, especially temperature and flow rate. This unusual behavior is most pronounced in the reversed-phase mode and to a lesser extent in the polar organic mode. The following table is a summary of the controlling parameters for each three mobile phase type:

Polar organic mode	Reversed-phase mode	Normal phase mode
Proper choice CD	Proper choice CD	Mobile phase composition
Mobile phase composition	pH	Temperature
Flow rate	Buffer	
Temperature	Mobile phase composition	
	Flow rate	
	Temperature	

6.1 Temperature

In the reversed-phase mode temperature has played a significant role and should be evaluated. It has been observed that reduced temperature enhances chiral resolution in this particular mode with only a moderate effect on retention. These temperature effects have less of an impact in the polar organic mode. The degree to which temperature affects resolution is analyte dependent. In order to evaluate temperature effect, determine alpha at 5, 15, and 25°C. A plot of alpha versus 1/*T* will be helpful in optimizing a chiral separation. Lower temperatures have the effect of reducing heats of friction within the column especially for the mobile phases with higher surface tension as reversed phase and intensifying the strength of weak binding forces usually associated with chiral interactions. In reversed mobile phase compositions of 40–60% aqueous methanol, the high viscosity negates any beneficial effect from a lower temperature. For low temperature studies use acetonitrile as the organic modifier. In addition, higher temperatures can be used to reduce tailing and improve peak efficiency if sufficient selectivity is available. These phases are temperature stable and have no operational upper limit but a maximum of 40°C seems to handle most cases undoubtedly due to the weaker chiral interactions.

6.2 Flow Rate

In the RP mode this is a most valuable operating parameter. The phenomena again relates to the relatively slow formation of the inclusion complex. Molecular modeling indicates that a molecule entering the cavity crosses an energy barrier before it locks into the cavity. This energy barrier is intensified from an increase in heats of friction generated at higher flow rates and the resulting turbulence. Lower flow rates can result in a dramatic increase in resolution by several factors. The effect is less dramatic with the polar organic mode where inclusion complexation is a reduced influence and does not manifest itself at all with normal phase conditions. Higher flow rates, under normal phase conditions, can have the effect of reducing tailing, lowering retention, and thereby improve resolution.

7 Trouble Shooting Chiral Separations

Test performance: To avoid difficulties early in the development of a chiral separation it is best to run the column test parameters before beginning work on a project. For cyclodextrin phases each of the phases has its own chiral standard for evaluation. See the manufacturer for details. Mobile phases are typically prepared based on volume ratios. The addition of salts is based on weight per volume. For salts compensation has to be made for water content. All salts, acids, and bases are reagent grade. Special care has to be given for the use of bases like triethylamine. Small sealed vials stored in the refrigerator are best. Air causes color oxidation and ghost

peaks on a chromatogram. To prepare triethylamine acetate, an aqueous solution of triethylamine is made first and the pH adjusted with acetic acid.

Equilibration: A column is typically equilibrated with 20 column volumes of mobile phase at 1.0 mL/min. The void volume of a 250 × 4.6 mm column is considered ~3 mL meaning that an hour is a good equilibration time. Never store CSPs in contact with buffer present no matter the pH. Always wash the CSP with water and then with an anhydrous organic solvent like methanol, ethanol, or acetonitrile. In sequencing mobile phases, especially on the multimodal phases, proceed from the storage solvent to the polar organic mode and then to the RP mode. Follow the RP mode with water, then anhydrous ethanol (stabilized with IPA), and then proceed to heptane/ethanol NP mobile phase. Acetonitrile can then be used to remove the heptanes and the column can be stored or displaced with methanol to start the process over.

Sample solvents/concentrations: It is best to dissolve the sample in mobile phase until it can be determined if methanol or acetonitrile could be used as the universal solvent. There are a limited number of cases when either methanol or acetonitrile used to dissolve the sample may distort peak shape even if the mobile phase contains these components. Inject minimum amount of sample until you can determine the effect of load. Some samples are load sensitive. Typically 1–5 μL of a 5 mg/mL sample dissolved in mobile phase is a good starting point.

Noisy or drifting baselines: These phenomena are the result of a number of operational details including lack of UV transparency of the mobile phase, a weak (aging) UV lamp, and contaminated organic solvent or additive. Column contamination can be eliminated by washing the CSP at 0.5 mL/min with 50/50 v/v; ACN/50 mM ammonium acetate buffer followed by methanol. When switching mobile phases make sure you have removed all mobile phase components from the tubing, detector cell, and injector. This is especially true when going from reversed-phase conditions to normal phase.

If a separation from the literature cannot be matched, the first step is to make sure the column you are using matches the manufacturers test chromatogram used to approve the column. It is often found that a column dries out and the efficiency of the bed is lost and not recoverable. The manufacturers' chromatogram library often relies on up–to-date performance as new techniques, like the polar organic mode or a new phase is developed. It pays to review the manufacturers' library or contact their technical service group when beginning a new project.

8 Conclusions

The structure of cyclodextrins and its derivatives offer unique opportunities for chiral as well as achiral selectivity both in the aqueous reversed-phase liquid chromatography mode and the non-aqueous polar organic mode. The uniqueness and sensitivity of inclusion complex formation combined with a hydrogen bonding mechanism with the sterically fixed chiral hydroxyl groups of the CD offers

opportunities for selectivity not found in other chiral stationary phases. The stereoselectivity of the native bonded cyclodextrin and certain derivatives was enhanced in the polar organic mode while the reversed-phase mode dominated the reported applications on all other derivatives. The preparation of the bonded dimethyl β-cyclodextrin that effectively blocked the formation of a stereoselective complex, typically formed through hydrogen bonding, demonstrated clearly the powerful influence of steric hindrance in the chiral separation process. It is apparent from the published studies that the contribution of steric hindrance to the selectivity mechanism has been grossly underestimated in evaluating these structures and especially the contribution of some of the cyclodextrin derivatives like the methylated and hydroxypropylated versions. The utility of the π-basic and π-acidic derivatives to produce a multimodal phase with broad stereoselectivity capabilities in a variety of mobile phases including reversed phase, normal phase, and the polar organic mode has been outlined including the influence of the chemistry of attachment especially on steric hindrance with these more complicated derivatives.

References

1. Lantz AW, Wetterer SM, Armstrong DW (2005) Use of the three-phase model and headspace analysis for the facile determination of all partition/association constants for highly volatile solute-cyclodextrin-water systems. Anal Bioanal Chem 383:160–166
2. Mitchell CR, Armstrong DW (2004) Cyclodextrin-based chiral stationary phases for liquid chromatography methods in molecular biology. Chiral Separations: Methods Protocols 243:61–112
3. Simms PJ, Jeffries CT, Zhao X, Huang Y, Arrhenius T (2004) Gradient elution of organic acids on a β-cyclodextrin column in the polar organic mode and its application to drug discovery. J Chromatogr A 1052:69–75
4. Ilisz I, Sapi J, Tourwe D, Armstrong DW, Peter A (2006) LC enantioseparation of tryptophan analogs on α- cyclodextrin stationary phase. Chromatographia 63(13):S23–S27
5. Remsburg JW, Armstrong DW, Peter A, Toth G (2008) LC enantiomeric separation of unusual amino acids using cyclodextrin-based stationary phases. J Liq Chromatogr Relat Technol 31:219–230
6. Liu Y, Urgaonkar S, Verkade JG, Armstrong DW (2005) Separation and characterization of underivatized oligosaccharides using LC and LC-ESI-MS. J Chromatogr A 1079: 146–152
7. Berthod A, Berthod L, Armstrong DW (2005) Selectivity of a native β-cyclodextrin column in the separation of catechins. J Liq Chromatogr Relat Technol 28:1669–1678
8. Hinze WL, Riehl TE, Armstrong DW, DeMond W, Alak A, Ward T (1985) Liquid chromatographic separation of enantiomers using a chiral β-cyclodextrin-bonded stationary phase and conventional aqueous-organic mobile phases. Anal Chem 57:237–242
9. Wang C, Jiang C, Armstrong DW (2008) Considerations on HILIC and polar organic solvent-based separations: use of cyclodextrin and macrocyclic glycopeptides stationary phases. J Sep Sci 31:1980–1990
10. Zhou J, Yang Y, Wei F, Wu P (2007) Comparison of the performance of chiral stationary phase for separation of fluoxetine enantiomers. J Zhejiang Univ Sci B 8:56–59
11. Han X, Huang Q, Ding J, Larock RC, Armstrong DW (2005) Enantiomeric separation of fused polycycles by HPLC with cyclodextrin and macrocyclic glycopeptides chiral stationary phases. Sep Sci Tech 40:2745–2759

12. Han X, Yao T, Liu Y, Larock RC, Armstrong DW (2005) Separation of chiral furan derivatives by liquid chromatography using cyclodexrtrin-based chiral stationary phases. J Chromatogr A 1063:111–120
13. Schumacher DD, Mitchell CR, Rozhkev RV, Laroch RC, Armstrong DW (2005) Cyclodextrin-mediated enantiomeric separation of chiral dihydrofuroflavones, a class of compounds with promising pharmacological activity. J Liq Chromatogr Relat Technol 28: 169–186
14. Soukup RJ, Rozhkov RV, Larock RC, Armstrong DW (2005) The use of cyclodextrin-based LC stationary phases for the separation of chiral dihydrobenzofuran derivatives. Chromatographia 61:219–224
15. He H, Sun C, Wang XR, Pham-Huy C, Chikhi-Chorfi N, Galons H, Thevenin M, Claude JR, Warnet JM (2005) Solid-Phase extraction of methadone enantiomers and benzodiazepines in biological fluids by two polymeric cartridges for liquid chromatographic analysis. J Chromatogr B 814:385–391
16. Han X, Zhong Q, Yue D, Della Ca N, Larock RC, Armstrong DW (2005) Separation of enantiomers of isochromene derivatives by HPLC using cyclodextrin based stationary phases. Chromatographia 61:205–211
17. Donovan JL, Crespy V, Oliveira M, Cooper KA, Gibson BB, Williamson G (2006) (+)-Catechin is more bioavailable than (-)-Catechin: relevance to the bioavailability of catechin from cocoa. Free Radic Res 40:1029–1034
18. Lipka E, Bonte JP, Vaccher C (2006) Direct enantiomeric separation and determination of enantiomeric purity of methoxytetrahydro-naphthylene derivatives and melatonin ligands by HPLC using RSP-β-cyclodextrin as chiral stationary phase. J Liq Chromatogr Relat Technol 29:1235–1245
19. Sun P, Krishnan A, Yadav A, Singh S, MacDonnell FM, Armstrong DW (2007) Enantiomeric separations of ruthenium (II) polypridyl complexes using high-performance liquid chromatography with cyclodextrin chiral stationary phases. Inorg Chem 46:10312–10320
20. Warnke MM, Mitchell CR, Rozhkov RV, Emrich DE, Laroch RC, Armstrong DW (2005) Use of native and derivatized cyclodextrin based and macrocyclic glycopeptides based chiral stationary phases for the enantioseparation of pterocarpans by HPLC. J Liq Chromatogr Relat Technol 28:823–834
21. Berkecz R, Ilisz I, Ivanov-Sztojkov A, Szatmari I, Fulop F, Armstrong DW, Peter A (2007) HPLC enantioseparation of 1-(α-aminobenzyl)-2-naphthol and 2-(α-aminobenzyl)-1-naphthol analogs on a β-cyclodextrin-based chiral stationary phase. Chromatographia 65:337–341
22. Chen S, Ward T (2004) Comparison of the chiral separation of amino acid derivatives by a teicoplanin and RN-β-CD CSPs using waterless mobile phases: factors that enhance resolution. Chirality 16:318–330
23. Torok R, Bor A, Orosz G, Lukacs F, Armstrong DW, Peter A (2005) High-performance liquid chromatographic enantioseparation of bicalutamide and its related compounds. J Chromatogr A 1098:75–81
24. Zhong Q, He L, Beesley TE, Trahanovsky WS, Sun P, Wang C, Armstrong DW (2006) Development of dinitrophenylated cyclodextrin derivatives for enhanced enantiomeric separations by high performance liquid chromatography. J Chromatogr A 1115:19–45
25. Zhong Q, He L, Beesley TE, Trahanovsky WS, Sun P, Wang C, Armstrong DW (2006) Optimization of the synthesis of 2,6-dinitro-4-trifluoromethylphenyl ether substituted cyclodextrin bonded chiral stationary phases. Chromatographia 64:147–152
26. Ilisz I, Berkecz R, Forro E, Fulop F, Armstrong DW, Peter A (2009) The role of π-acidic and π-basic chiral stationary phases in the high performance liquid chromatographic enantioseparation of unusual β-amino acids. Chirality 21:339–348
27. Gahm KH (2006) Evaluation of the new chiral stationary phase on π-acidic 2,6-dinitro-3-trifluoromethylphenyl β-cyclodextrins by supercritical fluid chromatography. Poster 78 at the 18th International Conference on Chirality, ISCD-18, Busan, S Korea, June 24–28

28. Sun P, Wang C, Armstrong DW (2006) Separation of Enantiomers of β-lactams by HPLC using cyclodextrin-based chiral stationary phases. J Liq Chromatogr Relat Technol 29(13–16):1847–1860
29. Berkecz R, Torok R, Ilisz I, Forro E, Fulop F, Armstrong DW, Peter A (2006) LC enantioseparation of β-lactam and β-amino acids stereoisomers and a comparison of macrocyclic glycopeptides and β-cyclodextrin based columns. Chromatographia 63:S37–S43
30. Chen J, Korfmacher WA, Hsieh Y (2005) Chiral liquid chromatography-tandem mass spectrometric methods for stereoisomeric pharmaceutical determinations. J Chromatogr B 820:1–8
31. Armstrong DW, Lee JT, Chang LW (1998) Enantiomeric impurities in chiral catalysts, auxiliaries and synthons used in enantioselective synthesis. Tetrahedron Asymmetry 9:2043–2064
32. Armstrong DW, He LF, Yu T, Lee JT, Liu YS (1999) Enantiomeric impurities in chiral catalysts, auxiliaries, synthons and resolving agents. Part 2. Tetrahedron Asymmetry 10:37–60
33. Huang K, Breitbach ZS, Armstrong DW (2006) Enantiomeric impurities in chiral synthons, catalyst and auxiliaries. Part 3. Tetrahedron Asymmetry 17:2821–2832
34. http://wwwsigmaaldrichcom/analytical-chromatography/hplc/columns/chiral/cyclobondhtml consulted on June 2009

Cyclofructans, a New Class of Chiral Stationary Phases

Chunlei Wang, Ping Sun, and Daniel W. Armstrong

Contents

Abstract Cyclofructans are cyclic oligosaccharides consisting of β-2,1 linked D-fructofuranose units. Although known for more than 20 years, they were only recently proposed as powerful chiral selectors after alkylation or aryl derivatization. The mechanism of chiral recognition of derived cyclofructan is investigated. A structural comparison of the 18-crown-6 ether and the cyclofructans with six fructofuranose units (CF6) and the derivatized CF6 is made taking into account the similarities between the internal ether macrocycles. Clear differences are seen that offer possible explanations for the very different enantioselectivities of the three selectors. The structural modification brought by the alkyl derivation of CF6 allows for a remarkable enantiorecognition of all native chiral amines. Aryl-derivatized CF6 are less selective for amines but have a broad enantiorecognition for other classes of enantiomers. This new type of chiral selector has a high loading potential, allowing it to purify large amounts of materials.

D.W. Armstrong (✉)
Department of Chemistry and Biochemistry, the University of Texas at Arlington, Arlington, TX 76019, USA
e-mail: sec4dwa@uta.edu

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_4,

1 Introduction

Cyclofructans are cyclic oligosaccharides consisting of β-2,1 linked D-fructofuranose units (Fig. 1). As first reported by Karvamura and Uchiyama in 1989, they were produced by fermentation of inulin using an extracellular enzyme from a strain of *Bacillus circulans* OKUMZ31B [1]. In 1994, Kushibe et al. reported a different strain of *B. circulans* (MCI-2554), which enabled more efficient cyclofructan production [2]. Cyclofructans with degrees of polymerization from six to eight have been produced, and they are commonly abbreviated as CF6, CF7, and CF8, respectively. CF6 can be crystallized from aqueous methanol solutions [3, 4] and is available with high purity. Analytical separation methods for cyclofructans of different degrees of polymerization have been reported [5], and high purity CF7 (>99%) can be produced via preparative separations using methods slightly modified from the reported analytical approaches.

There are two general types of applications of cyclofructans. First, they have been used as bulk additives in various industrial formulations, in much the same manner as cyclodextrins, another type of cyclic oligosaccharides, have. For example, cyclofructans have been be used as the coating material for inkjet recording media [6, 7] and silver halide photographic materials [8–11], as food additives [12–16], and as excipients in pharmaceutical applications [17–21]. Second, cyclofructans have

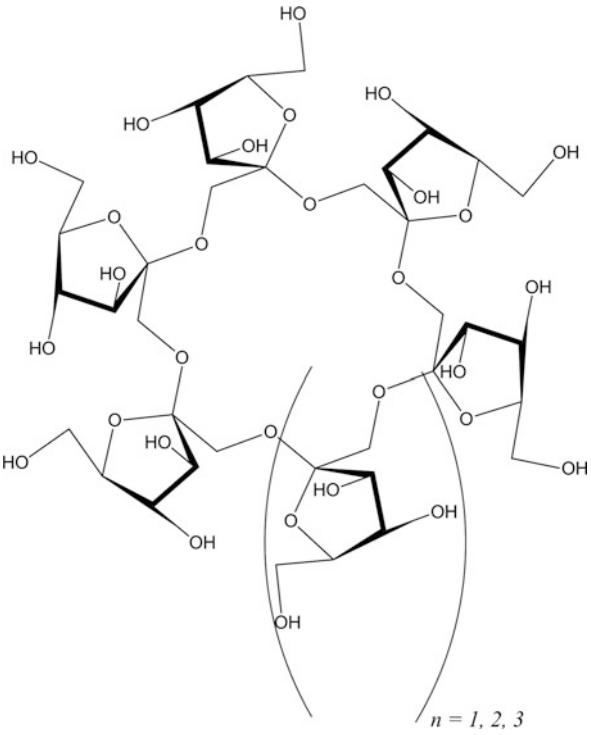


Fig. 1 Molecular structure of cyclofructans. $n = 1$, CF6; $n = 2$, CF7; $n = 3$, CF8

been used as ion trapping reagents due to their ability to form complexes with many metal cations [22, 23].

The first study on chiral recognition of cyclofructans was reported by Sawada et al. in 1998 [24, 25]. They found that permethylated CF6 and CF7 were able to discriminate between enantiomers of several amino acid esters in the gas phase using a direct FAB mass spectrometric approach. The highest enantioselectivity factor, 1.38, was observed for D,L-tryptophan isopropyl esters using permethylated CF6. There was no follow-up on this topic nor were there any other reports on any type of "chiral applications" of cyclofructans during the following 11 years. It was not until very recently that we employed native and/or derivatized cyclofructans as chiral selectors for the capillary electrophoresis (CE), gas chromatography (GC), high performance liquid chromatography (HPLC), and supercritical fluid chromatography (SFC) separations of enantiomers. In this chapter, we consider the enantiomeric separations obtained using cyclofructan-based selectors by all of the aforementioned approaches and then examine the possible chiral recognition mechanisms for this new class of chiral selectors with the help of computational tools.

2 Structural Characteristics of Native Cyclofructan 6

Cyclofructans are isomers of cyclodextrins of the same degree of polymerization. However, their structures are substantially different. Cyclofructans do not have hydrophobic cavities. Cyclodextrins are linked via α-1,4 glycosidic bonds, and α-cyclodextrin (α-CD) is made of six glucose units (six atom ring). The hydrophobic macrocyclic CD ring is made by 30 atoms (24 carbon and 6 oxygen atoms). In contrast, cyclofructans are composed of fructose units (five atom ring) linked via β-2,1 glycosidic bonds. Cyclofructan 6 (CF6) is made of six fructose units, being linked together by a crown ether-like ring with 18 atoms (12 carbon and 6 oxygen atoms) in the central macrocycle (Fig. 1) [3, 4]. The distance between opposing oxygens on the macrocyclic ring is 6.1 Å for CF6 as compared to 8.5 Å for α-CD [26]. In addition, the six glucose units in α-CD are uniformly tilted toward the molecular center, whereas the six fructofuranose units in CF6 are oriented alternatively toward ("inward" inclination) and away from ("outward" inclination) the molecular center to minimize steric repulsion on the macrocyclic rim (Fig. 2) [27]. The alternating inward/outward inclination of the fructofuranose units are more like chiral propellers attached to the CF6 central macrocycle.

The CF6 central macrocycle is a natural 18-crown-6. However, the conformation of macrocycle is different from that of synthetic crown ethers. When crown ethers are complexed with potassium ion, the six -O-C-C-O- units adopt a gggggg (g stands for gauche) conformation (Fig. 3a) [28], and are evenly distributed above and below the crown ether mean plane. In CF6, the six oxygen atoms are all aligned toward one side of the macrocycle due to the gtgtgt (g stands for gauche; t for trans) alternating configuration of the six -O-C-C-O- units in the center 18-crown-6 core as shown in Fig. 3b [4]. In addition, all 3-OH and 4-OH groups of the fructose units also

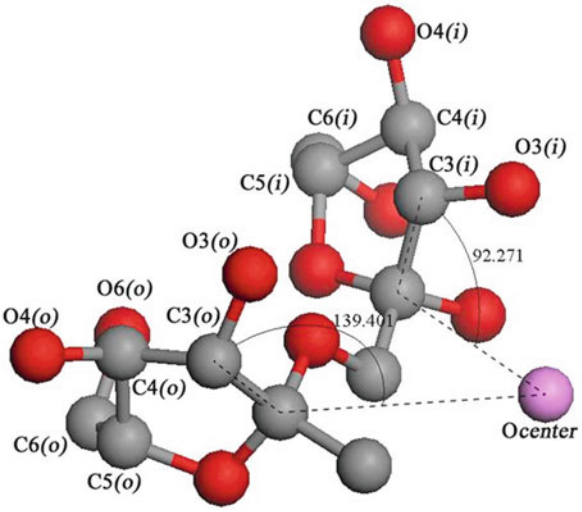


Fig. 2 An example of inward- and outward-inclined fructofuranose units on two successive units of the crown ether rim of CF6. The angle O_{center}-C_2-C_3 is 92.271° and 139.401° for inward- and outward-inclined fructofuranose units, respectively. O_{center} is the average position of six oxygen atoms on the crown ether internal rim and is colored *pink*. Only two fructofuranose units and part of the crown ether rim are shown for clarity reason. Carbon atoms are colored *gray*, oxygen atoms *red*, and O_{center} is *pink*. The suffix "(i)" and "(o)" are included in atom labels to indicate that the atom is in the "inward"-inclined and "outward"-inclined fructofuranose units, respectively

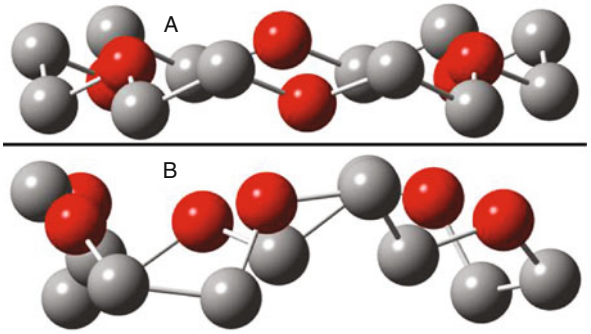


Fig. 3 Comparison of the internal ether macrocycle in CF6 with that in 18-crown-6: (**a**) the oxygen atoms are in the **gggggg** conformation in 18-crown-6 and K^+ complexes. (**b**) The oxygen atoms are alternatively in the **gtgtgt** conformation in the macrocycle in CF6. Color coding: carbon, *gray*; oxygen, *red*. Hydrogen atoms are left out for clarity purpose

are aligned on the same side of the macrocycle, making this side "pronouncedly hydrophilic" [27]. The other side of the macrocycle is "distinctly hydrophobic" due to occupation of the ethylene groups in the 18-crown-6 core, the 6-methylene groups, and the ether O5-C5-H5 fragments [27].

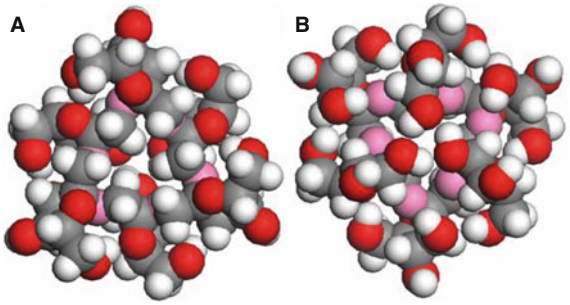


Fig. 4 Space filling model of CF6: (**a**) view from the hydrophobic side; (**b**) view from the hydrophilic side. Color coding: carbon, *gray*; oxygen, *red*; oxygen on the crown ether internal ether ring, *pink*; hydrogen, *white*

Figure 4a shows the space filling model viewed from the hydrophobic side of CF6. It is clear that the oxygen atoms are all hidden by the ethylene groups in the 18-crown-6 core, and the inner diameter of the macrocycle is around 1.5 Å (as compared to 2.6 Å in 18-crown-6 [29]). Consequently, any solute interaction with the six oxygen atoms on the 18-crown-6 moiety from the hydrophobic side of CF6 is not likely. On the hydrophilic side, 3-OH groups from the three inward-inclined fructofuranose units (the O3*(i)* groups in Fig. 2) are hydrogen bonding with each other and so, effectively blocking the access to the macrocycle cavity from this side (Fig. 4b). Consequently, the 18-crown-6 core of the native CF6 in cavity is effectively folded inside the molecule and is relatively inaccessible from both sides.

Overall, CF6 has distinctively different hydrophilic and hydrophobic surfaces on the two sides of the central macrocycle. It also has propeller-oriented fructofuranose units along the rim of the macrocycle. These loci are three possible docking sites for molecular recognition by CF6 units.

3 Chiral Recognition by Cyclofructans

3.1 Chiral Recognition by Native Cyclofructan 6 (CF6)

The native CF6 chiral stationary phase produced chiral separations of a few primary amines and binaphthyl-type molecules using organic solvents as mobile phases (as shown in Fig. 5). The fact that no enantiomeric separations were observed in the reversed-phase mode indicates that effective chiral recognition at the hydrophobic surface of CF6 is not prevalent. Previous NMR studies of metal complexation of native and permethylated cyclofructans also reveal that the 3-OH and 4-OH groups are responsible for cyclofructan–metal cation interactions [30–32].

In the X-ray structure of permethylated CF6 with Ba^{2+}, the Ba^{2+} is positioned on the molecular rotational axis on the hydrophilic side of the molecule [32]. Protonated primary amines could approach CF6 in a similar way. In fact, the three O3*(i)* atoms form a triangular plane, which is similar to the three alternating

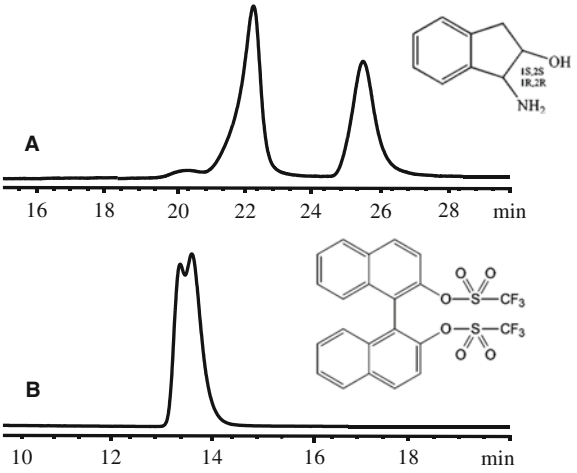


Fig. 5 Two out of a few enantiomeric separations observed on a native CF6 CSP (column 25 cm, 4.6 mm i.d.): (**a**) primary amine-type compound; (**b**) binaphthyl-type compound. Mobile phases: (**a**) heptane/ethanol/trifluoroacetic acid 70/30/0.1 (% v/v); (**b**) heptane 100%. Flow rate 1 mL/min, UV detection 254 nm

oxygen atoms in 18-crown-6. Primary amines are likely to form tripodal hydrogen bonding with these O3*(i)* atoms as they do with 18-crown-6. However, these three oxygen atoms are much closer together (2.8 Å as compared to 4.9 Å in 18-crown-6; see Fig. 6a and b) and they are hydrogen bonding with one another. As a result, the tripodal hydrogen bonding with three O3*(i)* atoms in CF6 is much weaker than that with 18-crown-6. As will be discussed in the following sections, partial derivatization of native CF6 very likely disrupts the CF6 internal hydrogen bonding thereby causing the remaining free hydroxyl groups to reorient to different geometries. In addition, the hydroxyl groups in CF6 can also act as hydrogen bonding donors, and enantiomeric separation of secondary and tertiary amines are common on cyclofructan-based CSPs, whereas crown ether-based CSPs separate almost exclusively primary amines. Tripodal hydrogen bonding alone is not sufficient for chiral recognition. After the docking of amines by the tripodal hydrogen bonding with 3-OH groups, surrounding chiral interaction sites and/or simply chiral barriers (i.e., C3-C4-O4 fragments on fructofuranose units), would provide enantioselective interactions for chiral amines.

There is a total of 18 hydroxyl groups in CF6, two-thirds of which are attached to chiral centers and are able to provide different hydrogen bonding with chiral analytes. In addition, some of these oxygen atoms adopt a similar geometry to 18-crown-6. As shown in Fig. 6c and d, the triad formed by O4*(i)*-O3′*(o)*-O3″*(i)* and O3*(o)*-O6*(o)*-O4′*(i)* are more similar to 18-crown-6 than the three O3*(i)* atoms are in the perspective of O-O distances among each other. Density functional theory calculations at the 6–31 g level were employed to validate the binding of potassium cations at these sites. Surprisingly, these two triangular planes provide stronger K^+ binding than the O3*(i)* triangular plan does (refer to Fig. 6 caption). When a potassium cation approaches these two sites (Fig. 6c and d), it is also able to have electrostatic–dipole interaction with one oxygen atom on the crown ether skeleton of CF6. Despite the energetically favored binding at these two sites (Fig. 6c and d), there are not many chiral interaction sites/barriers above these triangular planes

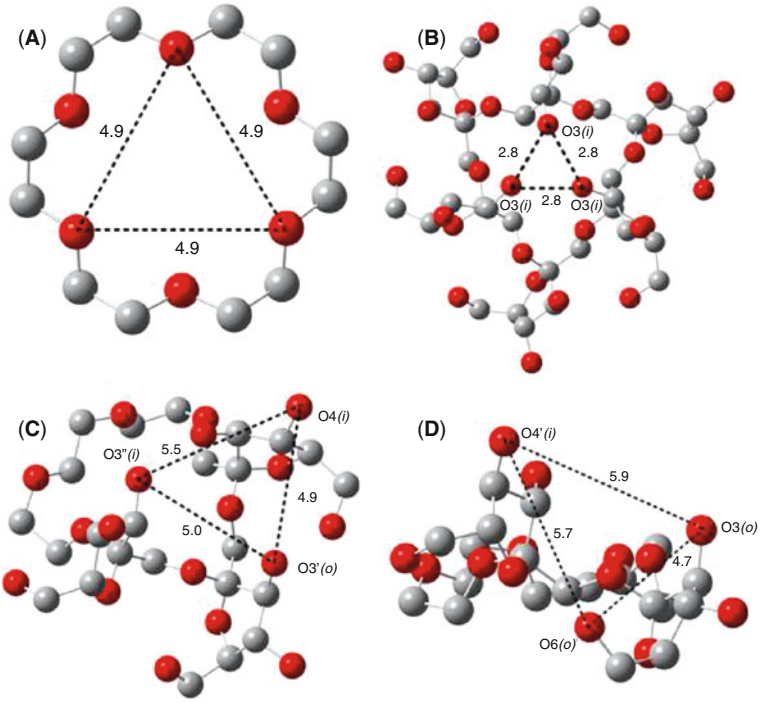


Fig. 6 Possible tripodal hydrogen bonding sites for ammonium cations: (**a**) alternating oxygen atoms in 18-crown-6 (internal ether ring); (**b**) three alternating O3 on inward-inclined fructofuranose units (O3*(i)* atoms); (**c**) O4*(i)*, O3′*(o)* and O3″*(i)* on three neighboring fructofuranose units; (**d**) O3*(o)*, O6*(o)* and O4′*(i)* on two neighboring fructofuranose units. The distance between those oxygen atoms are labeled on the figures in Å (0.1 nm). The binding energy of potassium cation (having similar size as ammonium cation) at these sites (calculated at B3LYP/6–31 g level) are −364, −195, −261, and −210 kJ mol^{-1}, respectively. Color coding: carbon, *gray*; oxygen, *red*. Hydrogen atoms are left out for clarity purpose

in native CF6 to afford chiral recognition. Derivatization could provide extra interactions surrounding those triangular planes for chiral recognitions. One thing to note is that, the hydroxyl groups can also reorient themselves upon interacting with a guest molecule. This "induced-fit" by hydroxyl groups could possibly provide even more interaction sites between cyclofructans and amine compounds. The abundance of hydroxyl groups and suitable interaction sites may account for the high loading capacity that is observed for cyclofructan-based CSPs as will be discussed in Sect. 3.3.

3.2 Chiral Recognition by Derivatized Cyclofructan 6

Despite the limited success of native CF6 as a chiral selector in both HPLC and CE, certain derivatized CF6 s show great potential as chiral selectors. During the

discussion of structural properties of native CF6, several advantages of derivatization have already been mentioned: (1) derivatization could disrupt the hydrogen bonding between 3-OH groups (as shown in Fig. 6b) and increase their hydrogen bonding interactions with guest molecules; (2) derivatized groups could provide additional interaction sites surrounding effective amine binding sites, i.e., sites as shown in Fig. 6c and d. In addition, the C3 symmetry of native CF6 could be broken. Finally, addition of aromatic groups to CF6 can increase $\pi-\pi$ and steric interactions, which are important for many successful chiral selectors [33–38].

3.2.1 Aliphatic-Derivatized CF6

Cyclofructan 6 derivatized with only a few aliphatic groups showed exceptional enantioselectivity for primary amine-containing compounds [39]. Organic solvents are the best mobile phases for these types of separations. As discussed in Sect. 3.1, hydrogen bonding is considered to be the most important primary interaction for chiral recognition of amines. This type of interaction is known to be enhanced in solvents of low dielectric constants (acetonitrile, heptane, etc.). It is also observed experimentally that better enantioselectivity is observed in the normal phase mode (heptane/alcohol solvents) than in the polar organic mode (acetonitrile/alcohol solvents) (Fig. 7). The efficiency, however, is typically better in the polar organic mode as indicated by the sharp and symmetric peaks observed, as compared to usually broad and fronting peaks in the normal phase mode. Overall, faster separations and better enantiomeric resolution (due to enhanced efficiency) are obtained in the polar organic mode (Fig. 7). In contrast, crown ether-based CSPs operate almost exclusively in the reversed-phase mode using acidic aqueous solvents.

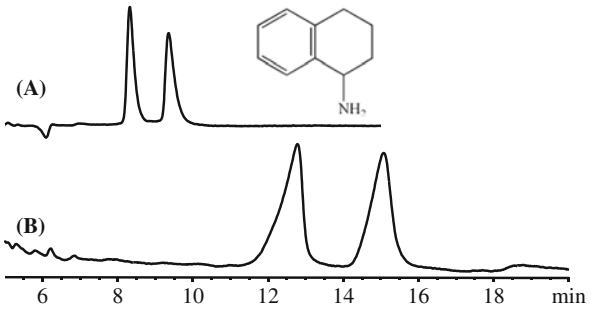


Fig. 7 The polar organic mode (**a**) and the normal phase (**b**) mode separations of the chiral primary amine tetrahydronaphthylamine on a methylated CF6 CSP (column 25 cm, 4.6 mm i.d.). Chromatographic parameters: (**a**) acetonitrile/methanol/acetic acid/triethylamine 60/40/0.3/0.2 (% v/v), $k_1 = 1.77$, $\alpha = 1.19$, $N = 8,800$, Rs $= 2.8$; (**b**) heptane/ethanol/trifluroacetic acid 70/30/0.1 (% v/v), $k_1 = 4.33$, $\alpha = 1.22$, $N = 3,100$, Rs $= 2.2$. Flow rate: 1 mL/min, UV detection 254 nm

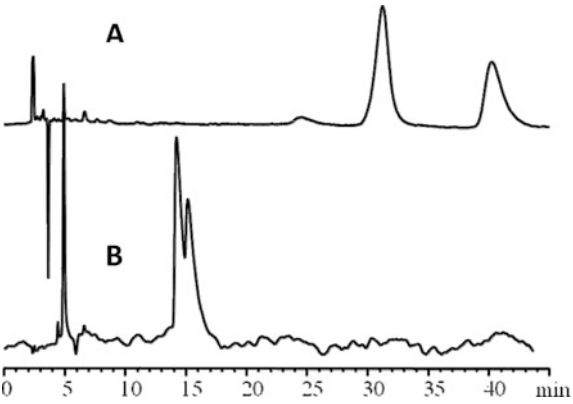


Fig. 8 Separation of *trans*-1-amino-2-indanol on a methyl carbamate derivatized CF6 CSP (column 25 cm, 4.6 mm i.d.) in a heptanes/ethanol 70/30% v/v mobile phase with different additives: (**a**) 0.1% trifluroacetic acid, 1 mL/min; (**b**) 0.1% triethylamine, 1 mL/min. UV detection 254 nm

Additives are often necessary to obtain an acceptable elution of chiral primary amines from aliphatic-derivatized CF6 in the normal phase mode. Figure 8 shows the separation of *trans*-1-amino-2-indanol with different additives in a heptane/ethanol 70/30 (v/v) mobile phase. Without additives, the analyte was strongly retained and did not elute in 2 h. When acidic additives were added to the mobile phase, peaks were eluted faster and enantiomeric separation was observed. The magnitude of the retention decrease is linked to the strength (p*K*a) of the acidic additive used. For example, the first peak was eluted at 91 min when using 0.1% acetic acid (chromatogram not shown) and at 31 min when using the same 0.1% amount of the stronger trifluoroacetic acid (Fig. 8a). Basic additives caused an even more dramatic decrease in retention times, yet amino-indanol enantioseparation was still observed when 0.1% triethylamine was used (Fig. 8b). The enantiomeric separation of a primary amine in its free form (when only basic additives are used) implies that the prevalent hydroxyl groups in CF6 could effectively act as hydrogen bond donors when interacting with free primary amines. In contrast, there are no hydrogen bonding donating groups on crown ether rings and strong acidic additives, i.e., sulfuric and perchloric acid, are required to protonate primary amines and to afford enantiomeric separation on synthetic crown ether-based CSPs.

A combination of acidic and basic additives is used for the separation of primary amines in the polar organic mode. Table 1 lists the chromatographic data obtained using different combinations of acidic and basic additives. The optimized additive composition is 0.3/0.2 (% v/v) acetic acid/triethylamine in pure methanol, which happens to be the recommended buffer for the polar organic mobile phase separation on cyclodextrin-based CSPs [40, 41].

Different aliphatic groups, i.e., methyl, ethyl, isopropyl, and tert-butyl groups, have been used to derivatize CF6. Slightly different separations are observed for these four aliphatic-derivatized CF6 CSPs due to the different bulkiness and geometry of the selected aliphatic groups. Overall, the isopropyl-derivatized CF6 produced the best enantiomeric separations for primary amines.

Table 1 The effect of acid and basic additives on the chromatographic separation of (±) *trans*-1-amino-2-indanol in the polar organic mode on an isopropyl carbamate derivatized CF6 25 cm × 4.6 column

No.	Additives (in volume percentages)	k_1	α	Rs
1	Acetic acid (0.30%)/trimethylamine (0.13%)	3.36	1.29	5.3
2	Acetic acid (0.30%)/ethanolamine (0.08%)	1.97	1.14	2.6
3	Acetic acid (0.30%)/butylamine (0.14%)	2.36	1.16	2.3
4	Acetic acid (0.30%)/diethylamine (0.14%)	3.67	1.29	1.6
5	Acetic acid (0.30%)/triethylamine (0.20%)	2.85	1.31	4.0
6	Acetic acid (0.20%)/triethylamine (0.30%)	2.69	1.24	3.9
7	Acetic acid (0.25%)/triethylamine (0.25%)	3.24	1.27	4.4

The mobile phase is composed of 60% acetonitrile/40% methanol (v/v). Different volume percentages of basic additives in entry 1–5 are chosen to afford same 14 mM molar concentration of basic additives.

3.2.2 Aromatic-Derivatized CF6

Derivatization of native chiral molecules with aromatic moieties is a common strategy used to enhance their chiral recognition abilities. Examples are oligo- and polysaccharide-based selectors that lack delocalized π-electrons, e.g., (*R* or *S*)-naphthylethyl derivatized cyclodextrins, 3,5-dimethylphenyl, along with many other different aromatic moieties, derivatized cellulose, and amylose. The optimal derivatization degree is usually different for different type of native selectors. A low degree of derivatization, i.e., 6, was found to be the best for β-cyclodextrins because these aromatic groups were able to effectively provide extra interactions while not completely blocking the cyclodextrin cavity or removing all free hydroxyl groups [36]. On the other hand, cellulose and amylose-based CSPs favor high degrees of derivatization and even complete derivatization of all hydroxyl groups [42].

Aromatic-derivatized CF6 was thus prepared in both low and high degrees of derivatization. Interestingly, low- and high-degree-derivatized CF6 showed dramatically different chiral recognition properties. When CF6 is derivatized with three to five aromatic groups, the general enantioselectivity for primary amines were also observed, quite analogous to that found on aliphatic-derivatized CF6. The CF6 aromatic moieties play essentially the same role as the aforementioned aliphatic moieties did to enhance chiral recognition for primary amine compounds. Figure 9a and b compare the separation of *trans*-1-amino-2-indanol on similarly substituted aliphatic- and aromatic-derivatized CF6 columns. Higher enantiomeric selectivity, efficiency, and better separations were always observed on aliphatic-derivatized CF6 columns for primary amines compared to aromatic counterparts. However, in addition to primary amines, other types of racemic compounds were starting to show enantioselectivity on the aromatic-derivatized CF6 CSPs (Fig. 9d and e).

When most of the accessible hydroxyl groups in CF6 are derivatized with aromatic groups, however, the general enantioselectivity for primary amines is almost completely lost (Fig. 9c), whereas enhanced separations of a great variety of other chiral compounds can be obtained (Fig. 9f). These observations are readily

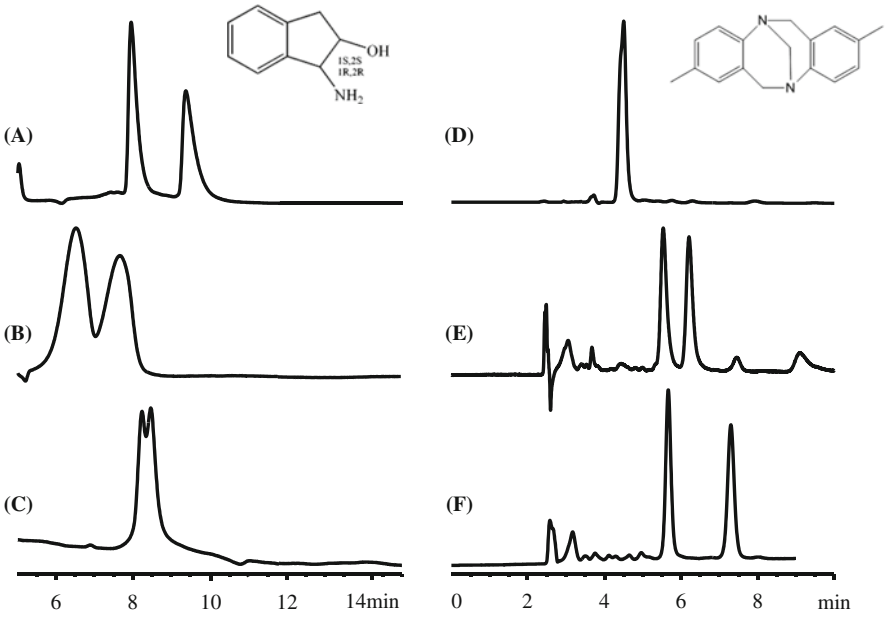


Fig. 9 Separation of *trans*-1-amino-2-indanol (*left*) and Troger's base (*right*) on CF6 CSPs (columns 25 cm, 4.6 mm i.d.) derivatized in three different ways: (**a**) and (**d**), low-degree methyl carbamate derivatization; (**b**) and (**e**), low-degree 3,5-dimethylphenyl carbamate derivatization; (**c**) and (**f**), high-degree 3,5-dimethylphenyl carbamate derivatization. Mobile phases: *left*, acetonitrile/methanol/acetic acid/triethylamine 60/40/0.3/0.2 (70/30/0.3/0.2% v/v for (**c**)); *right*, heptane/ethanol 70/30% v/v and 1 mL/min. UV detection 254 nm

explained by looking at the optimized (at HF/6–31 g level) structure of the all-*O*-3,5-dimethylphenyl carbamate derivatized CF6 as shown in Fig. 10. The bulky aromatic groups block the access to the tripodal hydrogen bonding sites (shown in Fig. 6), canceling, and negating the chiral recognition for primary amines. On

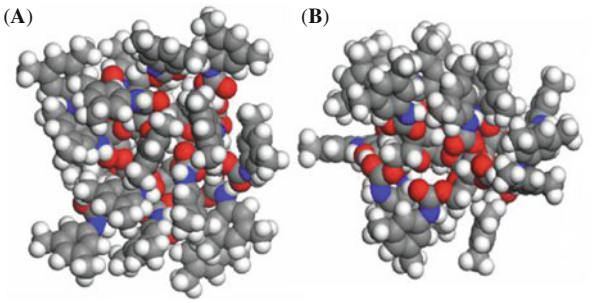


Fig. 10 Space filling model of all-*O*-3,5-dimethylphenyl carbamate CF6 calculated at HF/6–31 g level: (**a**) top view from the hydrophilic side of CF6; (**b**) side view. Color coding: carbon, *gray*; hydrogen, *white*; oxygen, *red*; and nitrogen, *blue*

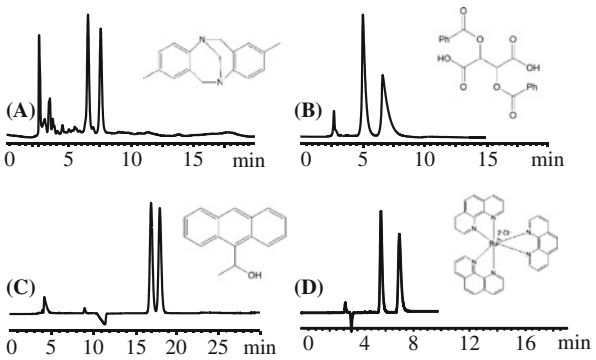


Fig. 11 Separation of compounds belonging to different classes on aromatic-derivatized CF6 CSPs (Columns 25 cm, 4.6 mm i.d.). Aromatic derivatization groups: (**a**) 3,5-dichlorophenyl carbamate; (**b**) 3,5-bis(trifluoromethyl)phenyl carbamate; (**c**), and (**d**) *R*-naphthylethyl carbamate. Mobile phases: (A) heptane/ethanol 80/20; (**b**) acetonitrile/methanol/acetic acid/triethylamine 75/25/0.3/0.2; (**c**) heptane/isopropanol/trifluoroacetic acid 98/2/0.1; (**d**) acetonitrile/methanol 40/60 with 25 mM ammonium nitrate. All mobile phases in % v/v and used at 1 mL/min flow rate. UV detection 254 nm

the other hand, the propeller side arm along the crown ether rim of CF6 was not only preserved but also extended in space to adopt a chiral helical geometry with polar oxygen and nitrogen moieties aligned in the helical grooves. Similar chiral helical groove motifs have been proposed to contribute to high enantioselectivity observed for highly aromatic-derivatized polysaccharide-type CSPs [38, 43]. These extended side arms are likely to play a similar role in chiral recognition for a variety of compounds in the highly aromatic-derivatized CF6.

Ten different aromatic derivatizations of CF6 were made. Examples of the enantiomeric separation of different classes of racemates are shown in Fig. 11. Most of the observed enantiomeric separations could be obtained on several different aromatic-derivatized CSPs [39]. In addition, the same elution order is generally obtained for chiral compounds on *R*- and *S*-naphthylethyl carbamate derivatized CSPs. These observations suggest that the enantiomeric selectivities of the aromatic-functionalized CF6 CSPs are more dependent on the chirality of the base CF6 than on the chirality of substituent groups. However, aromatic moieties play an indispensable role in chiral recognitions because the separations of non-primary amine compounds on aromatic-derivatized CF6 CSPs are generally better than those on aliphatic-derivatized ones.

3.2.3 Sulfate-Derivatized CF6 for CE

Capillary electrophoresis (CE) is usually carried out in aqueous solutions. However, the interactions (mainly hydrogen bonding) between native CF6 and amino compounds are greatly attenuated in aqueous solvents. Minimal interactions between native CF6 and amine compounds were observed in aqueous buffers used for CE.

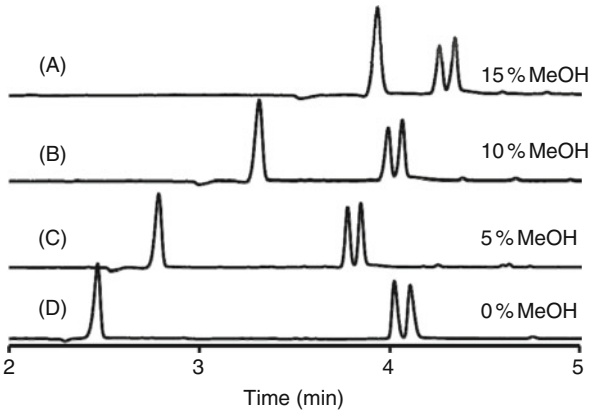


Fig. 12 Effect of MeOH addition (in volume percentages) on the capillary electrophoresis separation of *p*-chloroamphetamine using 5 mM sulfated cyclofructan as the chiral selector. CE conditions: buffer, 20 mM ammonium acetate +5 mM sulfated CF6; pH, 4.7; voltage, +25 kV; capillary length, 35 cm; capillary i.d., 50 μm. First peak is EOF marker. The unit for the time axis (horizontal axis) is minute. Reprinted by permission of Wiley-VCH GmbH, Weinheim [44]

On the other hand, sulfated CF6 was able to bind to basic analytes via strong electrostatic interactions in aqueous solvents at appropriate pHs [44]. This electrostatic interaction may not only facilitate initial docking of basic analytes but also contributes to their chiral separation, along with hydrogen bonding interactions. Figure 12 shows the enantiomeric separation of *p*-chloroamphetamine using sulfated CF6. The separation was virtually unaffected when 20% methanol was added to the background electrolyte (BGE) (Fig. 12). This was different from what was observed for enantiomeric separations using sulfated cyclodextrins, where methanol in the BGE competes for the hydrophobic cavity of cyclodextrin-based selectors and usually diminishes their enantiomeric separations [45, 46]. The fact that methanol has minimal effects on the enantiomeric separation by sulfated CF6 is a good indication that the hydrophobic side of CF6 is not responsible for the chiral recognition of chloroamphetamine, which is in accordance with the mechanisms deducted from HPLC separations.

Sulfated CF6 showed excellent enantioselectivity toward primary, secondary, tertiary, and quaternary amines [44]. Enantiomeric separations can be obtained in both normal and reverse polarity modes, although the reversed polarity mode usually produced electropherograms with better peak shapes [44]. In addition, 25 native amino acids were also separated in CE with the reversed polarity mode [44].

3.2.4 Derivatized CF6 for GC

Permethylated and partially pentylated CF6 have been used as chiral selectors for gas chromatography (GC) [47]. The geometry of these chiral selectors was optimized using Hatree–Fock theory calculations at the 6–31 g level. Computational

Table 2 O_{center}-C2-C3 angles (as shown in Fig. 2) for each of the six fructofuranose ring in native and derivatized CF6

Fructose ring index	CF6(X-ray)[a]	CF6[b]	PM-CF6 (X-ray with $Ba(SCN)_2$)[c]	PM-CF6[d]	PT-CF6[d,e]
1	93.9	92.6	98.1	103.4	79.4
2	139.2	139.4	121.3	124.6	130.6
3	93.9	92.3	139.2	152.2	101.6
4	139.2	139.2	98.1	101.2	105.9
5	93.8	92.4	121.3	126.1	106.9
6	139.2	138.6	139.2	159.6	117.6

[a]X-ray structure of CF6 (obtained from [4]);
[b]structure calculated at B3LYP/cc-pVDZ level;
[c]X-ray structure of permethylated CF6 when crystallized with $Ba(SCN)_2$ (obtained from [32]);
[d]structures calculated at HF/b-31 g level;
[e]all-4-O-all-6-O-pentylated CF6.

studies showed that the high degree alkylation of CF6 changed the alternating inward/outward arrangements on the CF6 macrocyclic rim. As shown in Fig. 2, the O_{center}-C2-C3 angle is 92° and 139° for inward- and outward-inclined fructofuranose units, respectively. After alkylation, the C3 symmetry of native CF6 is lost, the O_{center}-C2-C3 angles becomes larger on average (Table 2), the distances among the O3*(i)* atoms increase and thus the original intramolecular hydrogen bonding of the native CF6 is disrupted, and the crown ether skeleton becomes more exposed (Fig. 13). These structural changes after derivatization are also likely for other types of derivatizations of CF6.

Racemic compounds separated using alkylated CF6 as the selector in GC includes β-lactams, trifluoroacetyl derivatized amino acids, and tartaric acid esters [47]. Hydrogen bonding is of critical importance for enantiomeric separations in GC. On permethylated CF6, α-(trifluoromethyl)benzyl alcohol was baseline separated, whereas esters of this alcohol were less retained and no separation was observed (Table 3). Furthermore, no separation was observed for native α-methylbenzyl alcohol, which is a weaker hydrogen bond donor than α-(trifluoromethyl)benzyl alcohol. Likewise, enantiomers of *N*-acetylated amino acids were poorly separated or not separated, whereas *N*-trifluoroacetylated amino acids are very well separated [47].

3.3 Going Prep: Loading Tests on Cyclofructan-Based CSPs

The loading capability of a CSP is related to the available number of selector–analyte interaction sites. For example, protein-based CSPs are very vulnerable to overloading. Due to the high molecular mass of protein selectors, the number of sites available in a protein column is very limited. Since protein selectors generally interact enantioselectively with only one analyte at a time, there is no way

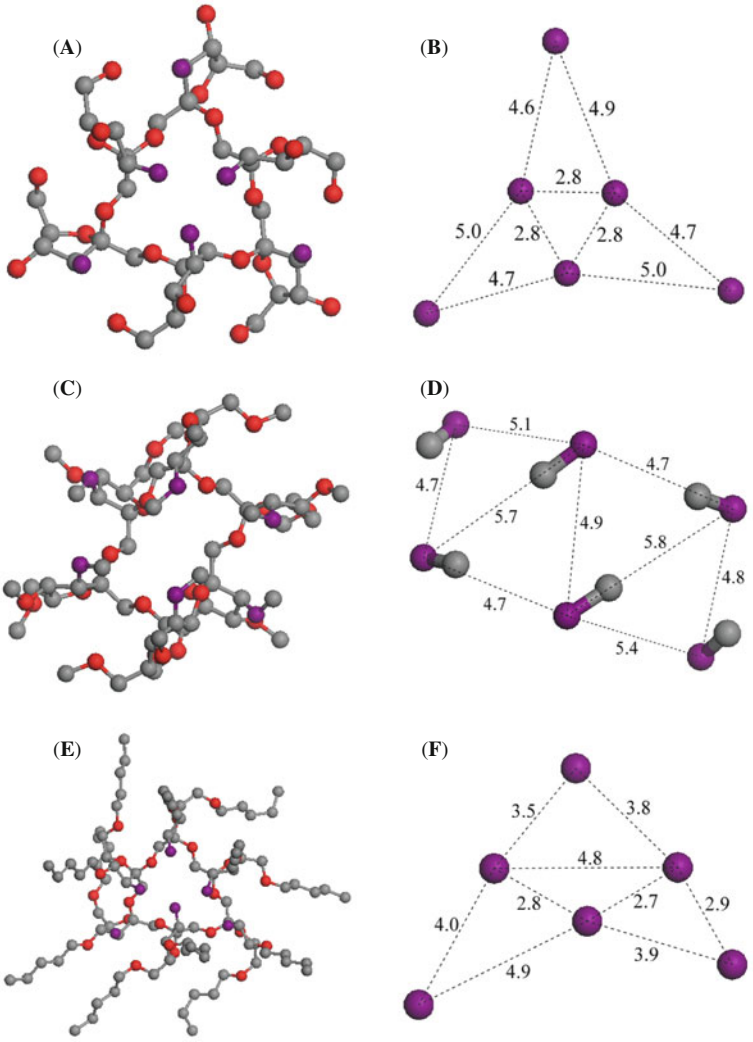
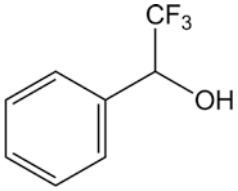
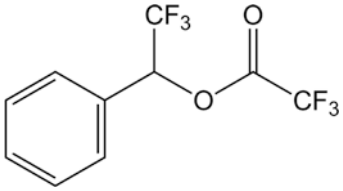
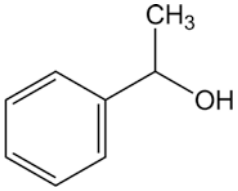


Fig. 13 Effect of fructose substitution on the ring structure and O3 spatial position. *Top* view of native and alkylated CF6: (**a**) and (**b**), native CF6 optimized at B3LYP/6–31 g level; (**c**) and (**d**), permethylated CF6 optimized at HF/6–31 g level; (**e**) and (**f**), all-4-*O*-all-6-*O*-pentylated CF6 optimized at HF/6–31 g level. Figures on the *right*side are displaying relative positions of O_3 atoms on each fructofuranose units. The distance between neighboring oxygen atoms are labeled in Å (0.1 nm). Color coding: *gray*, carbon; oxygen, *red*; O_3 oxygen atoms, *purple*. Hydrogen atoms are left out for clarity purpose. Reprinted by permission of the Royal Society of Chemistry [47]

to inject large amounts of analyte. On the other hand, polymeric CSPs usually have high loading capabilities because its repeating units can provide multiple interaction sites for chiral recognition. Cyclofructans are oligomers. As shown in Fig. 6, cyclofructans and derivatized cyclofructans can interact with chiral primary

Table 3 Chromatographic data for three structurally similar compounds on permethylated-CF6 chiral GC phase at the same chromatographic conditions:

No.	Compound	Structure	k_1'	α
1	α-(Trifluoromethyl) benzyl alcohol		93.2	1.05
2	Trifluoroacetyl α-(trifluoromethyl) benzyl alcohol		4.2	1.00
3	α-Methylbenzyl alcohol		34.4	1.00

Column 10 m, 200 μm, i.d., 0.5 μm film thickness, isothermal separation at 130°C, He carrier gas, FID detection. Data from [47]

amines via tripod hydrogen bonding through several interaction sites. In addition, adducts of one CF6 with two metal cations or multiple metal chloride salts (up to nine, see Fig. 14) were observed in ESI-MS [48]. In contrast, crown ethers can only interact with one guest molecule at a time, and only 1:1 crown ether–metal cation complexes were observed in ESI-MS [48]. Consequently, cyclofructan-based CSPs are expected to have superior loading capabilities than crown ether-based CSPs.

Loading test experiment was performed on a methylcarbamate CF6 CSP with *trans*-1-amino-2-indanol in the polar organic mode. As shown in Fig. 15, 3.37 mg of the primary amine can be baseline separated in 20 min on an analytical column (250×4.6 mm). Furthermore, cyclofructan-type CSPs operates best in organic solvents and supercritical fluid solvents as well, which makes the sample recovery much easier than with aqueous mobile phases. These characteristics make the cyclofructan-type CSPs viable candidates for the preparative separations of primary amines. In addition, high loading of N-blocked amino acids were also reported on aromatic-derivatized CF6 CSPs [39].

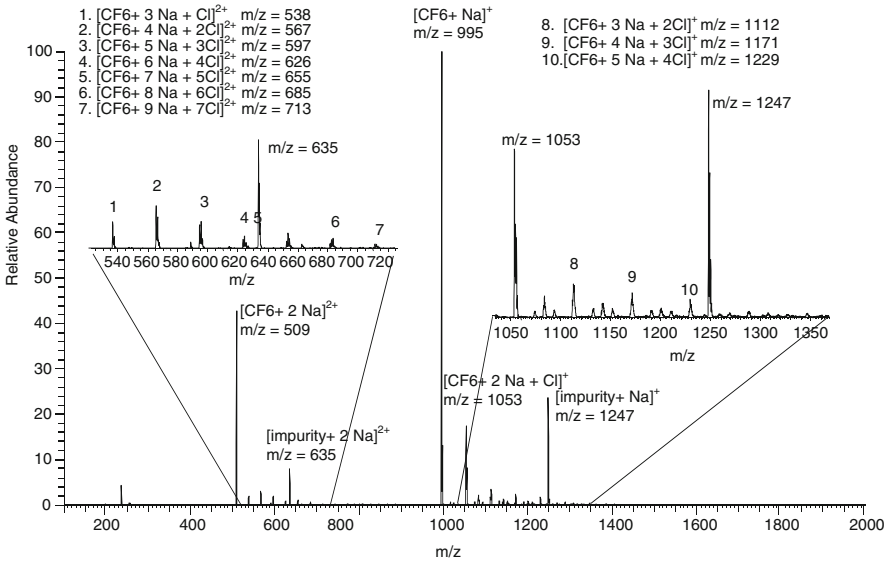


Fig. 14 ESI-MS spectrum of CF6 in NaCl solution showing adduct ions of one CF6 with multiple sodium cations (see [48] for detailed experimental conditions)

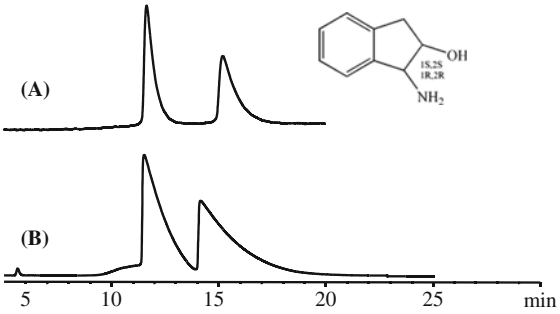


Fig. 15 Loading test of a methyl carbamate CF6 CSP 25 cm × 4.6 mm i.d. column in the polar organic mode. (**a**) 13.5 μg and (**b**) 3.37 mg of *trans*-1-amino-2-indanol were injected. Mobile phase: acetonitrile/methanol/acetic acid/triethylamine 75/25/0.3/0.2% v/v, 1 mL/min. UV detection 254 nm

4 Conclusions

Cyclofructans are a new class of chiral selectors. Despite the fact that they are cyclic oligosaccharides and have a crown ether core, their chiral recognition capabilities and mechanisms are completely different from those of either cyclodextrins or synthetic crown ethers. Two chiral recognition mechanisms are proposed for cyclofructan CSPs. Tripodal hydrogen bonding between the hydroxyl groups on

the hydrophilic side of CF6 and hydrogen bonding donor and/or acceptors in analytes is of critical importance for enantiomeric separations of primary amine-type compounds. On the other hand, chiral helical grooves on the side of CF6, along with the carbamate linked aromatic substituents, can provide steric and π–π interactions between the CSP and analytes. These interactions contribute to chiral recognition for aromatic non-primary amine compounds separated on the aromatic-derivatized CF6 CSPs.

While native CF6 showed only limited enantioselectivity to a few compounds, derivatized CF6s appeared to be versatile chiral selectors and can be tuned for the best HPLC separation of different types of compounds. Aliphatic-derivatized cyclofructans operating in the polar organic mode provide best enantiomeric separations for primary amines. On the other hand, extensively aromatic-derivatized CF6 were able to separate a variety of different classes of enantiomers. Cyclofructans are the newest types of chiral selectors, and their further development and their applications in both analytical and preparative enantiomeric separations are not yet fully explored and are expected to grow significantly in the future.

Acknowledgments We gratefully thank the Robert A. Welch Foundation (Y.0026) for financial support of the work and Mitsubishi Kagaku Co. and Zachary S. Breitbach for providing us with cyclofructans. We also thank Dr. Alain Berthod, Dr. Chunxia Jiang, and Ying Zhang for helpful discussions.

References

1. Kawamura M, Uchiyama T, Kuramoto T, Tamura Y, Mizutani K (1989) Enzymic formation of a cycloinulo-oligosaccharide from inulin by an extracellular enzyme of Bacillus circulans OKUMZ 31B. Carbohydr Res 192:83–90
2. Kushibe S, Sashida R, Morimoto Y (1994) Production of cyclofructan from inulin by Bacillus circulans MCI-2554. Biosci Biotechnol Biochem 58:1136–1138
3. Sawada M, Tanaka T, Takai Y, Hanafusa T, Hirotsu K, Higuchi T, Kawamura M, Uchiyama T (1990) Crystal structure of cycloinulohexaose. Chem Lett 2011–2014
4. Sawada M, Tanaka T, Takai Y, Hanafusa T, Taniguchi T, Kawamura M, Uchiyama T (1991) The crystal structure of cycloinulohexaose produced from inulin by cycloinulo-oligosaccharide fructanotransferase. Carbohydr Res 217:7–17
5. Wang C, Breitbach ZS, Armstrong DW (2010) Separations of cycloinulooligosaccharides via hydrophilic interaction chromatography (HILIC) and ligand-exchange chromatography. Sep Sci Technol 45:447–452
6. Matsubara M, Kaneko T, Chiba M, Oota M (1994) Ink jet recording medium and ink jet recording method using same. JP Patent 06-071903
7. Ono H, Ando O, Takeuchi M (1994) Electrophotographic toners containing cyclic oligosaccharide. JP Patent 06-124002
8. Meji I (1994) Silver halide photographic materials. JP Patent 06-043573
9. Nishio S (1994) Processing of silver halide photographic material by preventing formation of silver sludge. JP Patent 06-250347
10. Nishio S (1995) Processing of silver halide photographic material and fixing solution for it. JP Patent 07-064249
11. Nishio S, Komatsu H, Shimizu H (1996) Developing solution for silver halide photographic materials and processing method. JP Patent 08-029927

12. Nishioka M, Mori H, Nanjo F (2004) Cyclofructans as inhibitors for odor and taste of iron, and products containing them for reduced odor and taste of iron. JP Patent 2004-337133
13. Nishioka M, Mori H, Nanjo F (2004) Bitterness or astringency inhibitors containing cyclofructans, and oral compositions containing them. JP Patent 2004-337132
14. Mori H, Nishioka M, Nanjo F (2006) Cyclodextrin and cyclofructan for inhibition of bitterness and astringency of foods and beverages. JP Patent 2006-67894
15. Mori H, Nishioka M, Nanjo F (2006) Cyclodextrin and cyclofructan for inhibition of bitterness and astringency of foods and beverages. JP Patent 2006-67895
16. Mori H, Nishioka M, Nanjo F (2006) Cyclofructan for prevention of browning in beverages. JP Patent 2006-67896
17. Ishikawa K, Nanjo F (2005) Cyclofructan and inulohexaose as improvement drugs and health foods for intestinal function. JP Patent 2005-154388
18. Ishikawa T, Nanjo F (2005) Mucosal immunity stimulants and secretory IgA production inducers containing cyclofructan and preparations containing the stimulants applied to oral cavity. JP Patent 2005-179195
19. Schoch C, Bizec JC, Kis G (2007) Cyclodextrin derivatives and cyclofructan as ocular permeation enhancers. J Incl Phenom Macro 57:391–394
20. Ishikawa K, Nanjo F (2008) Cyclofructan as blood lipid improver and health food. JP Patent 2008-69095
21. Ishikawa K, Nanjo F (2008) Cyclofructan as antiobesity agent and health food. JP Patent 2008-69094
22. Uchama T (1993) Trapping agents for metal ions. JP Patent 05-076756
23. Imaki S, Takuma J, Aiura M, Hosono E (1994) Collecting agents for metal ions. JP Patent 06-121927
24. Sawada M, Takai Y, Yamada H, Hirayama S, Kaneda T, Tanaka T, Kamada K, Mizooku T, Takeuchi S, Ueno K, Hirose K, Tobe Y, Naemura K (1995) Chiral recognition in host-guest complexation determined by the enantiomer-labeled guest method using fast atom bombardment mass spectrometry. J Am Chem Soc 117:7726–7736
25. Sawada M, Takai Y, Shizuma M, Takeda T, Adachi H, Uchiyama T (1998) Measurement of chiral amino acid discrimination by cyclic oligosaccharides: a direct FAB mass spectrometric approach. Chem Commun 1453–1454
26. Harata K (1997) The structure of the cyclodextrin complex. V. Crystal structures of α-cyclodextrin complexes with p-nitrophenol and p-hydroxybenzoic acid. Bull Chem Soc Jpn 50:1416–1424
27. Immel S, Schmitt GE, Lichtenthaler FW (1998) Cyclofructins with six to ten -linked fructofuranose units: geometries, electrostatic profiles, lipophilicity patterns, and potential for inclusion complexation. Carbohydr Res 313:91–105
28. Fehlhammer WP, Schrölkamp S, Hoyer M, Hartl H, Beck W (2005) Alkali-isocyanaoacetates. synthesis and structure of [K(18-crown-6)](O_2CCH_2NC). Z Anorg Allg Chem 631:3025–3029
29. Pedersen C (1970) Crystalline salt complexes of macrocyclic polyethers. J Am Chem Soc 92:386–391
30. Yoshie N, Hamada H, Takada S, Inoue Y (1993) Complexation of cycloinulonexaose with some metal ions. Chem Lett 22:353–356
31. Shizuma M, Takai Y, Kawamura M, Takeda T, Sawada M (2001) Complexation characteristics of permethylated cycloinulohexaose, cycloinuloheptaose, and cycloinulooctaose with metal cations. J Chem Soc Perkin Trans 2:1306–1314
32. Takai Y, Okumura Y, Tanaka T, Sawada M, Takahashi S, Shiro M, Kawamura M, Uchiyama T (1994) Binding Characteristics of a new host family of cyclic oligosaccharides from inulin: permethylated cycloinulohexoase and cycloinuloheptaose. J Org Chem 59: 2967–2975
33. Pirkle WH, Welch CJ, Lamm B (1992) Design, synthesis, and evaluation of an improved enantioselective naproxen selector. J Org Chem 57:3854–3860

34. Gasparrini F, Misiti D, Villani C, La Torre F, Sinibaldi M (1988) High-performance liquid chromatography on chiral packed microbore columns with the 3,5-dinitrobenzoyl derivative of trans-1,2-diaminocyclohexane as selector. J Chromatogr A 457:235–245
35. Uray G, Lindner W (1990) (S,S)-diphenyl-ethanediamine (DPEDA) derivatives as chiral selectors Part I. Undecenoyl bound dinitrobenzoyl-DPEDA as a broadly applicable chiral stationary phase. Chromatographia 30:323–327
36. Armstrong DW, Stalcup AM, Hilton ML, Duncan JD, Faulkner JR, Chang SC (1990) Derivatized cyclodextrins for normal-phase liquid chromatographic separation of enantiomers. Anal Chem 62:1610–1615
37. Armstrong DW, Tang Y, Chen S, Zhou Y, Bagwill C, Chen JR (1994) Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography. Anal Chem 66:1473–1484
38. Okamoto Y, Ikai T (2008) Chiral HPLC for efficient resolution of enantiomers. Chem Soc Rev 37:2593–2608
39. Sun P, Wang C, Breitbach ZS, Armstrong DW (2009) Development of new chiral stationary phases based on native and derivatized cyclofructans. Anal Chem 81:10215–10226
40. Chang SC, Reid Iii GL, Chen S, Chang CD, Armstrong DW (1993) Evaluation of a new polar – organic high-performance liquid chromatographic mobile phase for cyclodextrin-bonded chiral stationary phases. Trends Anal Chem 12:144–153
41. Mitchell CR, Armstrong DW (2004) Cyclodextrin-based chiral stationary phases for liquid chromatography: a twenty-year overview. In: Gübitz G, Schmid MG (eds) Chiral separations: methods and protocols, methods in molecular biology, vol 243, Humana press, Totowa, NJ
42. Ikai T, Yamamoto C, Kamigaito M, Okamoto Y (2006) Immobilized polysaccharide-based chiral stationary phases for HPLC. Polym J 38:91–108
43. Okamoto Y, Yashima E (1998) Polysaccharide derivatives for chromatographic separation of enantiomers. Angew Chem Int Ed 37:1020–1043
44. Jiang C, Tong MY, Breitbach ZS, Armstrong DW (2009) Synthesis and examination of sulfated cyclofructans as a novel class of chiral selectors for CE. Electrophoresis 30:3897–3909
45. Fanali S (2000) Enantioselective determination by capillary electrophoresis with cyclodextrins as chiral selectors. J Chromatogr A 875:89–122
46. Jiang C, Tong MY, Armstrong DW, Perera S, Bao Y, MacDonnell FM (2009) Enantiomeric separation of chiral ruthenium(II) complexes using capillary electrophoresis. Chirality 21:208–217
47. Zhang Y, Breitbach ZS, Wang C, Armstrong DW (2010) The use of cyclofructans as novel chiral selectors for gas chromatography. Analyst 135:1076–1083
48. Wang C, Yang SH, Wang J, Kroll P, Schug KA, Armstrong DW (2010) Study of complexation between cyclofructans and alkali metal cations by electrospray ionization mass spectrometry and density functional theory calculations. Int J Mass Spect 291:118–124

Chiral Recognition and Enantioseparation Mechanisms in Capillary Electrokinetic Chromatography

Bezhan Chankvetadze

Contents

B. Chankvetadze (✉)
Department of Physical and Analytical Chemistry, School of Exact and Natural Sciences, Tbilisi State University, Chavchavadze Ave 3, 0179 Tbilisi, Georgia
e-mail: bezhan_chankvetadze@yahoo.com

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_5,

Abstract This chapter deals with the basic theory of enantiomeric separations in electrokinetic chromatography (EKC) in general and with the relationships between the recognition and the separation of enantiomers in EKC, in particular. It is important to note that the dependence between recognition and separation is not as straightforward in EKC as it is in chromatographic separation techniques. Therefore, a clear understanding of these dependences is very important for the explanation of experimentally observed results, as well as for a design of new powerful separation systems, technologies, and materials. Cyclodextrins (CDs) are mainly discussed as chiral selectors not only because the author has a long-term experience of working with these multifunctional macrocycles but also because CDs belong to the most widely used chiral selectors in EKC. In addition, these materials are quite well-characterized molecules of medium size. In addition, CDs are used for separation of enantiomers almost in all analytical separation techniques, as well as for determination of the enantiomeric excess in nonseparation techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. This chapter does not address applications of chiral EKC in chemistry, pharmaceutical and biomedical, environmental, and food analyses.

1 Fundamentals of Enantioseparations in Electrokinetic Chromatography

1.1 Separation Principle in Chiral CE: Electrophoretic or Chromatographic?

The important conceptual point in chiral CE is to consider that the enantioseparation in this technique is commonly not based on the classical principle of zonal electrophoretic separation. This principle postulates the separation as a result of different migration velocities caused by different charge densities of analytes. The enantiomers of a chiral compound possess the same charge densities. Therefore, none of the potential migration forces in CE, such as the electrophoretic mobility of the analyte, the EOF, their combination, or a transport by a nonenantioselective carrier, is, in principle, able to differentiate between the enantiomers.

The prerequisite for enantioseparations in CE is the enantioselective interaction of the analyte with a chiral selector. This interaction is analogous to differential partition of analytes between different phases in chromatographic techniques. The immiscibility of two phases as stated in the definition of chromatography according to the IUPAC nomenclature [1] is the secondary prerequisite. It is introduced in the definition of chromatography because the pressure as a migration force does not make possible the different velocities of miscible phases or different velocities of the components residing in the same phase. In contrast, the electrophoretic migration mechanism allows species residing in the same phase to migrate with

different velocities. It must be stated that due to this reason, even the enantioseparations performed in a monophasic physical system are based on the selective distribution of the analyte enantiomers between at least two constituents of the liquid phase having a different mobility, i.e., the separation principle in vast majority of chiral CE separations can be considered as chromatographic. In other words, most of CE enantioseparations belong actually to EKC [2, 3].

At the first glance a small number of enantioseparations based on the mobility difference between the noncovalent analyte–chiral selector complexes in CE may appear to be based on true electrophoretic principles. However, this is not the case because the analyte–chiral selector interaction is a necessary prerequisite for the formation of transient diastereomeric complexes [2–7].

Some confusion still exists regarding the classification of enantioseparations into groups such as capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and electrokinetic chromatography (EKC). The separation principle in CZE is mainly a distribution of the analyte components in a free solution according to their charge density which is equal for the enantiomers. This means that it is impossible to resolve enantiomers based on the separation principle of CZE. The separation principle in gel electrophoresis is a sieving effect of the charged analyte molecules depending on their size. Again, the enantiomers as such do not differ in their size and therefore are irresolvable with achiral gels. The separation principle in CIEF is based on the pK_a difference between the analytes. The two enantiomers have exactly the same pK_a. Thus, the enantiomeric separation in all of these techniques relies basically on enantioselective noncovalent intermolecular interactions between the analyte and a chiral selector which may be expressed as the effective mobility difference (CZE and CGE), stereoselective shift of the acid–base equilibrium (CIEF), etc. Thus, most of enantioseparations in chiral CE may be unified under the term EKC. This term was introduced by Terabe and co-workers in 1985 [8]. Micellar electrokinetic chromatography (MEKC) represents a special mode of EKC.

1.2 Enantioseparations with Charged and Uncharged Chiral Selectors

In earlier papers, enantioseparations of charged analytes with neutral chiral selectors were attributed to CZE and the enantioseparations of neutral analytes with charged chiral selectors to EKC. However, from the mechanistic point of view, there is no principal difference whether an analyte or a chiral selector is charged. Actually, it is the subject of convention which counterpart of chiral recognition process will be named selectand and which one chiral selector. The reciprocal chiral recognition strategy for a design of effective chiral selectors proposed by Pirkle and co-workers in HPLC is based on this philosophy [9] and that principle certainly applies for enantioseparations in EKC also [10].

The enantioseparation of neutral chiral analytes with charged chiral selectors, which was considered impossible in the earlier studies on chiral CE, was successfully performed based on the understanding of the aforementioned concept [11, 12].

1.3 Enantioselective and Nonenantioselective Phenomena in Chiral EKC

For many years the electrophoretic mobility of analyte (μ_{ep}) was considered to be a selective transport able to differentiate between enantiomers, while the electroosmotic mobility (μ_{eo}) was considered to be a non-selective transport. This is not correct for chiral EKC, although it applies without any limitation for true electrophoretic separations, i.e., for the separations which are based on a different electric charge density of the sample components [2].

The analyte-specific quantities such as the effective charge (q) and molecular mass (M) are used in Eq. (1) for the calculation of μ_{ep} [13]:

$$\mu_{el} = kqM^{-\frac{2}{3}} \tag{1}$$

This means that the μ_{ep} is an analyte-specific property. On the other hand, μ_{eo} which can be calculated according to Eq. (2)

$$\mu_{eo} = \varepsilon_r \varepsilon_0 \zeta / \eta \tag{2}$$

depends on the dielectric constant of the medium (ε), permittivity of vacuum (ε_0), the zeta potential on the solid–liquid interface (ζ), and the dynamic viscosity of the medium (η). The terms used in Eq. (2) are system specific but none of them is explicitly analyte specific. Therefore, μ_{el} is selective and μ_{eo} is a non-selective transport in true electrophoretic separations. Enantioseparations in CE are mostly based on the chromatographic separation principle as mentioned above. The quantities entering Eq. (1) may be different for other charged analytes but they are equal for the enantiomers. For this reason, transport by μ_{eo} is as nonenantioselective as transport by μ_{EOF} [2, 3].

Only in those enantioseparations in which the binding constants of both enantiomers with the chiral selector are equal but the electrophoretic mobility, pK_a, or the size of transient diastereomeric associates are different, the role of the EOF and the electrophoretic mobility will be the same as it is in classical (achiral) electrophoretic separations [2].

Another reason for the above-mentioned misunderstanding of the role of μ_{el} and μ_{eo} in enantioseparations in CE seems to be the fact that in early studies it was possible to observe an improvement of enantioseparations in CE under the conditions when the EOF was suppressed. However, no attention was paid to the fact that the

same apparent effect may, in principle, be observed when one suppresses the μ_{ef} instead of μ_{eo} under appropriate conditions [2].

A principal difference between μ_{ef} and μ_{eo} is that the former is a substance-specific transport, whereas the latter is a system-specific transport [2].

1.4 Similarities and Differences Between Enantioseparations by Pressure-Driven Chromatography and EKC

As mentioned above, enantioseparations in EKC rely on a chromatographic separation principle. Despite this fact, there are significant differences between these techniques. Responsible for all differences between chromatographic and electrophoretic enantioseparations is the property of the electrophoretic mobility to be selective for the analytes residing in the same physical phase [2]. Another important point is that in chromatographic techniques, except in the case of a chiral mobile phase additive (CMPA), the analyte is virtually immobile when associated with a chiral selector. In EKC the analyte selector complex is commonly mobile.

Basic differences between chromatographic and electrophoretic enantioseparations can be derived by analyzing the equation proposed for the calculation of the observed electrophoretic mobility difference $\Delta\mu$ between enantiomers [14]:

$$\Delta\mu = \mu_1 - \mu_2 = \frac{\mu_f + \mu_{C_1} K_1 [C]}{1 + K_1 [C]} - \frac{\mu_f + \mu_{C_2} K_2 [C]}{1 + K_2 [C]} \tag{3}$$

where μ_1 and μ_2 are the observed electrophoretic mobilities of the first and the second migrating enantiomer, respectively. K_1 and K_2 are the binding constants between enantiomers 1 and 2 and the chiral selector, μ_f and μ_c are the electrophoretic mobilities of the free and the complexed analyte, respectively, and $[C]$ is the concentration of a chiral selector.

One important point obviously seen from Eq. (3) is the crucial role of the electrophoretic mobilities in enantioseparations in EKC. This parameter is absent in the major chromatographic techniques except the above-mentioned mode with CMPA. The contribution of the mobilities in chiral EKC separations may allow the observation of the following specific effects:

1. It is feasible in chiral EKC but not in chromatographic techniques that the selectivity of enantioseparation exceeds the thermodynamic selectivity of chiral recognition [3].
2. It is possible in chiral EKC to revert the enantiomer migration order without reversing the affinity pattern between the enantiomers of the analyte and a chiral selector. This is impossible in chromatographic techniques at least in the mode when the chiral selector is immobilized and not used as a CMPA.
3. The most striking difference between these two techniques seems to be the fact that EKC allows, in principle, an enantioseparation even in the case when the binding constants of both enantiomers with the chiral selector are equal [2, 3, 7].

Below, these differences between EKC and chromatographic enantioseparations are illustrated using examples from the literature.

As already mentioned, in chromatographic techniques the selectivity of enantioseparation is entirely defined by the chiral recognition, i.e., by the difference between the affinities of enantiomers toward the chiral selector. Therefore, the selectivity of enantioseparations in common chromatographic techniques may maximally approach the thermodynamic selectivity of the chiral recognition but will never exceed it. In contrast to this, in EKC the separation selectivity may exceed the thermodynamic selectivity of recognition. This is experimentally illustrated in Fig. 1 [15]. In all separations of the chlorpheniramine enantiomers with the carboxymethylated β-CD selector shown in Fig. 1, the components involved in chiral recognition at the molecular level are invariant. This means that chiral recognition itself does not change significantly. However, an enormous (in principle unlimited) enhancement of the separation selectivity becomes possible in the step of transforming the chiral recognition into a chiral separation. In this particular example this was achieved by applying a counterbalancing pressure to the separation capillary in the opposite direction to the analyte migration.

The principle of resolution (R_S) enhancement without any change in recognition selectivity is a decrease of the observed averaged mobility term $\mu_{av} = \frac{1}{2}(\mu_1 + \mu_2)$

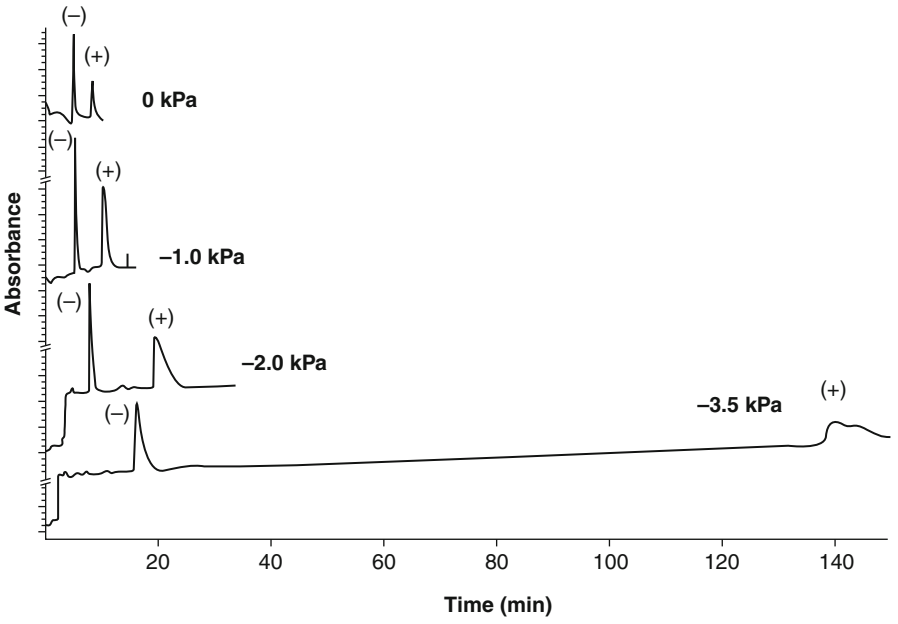


Fig. 1 Effect of increasing counterpressure on the separation of (±)-chlorpheniramine in the presence of 2 mg ml^{-1} CM-β-CD (reproduced with permission from [15])

while retaining the observed electrophoretic mobility difference ($\Delta\mu = \mu_1 - \mu_2$) constant in Eq. (4) [16]:

$$R_S = \frac{1}{4}\sqrt{N}\frac{\Delta\mu}{\mu_{av}} \tag{4}$$

As shown in [15] this concept may allow for the design of a separation system in such a way that two enantiomers certainly possessing the electric charge of the same sign will migrate toward opposite electrodes, which means that the enantioseparation factor becomes infinitely large (Fig. 2) [15]. This technique certainly may be applied for micropreparative purposes also as well as for separations of achiral analytes not only in a binary mixture but also in multicomponent mixtures.

Another important point is that a manipulation of the mobility terms in EKC allows not only an adjustment of the selectivity of enantioseparation but also a reversal of the enantiomer migration order without changing the affinity pattern of the enantiomers toward the chiral selector. This is again impossible in chromatographic techniques. This significant difference between chromatographic and electrophoretic separations from the viewpoint of the enantiomer migration order has been noted earlier [2, 3, 17–19].

Thus, taking into account that the mobility in CE is a vectorial quantity, one can imagine that just reverting the sign of $\Delta\mu$ even without any change in the chiral recognition will result in a reversal of the enantiomer migration order [17]. Similar to the case shown in Fig. 1, the components of a separation system immediately involved in chiral recognition are not significantly modified in the separation depicted in Fig. 3 [17]. However, the pH of the separation buffer is changed by a designed way to allow the detection of the analyte on the anodic or the cathodic ends of the separation capillary, alternatively. This means a reversal of the direction of the vector $\Delta\mu$ and, consequently, a reversal of the enantiomer migration order. The idea of the experiment shown in Fig. 3 is schematically described in [2, 17, 19].

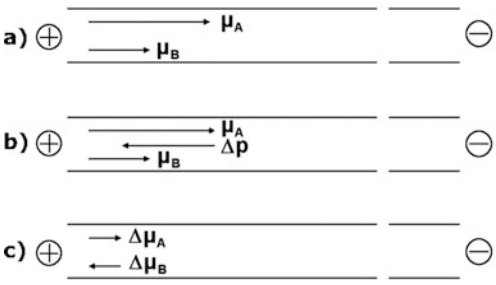


Fig. 2 Schematic representation of flow-counterbalanced separation principle in CE: (**a**) without counterbalanced flow; (**b**) with counterbalanced flow; (**c**) resulting mobilities (reproduced with permission from [15])

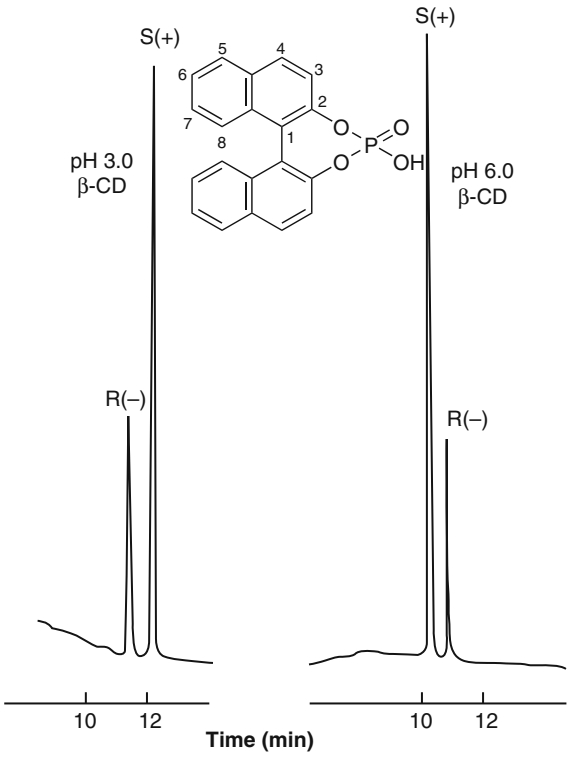


Fig. 3 Reversal of the enantiomer migration order in CE without principal modification of chiral recognition (reproduced with permission from [19])

A simplified form of Eq. (3) can be obtained when one assumes that the diastereomeric complexes of both enantiomers with a given chiral selector possess equal mobilities($\mu_{C_1} = \mu_{C_2} = \mu_C$) [14]:

$$\Delta\mu = \frac{C(\mu_f - \mu_c)(K_2 - K_1)}{1 + C\,[K_1 + K_2] + C^2 K_1 K_2} \tag{5}$$

This equation indicates ways of affecting the direction of $\Delta\mu$ without affecting the affinity characteristics between a chiral analyte and a selector. In particular, from Eq. (5) it is obvious that not only a reversal of the algebraic sign of $(K_2 - K_1)$ term but also that of $(\mu_f - \mu_c)$ term may result in a change in the algebraic sign of $\Delta\mu$, i.e., reversal of the enantiomer migration order. Changing the algebraic sign of $\mu_f - \mu_c$ may be achieved by affecting the effective mobility of the analyte or the chiral selector or both of them. The examples of the reversal of the enantiomer migration order based on mobility adjustments have been summarized in [17, 18]. Equation (5) does not explicitly contain separation parameters such as electric charge and concentration of the chiral selector, pH of the background electrolyte, and the direction and magnitude of the EOF. However, all of these parameters may affect the mobility term $(\mu_f - \mu_c)$ in Eq. (5). Therefore, all of them may affect the enantiomer migration order implicitly [17].

It should be noted that it is not the intrinsic mobility of the free and complexed analyte but the observed mobilities that determine the enantiomer migration order in EKC. Thus, if one can manage (even using external parameters such as pressure) the separation conditions requiring the polarity change of the high voltage supply for a detection of the analyte, then reversal of the enantiomer migration order will be observed [17, 19].

Another difference between enantioseparations in EKC and HPLC is the fact that an enantioseparation is, in principle, feasible in EKC even in the case when the binding constants of the enantiomers with the chiral selector are identical [2, 3, 7]. This conclusion can be made from Eq. (3). According to this equation, for the generation of a mobility difference between the enantiomers, e.g., an enantioseparation in EKC, the following are required:

(a) Formation of transient diastereomeric associates between the analyte and the chiral selector. This means that an enantioseparation is impossible in CE without a chiral selector.
(b) The mobilities must be different for the free and the complexed analyte ($\mu_f \neq \mu_c$). If both above-mentioned prerequisites apply, then enantiomers may be resolved with equal success using the following two alternative mechanisms:

(1) For a given overall migration time the ratio of residence times in the free and the complexed form is not identical for both enantiomers. The time fraction in which the enantiomers reside in the free and the complexed form is defined by the degree of complexation (which is dependent on the binding constants and the concentration of the chiral selector), e.g., in this case a difference in binding constants is required. This means that the enantioseparation will be based on the same principle as in chromatographic techniques.
(2) Alternatively, both enantiomers may + in the same time fraction in the free and the complexed form, e.g., $K_1 = K_2 = K$. Under these conditions, Eq. (3) may be rewritten in the following form:

$$\Delta\mu = \mu_1 - \mu_2 = \frac{K\,[C]\,(\mu_{C_1} - \mu_{C_2})}{1 + K\,[C]} \tag{6}$$

From Eq. (6) it is clear that the prerequisite for the enantioseparation in this case is the formation of the transient diastereomeric associates of both enantiomers with different mobilities μ_{C_1} and $\mu_{C_2} : \mu_{C_1} \neq \mu_{C_2}$. The enantioseparations based on the mechanism described by Eq. (6) are very close to classical zonal separations (EKC mechanism is not directly involved). However, considering the IUPAC nomenclature, these separations may also be attributed to EKC.

Thus either the binding constant difference or the mobility difference of the corresponding diastereomeric complexes may result in enantioseparations in CE [2, 3, 6, 7]. Rather common is the former case ($K_1 \neq K_2$) or the combination of both principles, although the possibility of enantioseparations solely based on the different mobilities of the diastereomeric associates has been unambiguously proven [7].

Thus, there are significant differences between enantioseparations in pressure-driven and electrically driven systems. On the one hand, these differences make the techniques complimentary. This is an advantage. On the other hand, the rules and dependencies observed in one technique should be applied to the other with some care in order to avoid mistakes in the interpretations of the experimental results.

2 Modes of Enantioseparations in EKC

The most traditional mode of enantioseparations in CE is when a separation capillary and both inlet and outlet vials are filled with a buffer solution containing a chiral selector and an analyte migrates with its own electrophoretic mobility, EOF, or their combination from the inlet vial toward the outlet vial passing a detector. Enantioselective interactions between the analyte and a chiral selector selectively affect the mobility of the enantiomers and this is the most common phenomenon responsible for enantioseparations in CE. Together with this mode, some alternative modes of CE enantioseparations have been proposed as briefly summarized below.

Thepartial filling technique has been proposed by Hjerten and co-workers [20] and involves the filling of a separation capillary only in part with a chiral selector. Later it has been shown that in the case when a chiral selector possesses sufficient self-electrophoretic mobility in the opposite direction to the chiral analyte, it is possible to fill the entire capillary with a chiral selector [11]. This technique was named *counter-current separation mode*. In both of the above-mentioned techniques, the outlet vial is free of chiral selector. The advantages of these techniques are the following: (a) UV-absorbing chiral selectors which would produce a significant detector response may be used for chiral separations. This not only relates to UV-absorbing chiral selectors as commonly described in the literature [21, 22] but also allows online CE–MS coupling [23–25] and, in principle, may allow to use chiroptical detectors also in chiral CE. (b) Expensive and exotic chiral selectors may be used in lower amounts in this mode. (c) Binding constants between an analyte and a chiral selector can be calculated by variation of plug length and concentration of a chiral selector in a plug [26, 27]. More comprehensive description of the partial filling and counter-current migration principles is given in a review by Amini and Westerlund [28].

Another promising mode of chiral CE separations seems to be the *mobility-counterbalanced mode* [15, 29–39]. Counterbalancing of analyte electrophoretic mobility by pressure has been applied by Culbertson and Jorgenson [29] for the enhancement of the detection sensitivity in achiral CE. Later the same technique was used for the separation of isotopomers of phenylalanine [30]. The potential advantage of flow-counterbalanced capillary electrophoresis (FCCE) can be seen from Eq. (7) [31]:

$$R_S = (\mu_1 - \mu_2)\frac{E\sqrt{t}}{4\sqrt{2D}} \tag{7}$$

where E is the electric field strength, D is the average effective diffusion coefficient of two analytes, and t is the electrophoretic migration time. In FCCE the sample is driven forward by electromigration and then backward by pressure-induced flow. The samples travel back and forth in the capillary until sufficient separation is obtained [32]. In the mode of FCCE as proposed by Culbertson and Jorgenson [29], the electric field and the pressure are applied alternatively as the driving forces.

In another mode of FCCE, the counterbalancing driving force such as pressure may be applied to the separation chamber continuously during the entire time of separation [15]. An enormous increase of the separation factor in chiral and achiral CE separations may be achieved using this technique as already mentioned above [15].

The difference between counter-current [11] and flow-counterbalancing CE [15] techniques is that in the latter case a chiral selector and a chiral analyte do not migrate in the opposite direction to each other, but the bulk flow moves with a definite velocity in the opposite direction to the effective mobility of the analyte zone [15].

The advantages of mobility-counterbalancing technique include the followings: (a) Enormous, in principle unlimited, enhancement of the separation factor may be achieved. (b) This technique allows to easily transform a discontinuous zonal separation of a binary mixture into a continuous separation with stepwise migration of the sample components from the inlet toward the outlet vial (Fig. 4) [15]. (c) The technique may be used for micropreparative purposes and offers significantly higher sample capacity compared to discontinuous separations. Other potential advantages of mobility-counterbalancing techniques are discussed in [15].

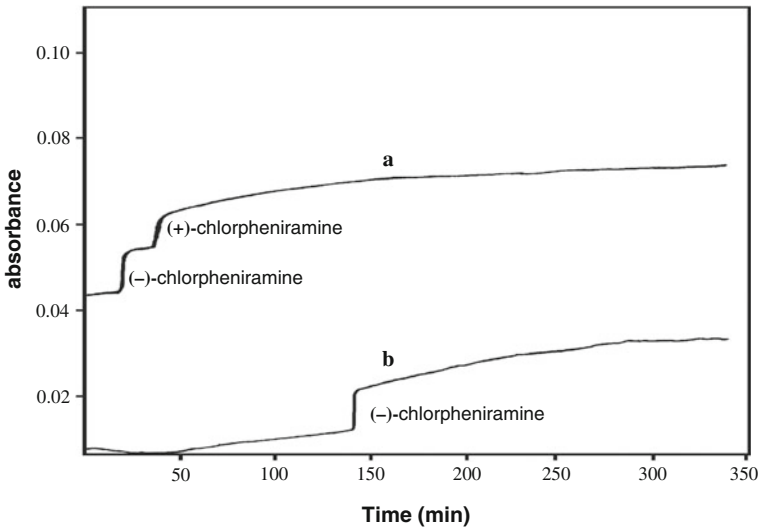


Fig. 4 Continuous CE separation of (±)-chlorpheniramine with 3.5 mg ml^{-1} CM-β-CD (**a**) in the absence and (**b**) in the presence of 689.6 Pa counterpressure (reproduced with permission from [15])

The mobility-counterbalancing technique is certainly not limited to binary mixtures and it can be easily applied in a stepwise manner for the separation of multicomponent samples.

The pressure/vacuum, the EOF, hydrodynamic pressure (leveling of the inlet and outlet vials), etc. may be used as a driving force for counter-mobilities in this technique [15]. An interesting remark regarding the potential of a technique similar to the above mentioned was made by Dovici et al. as early as 1990: "If pressure-induced flow balances the average velocity of two closely spaced analytes, then the resolution of the analytes becomes infinite" [32].

Microfluidic temperature gradient focusing, proposed by Ross and co-workers in 2002 and successfully applied for separation of fluorescent dyes, amino acids, DNA, proteins, particles [33], and enantiomers [34], represents one of the modes of FCCE, as is micellar affinity gradient focusing proposed by the same group in 2004 [35].

Ivory and co-workers developed earlier the electric field gradient focusing (EFGF) [36–39] technology enabling effects similar to those achieved in FCCE.

Synchronous cyclic capillary electrophoresis (SCCE) was proposed by Jorgenson's group as a technique which allows overcoming the dispersion problems in FCCE caused by the parabolic counterflow profile [31]. High resolution is achieved in SCCE by driving the samples in a virtually closed loop until desired resolutions are achieved. This technique was applied for isotopic and chiral separations. In the third cycle, the chiral compound (α-hydroxybenzyl) methyltrimethylammonium with a selectivity as low as 1.0078 was almost baseline separated in 3.5 h [40]. As mentioned by the authors, similar separations with a multicycle LC system as described in [41] would also be possible but would have taken 43 h.

The *carrier mode separation*, for the first time mentioned by Terabe [42], was developed further in the 1990s. In the carrier mode, a chiral selector not only is responsible for the enantioselectivity in the separation system but also transports the analytes to the detector. The most important advantage of this mode is that the analyte migrates to the detector only when associated with the chiral selector, i.e., when participating in the chiral recognition process. The uncomplexed analyte remains immobile or in certain circumstances may possess a self-mobility directed toward the inlet vial. Among the chiral selectors, charged ones possessing a self-electrophoretic mobility may be used as the carriers in EKC [43–45].

Carrier mode chiral separations offer significant advantages and may become a very useful technique for biomedical applications where structurally similar analytes, such as chiral drugs and their metabolites, must be separated and enantioseparated simultaneously. Such a rather complex separation problem implies higher enantio- and chemoselectivity requirements to a separation system (Fig. 5) [43].

Actually, there is no principal difference between carrier mode enantioseparations and the enantioseparations of weakly acidic anionic analytes in an uncoated capillary when the analytes are detected at the cathode. In the latter case a neutral or a cationic chiral selector (even an anionic chiral selector with a lower self-mobility compared to the analyte) also accelerates the analyte toward the detector. The difference between these two modes is that in the latter case the carrier ability of

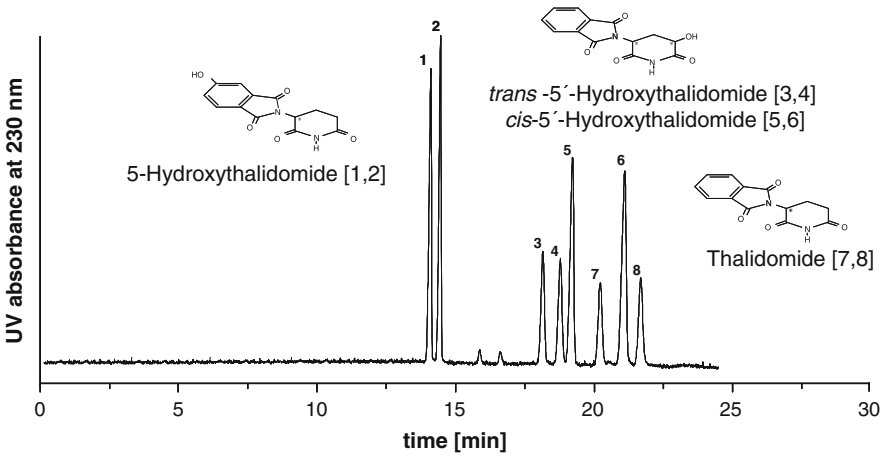


Fig. 5 Simultaneous carrier mode CE separation and enantioseparation of thalidomide and its hydroxylated metabolites (reproduced with permission from [43])

the selector is "assisted" by the EOF which in this particular mode may represent a "parasitic," nonselective migration toward the detector.

Combination of chiral selectors is a well-known approach in chiral EKC [2, 3, 43, 45–56]. A chiral separation in CE may be decoupled in two basic steps: (a) chiral recognition which occurs at the molecular level and (b) transformation of chiral recognition to a chiral separation [2]. In order to design a dual chiral separation system, it is useful to analyze both of the above-mentioned steps separately. When two chiral selectors cooperate in the first (recognition) step, for instance, when a mixed complex of one analyte and two selector molecules are formed, a design of the dual system becomes almost impossible without involving additional techniques and cannot be optimized according to the below-described simplified approach [45, 47].

An additional point is that both mobility and affinity effects must be considered and a conclusion about a favorable affinity pattern for an enhancement of a chiral separation may be drawn depending on the effect of both chiral selectors on the mobility of the analyte [12, 47]. Some of these points are illustrated in [48] using the anticoagulant drug warfarin as a chiral analyte.

In selected cases the application of a dual chiral separation system allows to observe extremely high selectivities of enantioseparations [49]. This technique also bears a certain potential for a better understanding of the fine mechanisms of chiral separations which are sometimes difficult to be observed in a single selector system [50–52].

In the papers dealing with a combination of chiral selectors in CE, basically multiple cyclodextrin (CD) systems are discussed. However, it shall be noted that CDs may be combined with other types of chiral selectors or a combined chiral selector system without a CD component can also be used.

Extending the scope of combined chiral selector systems beyond CDs, one may note that this mode has been used in CE for a rather long time [53–56]. The very first example of combined chiral selectors in CE seems to be the report by Fanali et al. [53] in 1989 when 15 mM L-(+)-tartaric acid buffer was used in combination with 15 mM β-CD in order to resolve the enantiomers of chiral cobalt complexes. CDs have also been combined with chiral surfactants such as cholic acids [54, 55] and synthetic micelle-forming agents [56]. In recent years, several studies were published on the combination of CDs with chiral [57, 58] and achiral [51, 59–61] crown ethers. The latter studies [59–62] where the achiral crown ether cannot contribute to enantioseparations independently clearly illustrate that the simplified approach described in [12, 47] may not be universally applied to all dual chiral separation systems in CE.

Nonaqueous enantioseparations in CE have been reported since 1994 [62]. Nonaqueous buffers offer certain advantages compared to aqueous buffers from the viewpoints of alternative chiral recognition mechanisms involved in the separation [63], lower electric current and Joule heat generation, higher solubility and stability of certain analytes and chiral selectors [64–67], easier online coupling to a mass spectrometer, etc.

Among the nonaqueous solvents, *N*-methylformamide (NMF), *N*,*N*′-dimethylformamide (DMF), dimethylacetamide (DMA), and lower alcohols represent certain interest as separation media in nonaqueous chiral CE. The solvents such as NMF and DMF possess advantages for online chiral CE–MS coupling because they do not necessarily require electrolytes as conductive additives. However, these solvents strongly inhibit inclusion complex formation and hydrogen-bonding interactions which are often essential contributors to chiral recognition. For this reason the concentrations of chiral selectors required for enantioseparations in these organic solvents are relatively high and sometimes it becomes questionable whether nonaqueous CE is really superior compared to the enantioseparation in an aqueous buffer with the same chiral selector.

It has been considered that pure water, alcohols, and many organic solvents require some ionizable additives which may create some problems for online coupling of chiral CE with MS. However, many chiral selectors work rather effectively in solvents such as alcohols [65–67] compared to more polar solvents (DMF, NMF, and DMA). Valko et al. [68] reported a significant EOF in methanol, ethanol, acetone, acetonitrile, and several other organic solvents without the addition of any electrolyte.

It is obviously of interest to compare the same chiral selector in both aqueous and nonaqueous buffers from a mechanistic point of view (enantiomer migration order, intermolecular forces involved in complex formation and chiral recognition, structure of complexes, etc.). Enantioseparations of many chiral analytes have already been described with the same chiral selector in aqueous and nonaqueous buffers [66–70]. Only very recently the enantiomer migration order of chiral β-blocker drug propranolol was studied in both aqueous and nonaqueous buffers. The enantiomer migration order was the same in the case of several CDs applicable

in both aqueous and nonaqueous buffers. However, the opposite migration order was observed with heptakis-(2,3-dimethyl-6-sulfo)- β-CD (HDMS-β-CD). The nuclear Overhauser enhancement (NOE) experiment showed clearly that the inclusion complex was formed between propranolol and HDMS-β-CD in aqueous buffer, while the complex was of external type in nonaqueous buffer [71].

Microfabricated devices offer important advantages from the viewpoint of analysis time, costs, and throughput capacity. The problems with pressure-driven microchip technologies are basically connected to the management of fluid flow with acceptable characteristics through the miniaturized channels. In contrast, an electrokinetically driven flow can be precisely controlled by regulating the applied potentials at the end of each microchannel. Using electric fields to direct and control fluid flow eliminates the need for micro-moving parts such as pumps and valves allowing for convenient integration process of complete assays. Due to the flat cross section of the channel and the large thermal mass of the glass chip, temperature dissipation on chips is greatly improved compared to conventional capillaries. This allows the application of higher electric fields, which in combination with short separation lengths enable fast separations [72, 73].

"Lab-on a chip" separations of enantiomers represent a further miniaturization of capillary techniques and offer advantages of ultra short separation times, high throughput, and miniaturization of instruments. High throughput is especially required due to the rapid development in combinatorial synthetic technologies, screening of chiral catalysts [73], etc.

Enantiomeric separations on micromachined electrophoretic devices (MED) have been reported since 1999. In all enantioseparations performed using MED, CDs have been used as chiral selectors [72–82] except one case [83] in which the chiral crown ether, (–)-(18-crown-6)-tetracarboxylic acid ($18C_6H_4$), was used as an effective chiral selector for resolving gemifloxacin in sodium-containing media.

Charged chiral analytes can be analyzed with neutral native or derivatized CDs as it is commonly done in chiral CE. However, derivatization might be required for some analytes in order to enhance a detection sensitivity which represents a serious challenge in separations using MED. The derivatization may turn analytes into neutral compounds which can be resolved only with charged CDs or with a combination of neutral (or charged) CDs and micelles. Another argument for using charged CDs as chiral selectors in MED-based enantioseparations can be their commonly higher enantiomer-resolving ability as illustrated in the studies by Belder and co-workers. The authors performed enantioseparations in a few seconds [80, 84]. One additional advantage of charged selectors in MED-based enantioseparations can be the directed mobility of these chiral selectors. The practical use of this advantage has still to be explored.

The MED technique described in [72] was applied to the analysis of hot water extracts taken from interior and exterior samples of the Murchison meteorite. The results were comparable to those previously obtained using GC/MS and HPLC techniques. Higher racemic ratios were found in the interior versus the exterior of the

meteorite. According to the authors this finding supports the hypothesis that there was contamination by terrestrial L-amino acids after the meteorite fell to the earth in 1969 [72].

Chiral CE is considered basically to be a technique suitable for analytical-scale enantioseparations. However, this technique possesses a certain potential for *micropreparative-scale enantioseparations* [15, 85–93].

In the studies by Stalcup and co-workers [87, 88] similar to [85], CE has been used basically in order to optimize enantioseparations in classical gel electrophoresis and to study the optical purity of collected fractions. The group has been the first who, by the example of the short-acting β_2-adrenergic agonist terbutaline, demonstrated that classical gel electrophoresis is indeed viable as a method for the separation of milligram quantities of chiral compounds [87]. In another study, the enantiomers of the chiral drug piperoxan were resolved on a micropreparative scale using the commercially available "Mini Prep Cell" from Bio-Rad (Hercules, CA, USA). This unit allowed under optimized conditions to resolve in a single run 0.5 mg of racemic piperoxan in the run time of 4.5–5.0 h. The electropherograms of fractions collected from the Prep Cell run indicated that the fractions were almost enantiomerically pure.

Lanz et al. [89] reported the enantiomeric separation of methadone by CD-based capillary and recycling isotachophoreses. The latter technique was also applied for micropreparative purposes using two commercially available units, the model RR3 and MiniPhor. Both systems allowed to achieve a partial separation of *R*-(–)-methadone and *S*-(+)-methadone being significantly enriched at the front and the back side.

Kaniansky et al. [90] have shown that similar to [15], electrophoresis in the capillary format may be directly applied for micropreparative purposes. Since commercial CE equipments with multiple capillary bundles (several hundreds of capillaries) are available, the techniques described in [15, 90] may gain more actuality. In the latter work, the authors initially showed on a theoretical basis that the sample capacity may be several orders higher when performing separations in the isotachophoretic mode compared to the CZE mode. The counter flow migration principle described in [15] for the capillary format has been applied by Glukhovskiy and Vigh [93] in the continuous free-flow electrophoretic system. Production rates of 2.8 mg h^{-1} were achieved for the enantiomers of terbutaline when using heptakis-6-sulfo-β-CD as a chiral resolving agent.

Ivory's group published very interesting examples of micropreparative enantioseparations based on the FCCE principle very similar to that described in [15]. This group also developed an electrophoretic separation system shown in Fig. 6 [91, 92, 94] as an analogue of the simulated moving bed (SMB) system well known in chromatography [95].

In all works devoted to micropreparative-scale enantioseparations using electrophoretic techniques, there are still many practical problems to be solved before these techniques become a viable alternative to other methods for obtaining enantiomerically pure compounds.

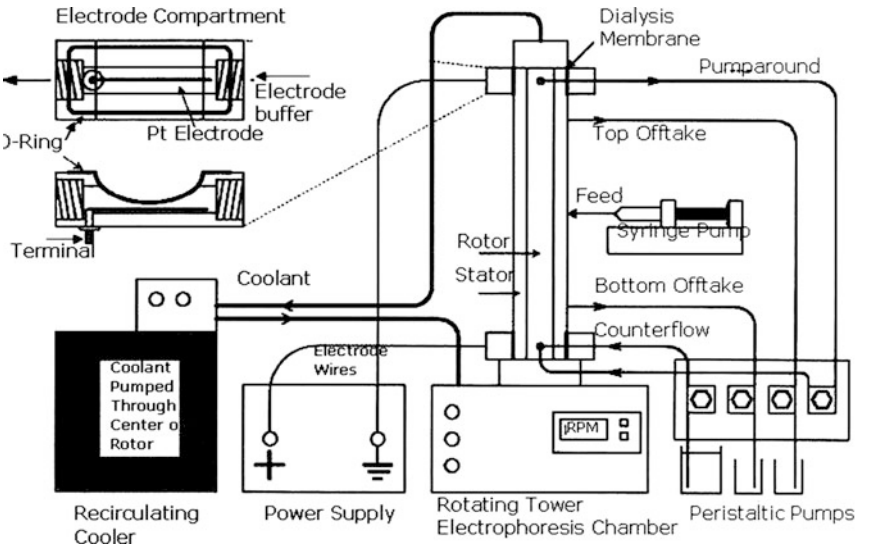


Fig. 6 A schematic diagram of the apparatus used for the continuous moving bed enantiomeric separation (reproduced with permission from [92])

3 Chiral Selectors

Although CDs and their derivatives remain the most widely used chiral selectors in CE, new chiral selectors are continuously proposed. Not all of the novel chiral selectors become widely used due to different reasons such as availability, costs, compatibility, and competitivity with established chiral selectors. However, some of them are interesting for mechanistic studies.

Several randomly and selectively substituted CD derivatives have been used as chiral selectors in CE. The suppliers of randomly substituted CD derivatives try to provide their products in a reproducible quality and well-characterized form. Nevertheless, the CD derivatives with a known derivatization pattern are recommended to be used for more or less deeply going into mechanistic studies as well as for the development of validated chiral CE assays. Derivatization of a CD in a designed way by selective activation and protection of the hydroxyl groups on the CD rim is a well-established procedure in carbohydrate chemistry.

The application of selectively substituted CDs in chiral CE has a relatively long history. The first charged CD derivative used for enantioseparation by Terabe was the single isomer 6-β-monoaminoethylamino-6-monodeoxy-β-CD [42]. Later, Nardi et al. [96] used also two single-isomer positively charged β-CD derivatives 6-monoethylamino-6-deoxy-β-CD and $6,^{A}6^{D}$-dimethylamino-dideoxy-β-CD for the enantioseparation of chiral 2-hydroxy acids. This topic attracted enhanced attention after the description of the syntheses and applications of the negatively charged

single-isomer CD sulfates by Vigh's group [97–103]. About 10 members of this family have been commercialized. Although useful for the enantioseparation of chiral cationic, anionic, neutral, and zwitterionic analytes, the single-isomer CD sulfates do not seem to be always superior from the viewpoint of chiral separation power compared to randomly substituted analogs such as commercially available β-CD sulfate or sulfobutyl-β-CD. However, unambiguous advantages of the single-isomer CD sulfates are better reproducibility of separation results achieved with this kind of chiral selector. Other advantage of these CDs is their perfect suitability for mechanistic studies. This relates not only to the reliable thermodynamic quantities obtained in binding studies or developing mathematical models but also to well-resolved signals in ^{1}H- and ^{13}C-NMR spectra. The latter makes single-isomer CD sulfates, especially well-suitable probes, for studies of structure of CD–analyte complexes in solutions based on intermolecular nuclear Overhauser NOE in NMR spectroscopy. One additional advantage of the CD derivatives described in [97–103], especially of heptakis-(2,3-diacetyl-6-sulfo)- β-CD [97, 104], seems to be its opposite enantiomer-binding pattern for enantiomers of many racemates compared to other CDs [18]. This property is interesting from both a practical and a theoretical point of view.

The syntheses and applications of novel single-isomer cationic derivatives of CDs have also been reported [105–113]. Few members of the family of single-isomer cationic CDs are commercially available from Cyclolab Ltd (Budapest, Hungary). Although these derivatives are rather expensive, the minute amounts of chiral selectors required in CE may facilitate a more extensive study of the commercially available single-isomer cationic CD derivatives as chiral selectors in CE.

Together with the above-mentioned cationic and anionic CD derivatives, few other representatives of this family such as the sodium salts of β-CD-6-monophosphate, the sodium salts of β-CD-6-monocarboxylic acid, and mono- and dicarboxymethyl-β-CDs were used for mechanistic studies [114].

Zwitterionic CD derivatives may represent some interest as chiral CE selectors. Several interesting applications have been reported [115, 116]. In these CDs, the anionic, neutral, and cationic forms may be switched by pH changes and this property seems to be attractive. However, it remains still to be proved that these derivatives may successfully cover the wide spectrum of cationic, anionic, zwitterionic, and neutral chiral analytes resolvable with the anionic or cationic CDs available. It seems noteworthy that zwitterionic mono-(6-δ-glutamylamino-6-deoxy)-β-CD described in [115] also represents a single-isomer chiral selector.

Neutral single-component CD derivatives are also commercially available. Cyclolab provides several single-component methylated, ethylated, and acetylated derivatives of α-, β-, and γ-CD. Chiari et al. [117] reported the use of one additional member of neutral CD polymer family for enantioseparations of cationic chiral analytes.

Summarizing this section it can be noted that there is no real urgent need to introduce new chiral selectors belonging to the CD family. In contrast, despite rather long-time studies, it seems that the binding and the chiral recognition mechanisms of CDs are still not well understood. Therefore, the designed synthesis of

CD derivatives with desired properties and the study of their interaction with chiral analytes using other instrumental and computational techniques in combination with CE seem to be rather important.

Together with CDs, other chiral selectors such as non-cyclic oligosaccharides and polysaccharides [118, 119], chiral surfactants [120–123], macrocyclic antibiotics [124, 125], proteins, peptides [126, 127], peptide libraries [128, 129], ligand exchange materials [130], and synthetic macrocyclic compound [131] can be used as chiral selectors in EKC.

4 Design and Adjustment of Enantioseparations in EKC

The rational design of a separation experiment is as complex as it is important. Once a decision has been made to perform an enantioseparation by EKC, then the questions concerning the most suitable mode, chiral selector, and separation conditions need to be answered. For the latter it is possible to use either more traditional single or multivariate approaches.

4.1 Enantiomer Migration Order

Enantiomer migration order (EMO) in chiral capillary electrophoresis represents a challenging issue. Observing a certain migration order of enantiomers might be required for practical reasons in the case of determination of the minor enantiomeric impurity in the presence of the major component. In addition, the determination of EMO may provide a very useful key for a better understanding of enantioselective intermolecular interactions. Despite the above mentioned, more than 80%, especially of earlier, publications devoted to the enantioseparations using CE do not touch the question of EMO. The main reason for this is that there is still no detector (polarimetric or circular dichroism) available for CE which can provide online information regarding EMO. First, the small capillary diameter requires a very sensitive detection system based on the energy-rich laser light sources and second, the presence of a chiral selector in the detection window commonly in more than a 100-fold excess creates serious problems for online determination of the EMO in CE. Thus, at least one of the enantiomers must be available in an optically pure or enriched form in order to obtain the information regarding the EMO by spiking the sample.

In chromatographic techniques, a reversal of the enantiomer elution order may take place only in the case when a chiral selector is exchanged with its counterpart having the opposite stereochemical configuration. This experience established in chromatographic techniques affected somewhat the philosophy regarding the EMO in early days of chiral EKC. Major chiral selectors in CE, such as cyclodextrins (CDs) and their derivatives, macrocyclic antibiotics, and peptides, are natural compounds available only in one stereochemical configuration. For this reason the reversal of enantiomer migration order was commonly unexpected at the first glance

for a given group of chiral selectors. In contrary to this assumption, multivariate scenarios may be observed for many chiral analytes from the viewpoint of EMO as it became obvious with the development of chiral CE. In many cases, even minor structural or chemical modification of a chiral selector may dramatically affect its chiral recognition properties and even revert the affinity pattern of analytes toward it. The reversal of EMO caused by opposite recognition pattern of a given mixture of enantiomers by two or more chiral selectors is discussed in this chapter later because this topic belongs more to molecular recognition than to separation science. These examples and their structural reasons at molecular level are summarized in many researches [132–139] and review papers [2, 17, 18, 139–141]. Below, at first the examples are overviewed in which a reversal of EMO is caused by the characteristics that are specific only for electrophoretic separation systems and cannot be observed in chromatographic techniques.

There are principal differences in the mechanisms determining the enantiomer migration order in CE and the enantiomer elution order in chromatographic techniques. The most distinct difference between CE and chromatographic techniques from the viewpoint of EMO is that in chromatographic techniques, the elution order of the enantiomers is solely determined by the affinity pattern of the enantiomers toward the chiral selector. This is not the case in CE. In the major chromatographic techniques with stationary chiral selectors, the more tightly bound enantiomer will elute as the second peak. However, in CE depending on mobility effects present in the separation system, the more strongly bound enantiomer may migrate either as the first or the second peak.

In chromatographic techniques, there is no alternative mechanism beyond the affinity reversal for a reversal of EMO. In contrast, CE offers various possibilities for a designed reversal of EMO without any change in the enantiomeric affinity pattern of the analytes toward the chiral selectors [2, 17, 18, 139–141].

The principal difference between chromatographic and electrophoretic techniques is the following. The electrophoretic (electrokinetic) migration mechanism makes it possible that the species residing in the same liquid phase migrate not only with different velocities but also to opposite directions [2]. The chromatographic (pressure-driven) migration mechanism does not allow different velocities for the species residing in the same phase and, moreover, opposite migration direction. Thus, separations can occur in electrokinetically driven systems in a monophase and the separation factors are determined not only by affinity coefficients but also by mobility vectors. The mobility term is responsible for basic differences between the enantioseparations in chromatographic techniques and EKC in general [2] as well as from the viewpoint of EMO [2, 17, 18, 139–141].

In order to achieve a separation in CE, a non-zero mobility difference ($\Delta\mu$)

$$\Delta\mu = \mu_1 - \mu_2 \neq 0 \tag{8}$$

must be generated between the enantiomers. The mobility difference being a velocity is a vectorial quantity and its numerical value can be determined based on Eq. (3) or its simplified version, Eq. (5).

The analysis of Eqs. (3) and (5) and the consequences related to the enantiomer migration order are discussed below.

One important finding already noted in the first overview of this topic [17] is that the vectorial property of $\Delta\mu$ allows the reversal of EMO just by reversing the polarity of the high voltage supply of the CE instrument. This means that if one can reverse the polarity of the high voltage supply and still manage to detect the enantiomers, then they will appear in the detection window in the opposite order compared to the original separation system (before reversing the polarity of the power supply) (Fig. 3) [19]. A short explanation is given below. If one considers that $\Delta\mu$ as a vectorial quantity has a positive sign of direction in the original separation system, then it must have a negative sign of direction in the separation system with reversed polarity. This means that

$$\text{if } \Delta\mu_{\text{original}} = \mu_R - \mu_S > 0, \text{ then } \Delta\mu_{\text{reverted}} = \mu_R - \mu_S < 0.$$

Thus, assuming $\mu_R > \mu_S$ in the original separation system, reversing voltage polarity will lead to $\mu_R < \mu_S$. The technical realization of this mechanism is shown in [17]. The most interesting feature is the fact that the migration order of enantiomers can be reversed without affecting enantioselective interactions in the separation system and by just applying an additional nonenantioselective migration force. Such forces are many and of different nature as it has been summarized in the earlier review article [17] and employed in many research articles published in the recent years [19, 47, 52, 142–149]. Some of these reversals of EMO were achieved by reversing the polarity of the high voltage supply.

As it can be seen from Eq. (5), not only the term $(K_R - K_S)$ but also the term $(\mu_f - \mu_c)$ determines the sign of $\Delta\mu$, i.e., the EMO in CE. In other words, the interaction with a stationary chiral selector always decelerates the analyte in chromatographic techniques. Therefore, the more tightly bound enantiomer elutes as the second peak. In contrast to this, a chiral selector may decelerate ($\mu_f > \mu_c$) as well as accelerate ($\mu_f < \mu_c$) analytes in CE. This means that the more tightly bound enantiomer may migrate as the first as well as the second peak in CE. This has been illustrated in the examples summarized in the review article [17] as well as in several research papers.

Charged chiral selectors can be used for separation of enantiomers at relatively low concentrations having decelerating effect on the migration of the analyte. Alternatively, after reversing the polarity of the high voltage supply, sometimes the same chiral selector can be used at higher concentration in the carrier mode transporting the analyte to the detector. The opposite migration order of the enantiomers will be observed as shown in [146–148].

One major difference between chromatographic and electrophoretic techniques is that in CE the enantioseparation can in principle be solely based on the mobility differences of the transient diastereomeric associates and does not necessarily require a difference in the equilibrium constants of the enantiomers with a chiral selector [2, 3, 7, 17].

In the particular case when there is no enantioselectivity present in the complex formation step, the EMO will be determined by the values of μ_{cR} and μ_{cS}. In the more general case, when both $K_R \neq K_S$ and $\mu_{cR} \neq \mu_{cS}$, the enantiomer migration order will be determined by the interplay of these factors also including the concentration of the chiral selector. One relatively old example of this kind of reversal of the EMO remained confusing for several years [150]. The sound explanation of this observation became possible after the studies by Rizzi and Kremser [4, 86] and the Scriba's group [151–158].

From Eq. (5), it is clear that if both the mobility and the affinity terms act in the same direction, i.e., $K_R > K_S$ and $\mu_{cR} < \mu_{cS}$, and the EOF does not affect the migration order, then the EMO is *S*-enantiomer before *R*-enantiomer. It will not be dependent on the concentration of a chiral selector. However, if $K_R > K_S$ and $\mu_{cR} > \mu_{cS}$, then an enantiomeric separation may not be observed at all or may be observed with the order *R* before *S* or the opposite, *S* before *R*. The difference in the binding constants and the mobilities of the noncovalent diastereomeric associates together with the concentration of the chiral selector will determine which of the aforementioned scenarios will actually be observed.

Charged chiral selector (CCS) may allow easy reversal of the enantiomer migration order depending on the pH of the background electrolyte, the concentration of the CCS, or the electric charge of the CCS. All these effects are based on the ability of CCS to migrate with an electrophoretic mobility that is different from the mobility of the bulk solution in a separation capillary. Thus, for example, the mobility of the weakly acidic carboxymethyl-β-CD (CM-β-CD) is pH dependent and increases with increasing pH of the background electrolyte, while the electrophoretic mobility of strongly acidic 1,1'-binaphthyl-2,2'-diyl-hydrogen phosphate (BHP) acid does not depend on the pH of the background electrolyte significantly. Based on the above mentioned, in the example shown in Fig. 7, the chiral selector migrates with lower, equal, and higher mobility compared with the chiral analyte depending on the pH of the background electrolyte. Based on this, the chiral selector decelerates, does not affect, or accelerates the velocity of the enantiomers of the chiral analyte. This results in a pH-dependent reversal of the enantiomer migration order [19].

Depending on the concentration of the CCS, it may decelerate the chiral analyte or accelerate it (usually at higher concentrations when used as a carrier). This will also result in a reversal of the enantiomer migration order in CE as this has been illustrated in the studies by Fanali's group for the cationic chiral analyte propranolol [159], by Mazzeo et al. for neutral analytes [44], and by our group for anionic analytes [160].

The reversal of EMO will be observed when two CCS' with the same affinity toward analyte enantiomers but with opposite electric charge are used for the enantioseparation of a given chiral analyte in the presence of the EOF in the separation system [160]. This is illustrated by Fig. 8 for the neutral chiral drug thalidomide. The origin of the reversal of EMO is that the negatively charged CM-β-CD decelerates the preferentially bound *S*-(–)-thalidomide (Fig. 8a), while the positively charged trimethylammonium salt of β-CD (TMA-β-CD) accelerates it (Fig. 8b).

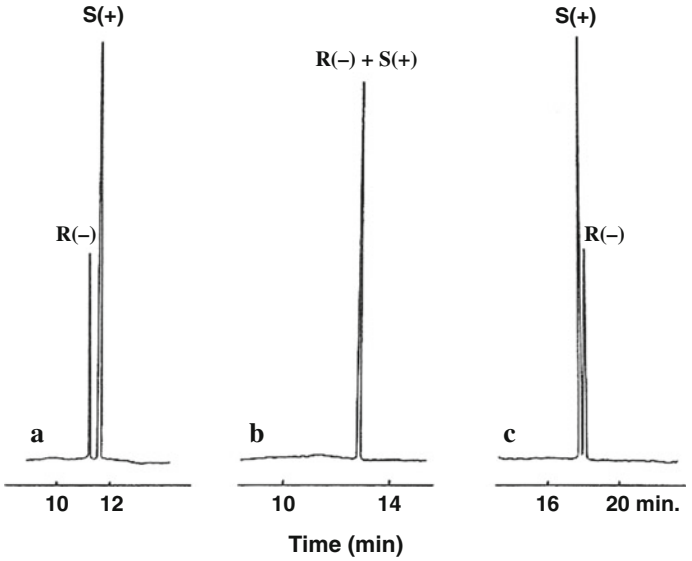


Fig. 7 Reversal of the enantiomer migration order of 1,1'-binaphthyl-2,2'-diyl-hydrogen phosphate (BHP) depending on the pH of the background electrolyte (reproduced from [19])

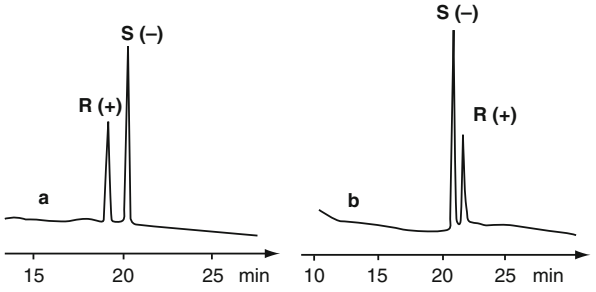


Fig. 8 Separation of enantiomers of thalidomide with 20 mg ml^{-1} CM-β-CD (**a**) and 20 mg ml^{-1} TMA-β-CD (**b**) (reproduced from [160])

The reversal of EMO is also feasible depending on the substitution degree of CCS. The example shown in Fig. 9 can be explained in the following way. Both the chiral analyte 1,1'-binaphthyl-2,2'-diamine (BNDA) and the chiral selector TMA-β-CD are positively charged. TMA-β-CD with a degree of substitution of approx. 1 possesses a lower self-mobility compared with the chiral analyte and decelerates it (Fig. 9a), while TMA-β-CD with the average degree of substitution of 3.5 possesses a higher self-mobility compared with the chiral analyte and accelerates it (Fig. 9b). Thus, in all of the above-mentioned examples, the affinity of the enantiomers to

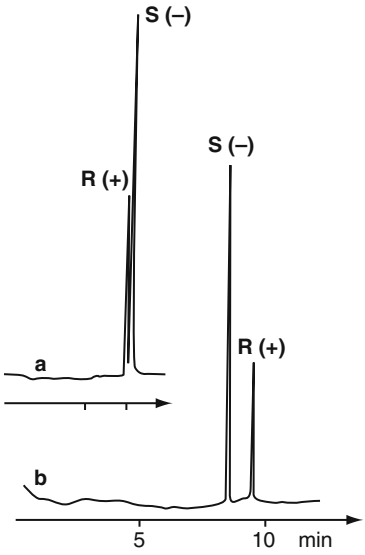


Fig. 9 Separation of enantiomers of 1,1'-binaphthyl-2,2'-diamine with TMA-β-CDs of average degree of substitution 1.0 (**a**) and 3.5 (**b**) (reproduced from [160])

pairs of chiral selectors was the same. The migration order reversal was based on the opposite effect of CCS on the mobility of analytes [140, 160].

4.2 Experimental Designs

4.2.1 One-Parameter Approach

Most commonly, enantioseparations in EKC have been optimized by changing one parameter at a time (univariate approach). The parameters optimized in chiral EKC are the nature and concentration of a chiral selector, the ionic strength, the pH, possible achiral additives to a separation buffer, the separation temperature, the material, dimensions, and the inner surface of the capillary. In many research papers, enantioseparations are studied depending on the above-listed or some other variables. Currently, these studies as well as simple screening of chiral selectors for a wide variety of chiral analytes have become somewhat less important because a large body of papers of this kind have already been accumulated. It seems rather more interesting to rationalize our knowledge in chiral EKC by studying structure–binding and structure–chiral recognition relationships in more details. For a particular separation problem, however, the adjustment of the separation by a variation of the separation conditions will still remain necessary. To this group of problems may belong a particular drug and its metabolites, environmental pollutants and their degradation products, important synthetic chemicals in combination with their precursors, and possible side and/or degradation products. This chapter does not summarize the effect of particular variables on the enantioseparation in CE because this topic has been extensively discussed in previous publications [141, 161].

As mentioned above, many papers concerning the effects of different variables on chiral CE separations are published. However, only few of them represent a systematic approach to cost- and time-effective method development based on the univariate approach. The subject has been reviewed by Fillet et al. [162], who, in analogy to other published works [163], also suggested a method development chart and illustrated its suitability for achieving the enantioseparation of 48 of 50 chiral analytes tested. These are quite useful schemes. However, one has to keep in mind that every chart is somewhat intuitive and based on empirical knowledge, even on the personal experience of the authors. Because the charts are a priori based on a limited number of experiments, its predictive power may be limited. Although widely used, the univariate approach requires a large number of independent experiments because typically chiral EKC involves a large number of different parameters that may have to be studied one after the other. This approach is experimentally reliable and, when properly designed, it provides besides the optimization of the separation conditions some direct or indirect information about the separation mechanism. However, the univariate approach is not ideal from the viewpoints of method development with respect to time and costs (personal and equipment time, reagents, etc.). Chemometric experimental designs may provide significant help in the fast development of chiral CE methods.

4.2.2 Chemometric Experimental Designs

The goal of statistical design techniques is the reduction of the number of experiments required for optimization and to consider the possible interdependence of parameters. This is very important in CE because the separation variables are usually interdependent in this technique.

Several types of designs are available, and the choice of design depends on the number of variables and how detailed the information has to be. A full factorial design is a good choice when the number of variables is four or less. When more than four variables are of interest, a fractional design is applicable. With a large number of variables, a Plackett–Burman design (PBD) [164, 165] is the preferred choice.

A factorial design is a statistical method in which all possible factor combinations are considered, allowing the calculation of the single effects of each factor and any factor interactions. The number of experiments required can be calculated using the following equation:

$$N_{\text{exp}} = m^n \tag{9}$$

where n is the number of factors and m is the number of levels (number of values of each factor). The number of experiments required for factorial designs increases quickly with an increasing number of factors to be optimized. If, for example, one needs to optimize the concentration of the chiral selector, the ionic strength and pH of the buffer, the applied voltage, and the amount of organic modifier in the

background electrolyte (five parameters), each one at three levels, the number of experiments required will be $N_{exp} = 3^5 = 243$.

Statistical techniques such as central composite designs (CCDs) developed by Box [166] and the PBD [164] require a smaller number of experiments and lead in general to the same result.

The number of experiments required in CCD is

$$N_{exp} = 2^n + 2n + 1 \tag{10}$$

where n is the number of factors to be optimized [167].

CCD provides data which are sufficient for the fitting of a linear polynomial model to a set of data. Regression analysis can be used for such a model which enables one to predict the response at levels of the variables within the factor space not investigated in the design. The response in the case of CE can be resolution or selectivity and factors can be the concentration of the chiral selector, the pH value, or the ionic strength of the buffer.

Small et al. [168] achieved a rapid optimization of the chiral CE separation of amlodipine using the CCD technique. In this study the response surface was modeled for three factors by fitting a second-order polynomial in four dimensions. The number of experiments was $N_{exp} = 2^3 + (2 \times 3) + 1 = 15$ by taking the pH of the background electrolyte, the separation temperature, and α-CD concentration as factors. The data acquired from this CCD were analyzed to model a four-dimensional response surface. The optimum conditions predicted by CCD were used experimentally and resulted in a baseline separation of the enantiomers. The experimental results observed for P_i and R_s are in excellent agreement with the predicted values. Although the response surface is four dimensional, it can be readily visualized as a three-dimensional graphic by presenting the response to two factors, while the third is kept constant at its optimum value.

Wan et al. [169] reported the use of a full factorial design for the optimization of pH and sodium dodecyl sulfate (SDS) concentration in the enantiomeric separation of amino acids (AAs) which were derivatized with (–)-1-(9-fluorenyl)ethyl chloroformate (FLEC). For the design, 10 experiments, including two center points, were performed utilizing a mixture of four different FLEC-AAs [threonine (Thr), isoleucine (Ile), valine (Val) and phenylalanine (Phe)]. The results obtained from the optimization indicated that a high pH was necessary for the enantiomeric separation of these four FLEC-AAs and that the different AAs showed optimal resolution at different SDS concentrations. For example, the first-eluted analyte, Thr, has a relatively high optimal SDS concentration of about 60 mM. In contrast, for the last-eluted analyte, Phe, the SDS concentration should be as low as possible. This result was explained by the higher hydrophobicity of Phe compared to Thr. A buffer containing an intermediate concentration of SDS, 20 mM, and with a high pH, 9.2, was therefore selected in order to facilitate the separation of a maximum number of FLEC-AAs. As a result, 11 of the 19 FLEC-AAs examined were baseline separated.

The important advantage of a full factorial design for the optimization of chiral EKC separations is that it considers nonlinear changes of parameters and the interrelation between them.

The most critical point of this technique is the selection of the low and high limits of the designed parameters. A good knowledge of the separation system is generally required to do this properly. In a situation where the high and low limits have been incorrectly selected, the experiment will be misleading as to the direction in which the optimum will be found [169]. CCD was shown to be a useful approach for optimizing EKC enantioseparation conditions of a mixture of five racemic amphetamine analogues. The same group illustrated that experimental designs offer an efficient test for the robustness of the analytical method [170]. Several papers have described the use of PBD [170–174] or Box–Behnken designs [175–178] for the optimization of CE enantioseparations.

A simultaneous application of a fractional factorial design and a central composite design for the optimization of the chiral EKC separation of epinephrine enantiomers has also been reported [173, 174]. In this study a compromise between conflicting goals, such as maximization of resolution and minimization of analysis time, was achieved by introducing a desirability function *D*. Balancing these goals, the most acceptable solution to the problem was found and the optimized method gave a fast separation with baseline separation of the epinephrine enantiomers.

The PBD focuses on the main effect of the factors and is especially effective when the number of variables is high. The limitation of this technique compared to full factorial designs is that the former does not allow considering easily the interdependence between the parameters. In addition, similar to all first-degree factorial designs, the Plackett–Burman design also assumes a linearity of the estimated variables over the whole range of the experiment. In chiral EKC, however, nonlinear dependencies are quite common.

5 Mathematical Models of EKC Enantioseparations

Mathematical models have the advantage that in an ideal case, they not only describe a separation as an entire process but also may allow to find out the effects which are difficult to be turned out based either on the intuitive or on the empirical approach. The first mathematical model proposed for an optimization of the selector concentration in chiral EKC was proposed by Wren and Rowe [14, 179]. This model is based on the same equation as an earlier developed model by Stepanova and Stepanov [180] for the electrophoretic separation of cations.

The model described in [14, 179] allows to optimize the concentration of a chiral selector which may result in a maximum mobility difference between the enantiomers. Although the model proposed by Wren and Rowe allows optimizing only one separation parameter, in particular the concentration of a chiral selector, among many variables, it attracted much attention by the researchers perhaps most likely

due to its relative simplicity. The second reason for a wide acceptance of this model seems to be the fact that the chiral selector concentration which this model allows to optimize represents one of the major variables in chiral CE.

Two critical points should be mentioned when applying the aforementioned model for optimization purposes in chiral CE: (1) the maximum mobility difference between the enantiomers does not a priori mean the maximum resolution and (2) the model does not cover several important parameters affecting chiral CE separations. However, this model without any doubt markedly contributed to the development of chiral CE and good correlations between the values of the optimal chiral selector concentrations calculated based on this model and observed experimentally have been reported [182–185].

The duoselective chiral separation model proposed by Vigh and co-workers [186–191] in the initial form covers besides the selector–selectand interactions the acid–base equilibrium of the analyte also and thus allows optimizing the pH of the background electrolyte also. This is a rather advanced model compared to that described in [14, 179]. The authors of the duoselective separation model made interesting predictions about the reversal of EMO based on the calculations. Although this effect in most cases may be predicted intuitively without any cumbersome calculations, the above-mentioned prediction proves the correctness and the power of the model. Later the duoselective separation model was extended to other separation parameters [182]; among them the inclusion of the effect of the EOF seems to be the most important point. The authors of [187–191] consider the EOF as a nonenantioselective transport in chiral CE. Although the mathematics is elegant as well as the examples are illustrative in the papers [187–191], we consider the EOF and the electrophoretic mobility of the analyte as equal transport phenomena from the viewpoint of enantioseparations [2, 3].

Further advancement of mathematical models of chiral CE was the development of a chiral charged resolving agent migration model (CHARM) by the same group [190]. This model allows predicting several nontrivial phenomena. Many of these predictions were experimentally verified mainly by the authors' group as well as by other research groups [190–193].

Gareil et al [194], Surapaneni et al. [195], Crommen and co-workers [196, 197], and Desbene et al. [198] have proposed mathematical models optimizing dual chiral separation systems. All of these models allow in principle a more or less complete description of the separation system if one suggests that the boundary conditions are correctly selected. However, the last is the bottleneck of any (mathematical) model which is a product of our imagination. When developing a model, criteria must be found allowing an estimation of the model approaching reality. A model in an ideal case should allow predicting effects which are nontrivial and impossible to be derived by a simple logistic or intuitive way. One of the most important requirements to a model is to be elegant, simple, and understandable for most of the researchers working in the field. Thus, the scientists involved in the development of models have to consider the old wisdom: "One of the principal objects of theoretical research in any department of knowledge is to find the point of view from which the subject appears in its greatest simplicity" [199].

6 Molecular Mechanisms of Selector–Selectand Interactions in CE

As already mentioned above, the enantioseparation can be split into two processes: chiral recognition and the transformation of a chiral recognition into a chiral separation, i.e., the generation of enantioseparation from enantiorecognition.

A correlation between recognition and separation is simpler in pressure-driven techniques compared to electrically driven techniques. As stated in our previous papers [2, 3], in CE, in contrast to chromatographic techniques, a chiral separation is possible even in the absence of a chiral recognition in the classical meaning of this definition. On the other hand, a chiral recognition not always leads to chiral separation in CE. Thus, enantioseparation in CE may be a result of stereoselective selector–selectand interactions and achiral selector–selectand interactions but different mobility of the diastereomeric associates formed, or the combination of both. Independent of which of the aforementioned three mechanisms is involved in a particular case, selector–selectand interactions are an unavoidable part of any chiral CE separation [200].

6.1 Determinations of Stereoselective Selector–Selectand Binding Constants Using CE

CE offers significant advantages for the determination of stereoselective selector–selectand binding constants in the way that the constants can be calculated under the same or very similar to the separation conditions. This means that the binding constants determined in CE may correlate best with separation.

The equation for the description of equilibrium in electrophoresis was introduced by Tiselius [201]. Rundlett and Armstrong [202, 203] summarized and updated this topic. In the review paper [204], a critical treatment of the subject is given and together with advantages of CE for the binding studies, such as high efficiency, ease of automation, short analysis time, small sample size, and buffer volume, limitations of this technique and possible error sources in CE binding constant determinations are also addressed. In order to avoid duplications with above-mentioned papers which are advised for additional reading, some fundamental aspects of the subject are omitted in this chapter.

One important advantage of CE which does not seem to be adequately addressed in earlier studies is the possibility to study the binding of a given solute to multiple hosts and vice versa, or the competitive binding of multiple guests to a single host or even the combination of both. This potential seems especially challenging with the increasing activity in the fields of combinatorial synthesis and high-throughput screening.

The dependence of a solute mobility on the concentration of a selector represents basic information for the calculation of the binding constants. Therefore, the experimental data must be refined in the way that only the effect of the binding with a

chiral selector on the mobility of analyte enantiomers are included in the final plot. Thus, the effects of increasing viscosity of the BGE with increasing concentrations of a chiral selector, a possible selector adsorption on the capillary wall, an increasing ionic strength of the BGE, and a variation of conductivity must be eliminated from the observed overall mobility. In addition, the equilibrium timescale must be faster than the CE separation timescale and the concentration of the chiral selector must be varied in a wide range in order to adequately cover binding isotherm [204]. Two additional points seem worth to be mentioned. First, as in most other techniques, concentrations are used instead of activities in CE. Therefore, the binding constants are not true thermodynamic equilibrium constants but apparent constants. Second, almost all equations used for equilibrium constant determinations in CE assume 1:1 stoichiometry while actually complexes with different stoichiometries can be encountered.

Several interesting studies on the determination of stereoselective binding constants using chiral CE have been published in last 2 years [7, 152]. A critical discussion on the general aspects of this technique was published by Vespalec and Bocek [204].

6.2 UV, NMR, and ESI–MS Studies of the Stoichiometry of Selector–Selectand Complexes

UV-VIS spectrometry has been established for long time as the useful technique in studies of intermolecular noncovalent interactions. Both the stoichiometry and the equilibrium constants of selector–selectand complexes may be determined using this technique. Although the shift of the absorbance maximum of a selectand (or selector) is usually affected by complexation, the more direct information on the involvement of both counterparts in the complex formation may be obtained from the changes of the molar absorption coefficient (ε). Experimental data are commonly treated according to the continuous variation plot (Job's plot) [139, 141, 205, 206] for obtaining the information on the stoichiometry of the complexes and according to Scott's technique for the determination of the equilibrium constants [139, 141, 207]. The advantage of UV-VIS spectrometry is the relative simplicity, availability, and universality. The disadvantage of this technique is that it commonly does not provide different signals for the diastereomeric complexes between the enantiomers and a chiral selector. For this reason, either pure enantiomers are required to be used in the experiment or the information obtained will not be enantioselective. This fact may be one of the reasons limiting the use of UV-VIS spectrometry for the description of selector–selectand associates related to chiral EKC [206, 208].

The most distinct advantage of NMR spectroscopy compared to UV and many other spectrometric techniques is that the former may in principle provide two sets of resonance signals for noncovalent diastereomeric associates between the selector and the selectand enantiomers. Thus, the NMR spectroscopy may allow the application of racemic samples or nonracemic mixtures of enantiomers for the

stereoselective determination of the stoichiometry and equilibrium binding constants of selector–selectand complexes. Besides the easier availability of racemic analytes, NMR spectroscopy offers the possibility of competitive binding studies. This means that the interaction of one of the enantiomers of an analyte with a chiral selector may be studied in the presence of the opposite enantiomer which approaches well the real conditions in chiral CE separations. An additional advantage of NMR spectroscopy is that it provides a multiple set of data based on a single set of experiments. UV-VIS spectrometry and most other instrumental techniques provide a change in averaged molecular characteristics (specific molar absorbance, shift of absorption maximum, solubility, etc.), while NMR spectroscopy provides distinct signals for each odd atom, mainly proton and C13, involved in different fragments and functional groups of a selectand and a selector molecule. Thus, NMR spectroscopy may provide statistically more reliable data for the characterization of selector–selectand complexes.

Technical aspects of stoichiometric determination of selector–selectand complexes based on NMR spectroscopy have been summarized in several works [132, 134, 137, 139, 141, 206, 208–215]. The most convenient way seems to be a preparation of equimolar solutions of a selector and a selectand and mixing them in the ratio 10:0; 9:1; 8:2; . . .;0:10 and the measurement of the NMR spectra of these samples. A plot of $\Delta\delta \times \frac{[\text{selector}]}{[\text{selector}]+[\text{selectand}]}$ vs. $\frac{[\text{selector}]}{[\text{selector}]+[\text{selectand}]}$ results in a continuous change as illustrated by Fig. 10 [114]. The maximum of this plot indicates the stoichiometry of the complex. Although the construction of a Job's plot is easy, certain experience is required in order to give a correct interpretation to the observed dependencies. Special care must be taken in order to differentiate between uniform and multiple complex formations. The experimental data cannot be treated according to a continuous variation plot in the latter case because the chemical shifts of

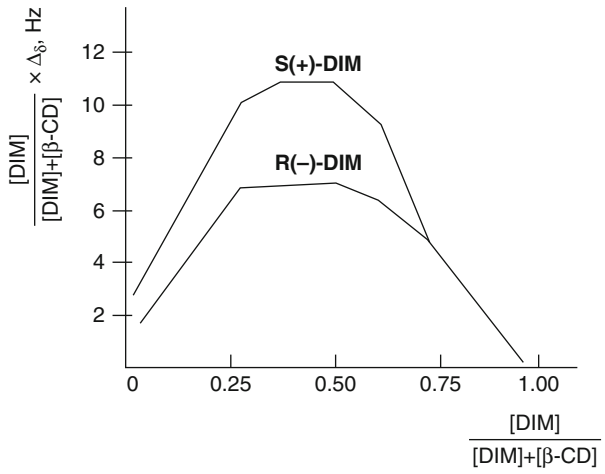


Fig. 10 Job's plot for (±)-DIM/β-CD complex (reproduced with permission from [114])

the resonance signals due to complex formation, so-called complexation-induced chemical shifts (CICS'), are not additive in this case. Additional care must be taken when a maximum is not sharp on a continuous variation plot. This may be the indication for the formation of a very weak complex, as well as multiple complexes. For instance, the Job's plot shown in Fig. 10 indicates that the stoichiometry of the complex between dimethindene (DIM) and β-CD is a simple 1:1 association. However, the plot does not have a characteristic sharp maximum. Further studies using ESI–MS confirmed the formation of a minor amount of the complex with 1:2 stoichiometry besides a complex of 1:1 stoichiometry (Fig. 11) [114].

Thus, as this example shows, NMR spectrometry, which is in general a very powerful technique, may not always be applicable in studies of selector–selectand complexes. One additional example of this kind is shown in Fig. 12 [114]. The data shown in this figure indicate that the continuous variation plot cannot be constructed due to multiple complexations between the DIM and the CM-β-CD. Despite this failure, the data shown in [117] are very informative. Besides the aforementioned multiple complex formation, these data indicate that the complexes formed have a different stoichiometry and in addition, the chiral recognition pattern in the complexes with different stoichiometry is opposite to each other. The latter seems to be the unique and the most interesting result of this experiment.

In certain cases, ESI–MS experiments can complement data obtained using NMR spectrometry as was mentioned above (see Fig. 11). The advantage of ESI–MS is that this technique provides the information about the *m*/*z* ratio of the complex and

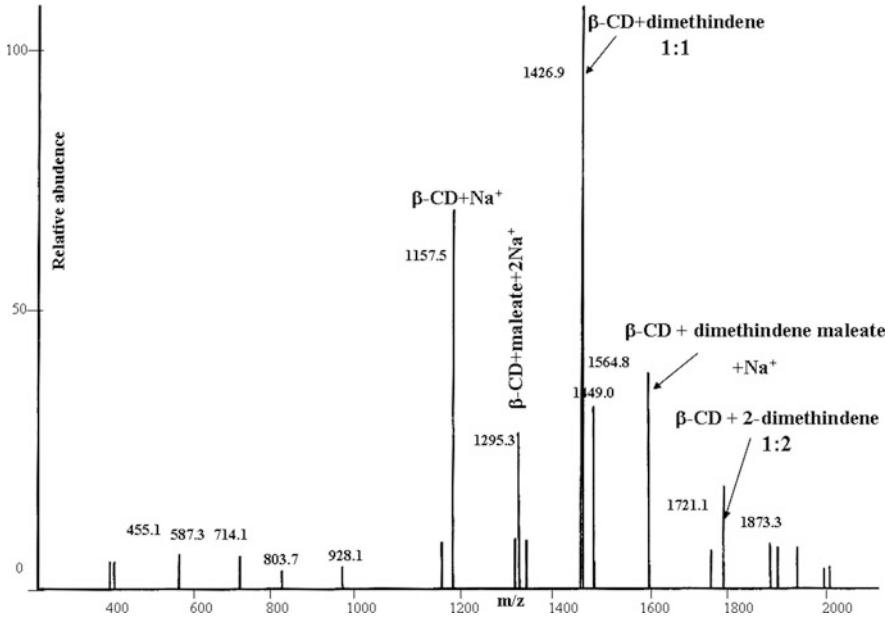


Fig. 11 ESI–MS spectrum of (±)-DIM/β-CD mixture (reproduced with permission from [114])

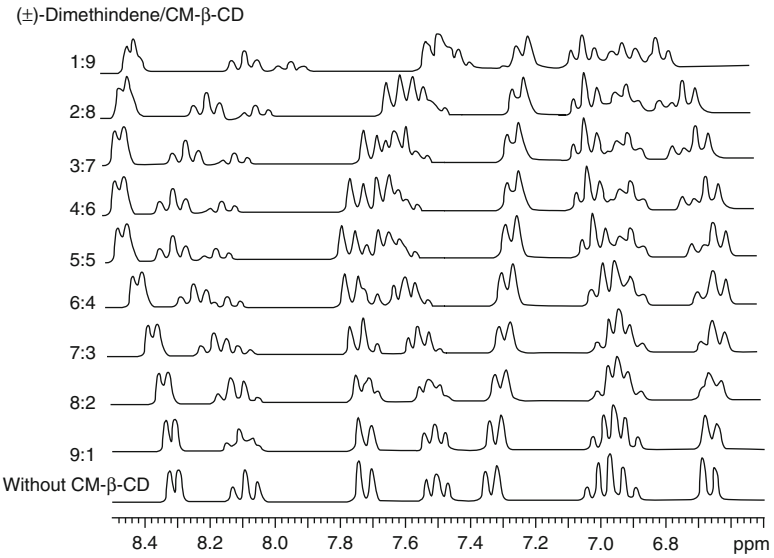


Fig. 12 H-NMR spectra of (±)-dimethindene/carboxymethyl-β-CD mixture with different molar ratios (reproduced with permission from [114])

this way the stoichiometry from a single experiment. This is impossible using other techniques mentioned above which require a set of experiments and therefore are time consuming and expensive. In the ESI–MS studies, care must be taken due to the possible formation of "false peaks" [216]. This problem can be avoided by optimization of experimental conditions and solved by the addition of some standard compounds [137].

Together with ESI–MS, other soft-ionization MS techniques such as matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and fast atom bombardment (FAB) MS may be used for the determination of the stoichiometry of selector–selectand complexes.

6.3 NMR Spectrometric Studies of the Enantioselective Selector–Selectand Binding Constants

Various techniques which are suitable for the determination of equilibrium constants in noncovalent interactions [207, 217, 218] may also be applied to chiral CE-related studies. The applications of fluorescence [219] and circular dichroism spectrometry [220] have been reported. The advantage of the latter is that it is a chiroptical technique. The applications of other techniques, such as microcalorimetry or electron-spin resonance, although it may be very useful, have not yet been reported in the studies related to chiral CE.

At present, NMR spectroscopy remains the major technique used for the determination of selector–selectand binding constants related to chiral CE. Techniques and treatment of experimental data have been described in several studies [7, 139, 141, 200]. The effect of the increasing amount of a selector on the CICS of an NMR-active nucleus of a selectand (or vice versa) such as ^{1}H, ^{13}C, and ^{19}F, is measured. It is important to cover the widest available concentration range of a chiral selector. Provided that CICS is stereoselective, a racemic or an optically impure selectand can be used and the competitive binding constants between enantiomers and the chiral selector may be determined.

Experimental data may be treated according to Scott's modification [207] of the Benesi–Hildebrand equation [221]. Technically easier and more exact may appear other methods described especially for the treatment of NMR data [222]. The known stoichiometry of the complex is a prerequisite for obtaining correct binding data. Almost all techniques described in the literature are suitable for 1:1 complexes. The formation of complexes of other stoichiometry may significantly complicate the treatment of the data or introduce a significant source of error in the calculations. Nonlinear curve-fitting techniques may avoid the problems of this kind.

Among NMR-binding studies performed in order to explain the results observed in chiral CE, the example of chiral Ca^{2+}-blocking drug verapamil (VP) seems to be rather illustrative [214]. The migration times of the enantiomers of VP in CE were much longer in the presence of β-CD compared to equimolar amounts of heptakis-(2,3,6-tri-*O*-methyl)- β-CD (TM-β-CD). However, the enantioseparation factor was higher in the latter case and the migration order of the enantiomers was opposite to each other in the presence of these two CDs [214]. The binding studies performed using NMR spectroscopy clearly indicated that the enantiomers of VP possess a higher affinity toward β-CD ($K_{(+)} = 279 \pm 34\ M^{-1},\ K_{(-)} = 207 \pm 59$) compared to TM-β-CD ($K_{(+)} = 6 \pm 1\ M^{-1},\ K_{(-)} = 30 \pm 7$). The enantiomers are more enantioselectively recognized by TM-β-CD (the enantioselectivities of the binding were 1.3 and 5.0 toward β-CD and TM-β-CD, respectively). In addition, (+)-VP possesses a higher affinity toward β-CD, whereas (−)-VP possesses a higher affinity toward TM-β-CD. Thus, the binding studies performed using NMR spectroscopy allowed in this particular case to explain rather unusual effects observed in chiral CE.

Although it is suitable, NMR spectroscopy is not recommended to be used just for screening of chiral selectors due to following reasons: (a) the NMR spectroscopic experiment is commonly more time consuming and expensive compared to CE; (b) enantioselective CICS' observed in NMR spectroscopy do not a priori translate to an enantioseparation in CE. The possible reasons for this discrepancy between the two techniques have been discussed earlier [139, 141].

In general, for a given pair of a chiral selector and a selectand, one may expect a higher enantioseparation power in CE compared to the enantiorecognition ability in NMR spectroscopy. In particular cases, however, NMR spectroscopy may provide an indication for a chiral recognition in those selector–selectand pairs which have been considered to be unsuccessful based on the CE experiment alone. For instance, native β-CD has been suggested to be not a suitable chiral selector for the enantioseparation of the cationic form of chiral cholinergic drug aminoglutethimide

(AGT) in contrast to other two native CDs (α and γ) which allowed baseline enantioseparation of AGT [223]. In contrast to the CE data, NMR studies indicated the most pronounced interactions (reflected in the strongest upfield shift of H-3 and H-5 CD protons) between AGT and β-CD among three native CDs (Fig. 13) [137]. These data were also supported by ESI–MS studies of comparative affinity of AGT enantiomers toward these CDs [137]. Careful optimization of the separation in CE allowed resolving the enantiomers of AGT also with β-CD. The migration times were longest in the presence of β-CD and in addition, the enantiomer migration order was opposite compared to two other native CDs (Fig. 14). Thus, in this particular case, NMR and ESI–MS studies allowed to optimize the enantioseparation in CE and to find an example of opposite affinity of the AGT enantiomers toward native CDs.

The binding constants determined by using NMR spectroscopy as an alternative technique can be very useful for explanation of some distinct effects observed in CE experiments. Thus, in the recent studies by Castro-Puyana et al., the reversal of EMO was observed for the enantiomers of some chiral antimycotic drugs depending on the concentration of a chiral selector, hydroxypropyl-β-CD (HP-β-CD) (Fig. 15) [224]. The binding constant determination performed with both CE and NMR spectroscopy (Table 1) together with mobility determinations performed by using CE clearly indicated that at the lower concentration of HP-β-CD, the binding of enantiomers with the chiral selector was a major contributor to enantiomeric separation. At higher concentration of HP-β-CD, the mobilities of transient diastereomeric associates (and not the affinities to the chiral selector) were a major contributor in observed enantiomeric separation [7]. This study also indicated that the binding

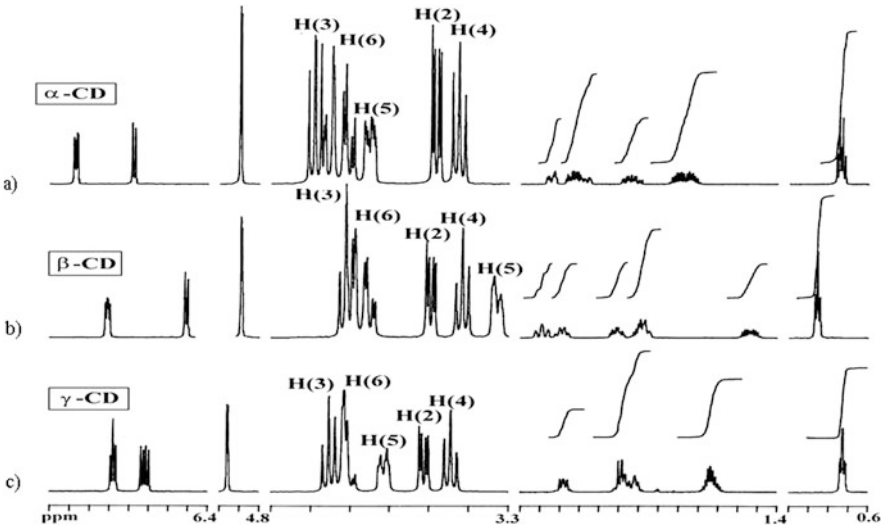


Fig. 13 The 600-MHz ^{1}H-NMR spectra of (±)-aminoglutethimide in the presence of two equivalents of α-CD (**a**), β-CD (**b**), and γ-CD (**c**) (reproduced with permission from [137])

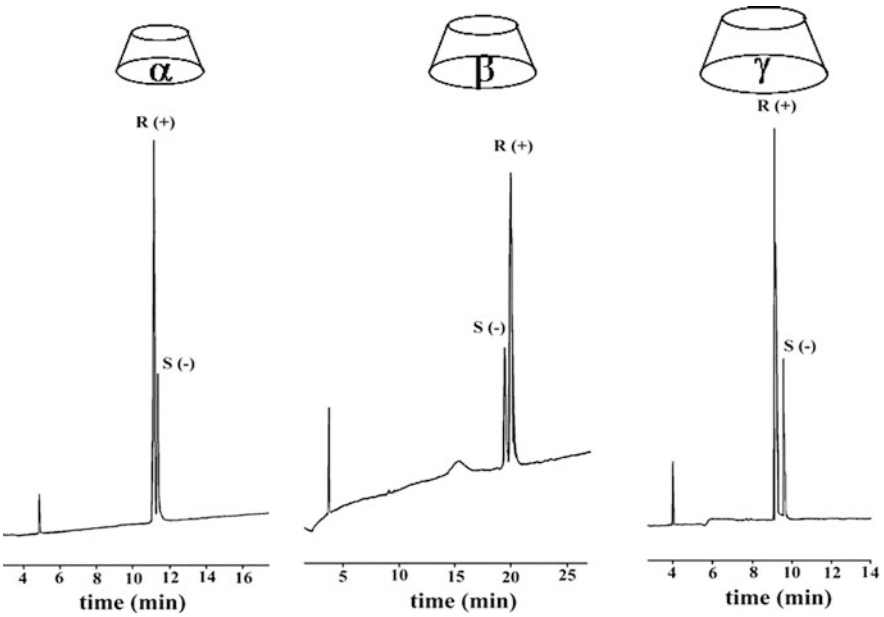


Fig. 14 Electropherograms of aminoglutethimide enantiomers [(+)/(−) = 2/1] in the presence of (**a**) 10 mg ml^{-1} α-CD (MW 973, 10.3 mM); (**b**) 10 mg ml^{-1} β-CD (MW 1135, 8.8 mM); and (**c**) 10 mg ml^{-1} γ-CD (MW 1297, 7.7 mM) (reproduced with permission from [137])

Table 1 Averaged and apparent equilibrium constants (K_a) and the electrophoretic mobilities (μ_c) of transient diastereomeric associates determined using CE (reproduced with permission from [7])

Temporary diastereomeric associate	Apparent equilibrium constant (M^{-1})	Electrophoretic mobility ($m^2V^{-1}\,s^{-1} \times 10^{-9}$)
2R4S-KC/HP-β-CD	427 ± 11	3.65 ± 0.09
2S4R-KC/HP-β-CD	456 ± 9	3.85 ± 0.08
(+)-TC/HP-β-CD	432 ± 6	3.70 ± 0.05
(–)-TC/HP-β-CD	452 ± 8	3.79 ± 0.04

constants determined using CE and NMR spectroscopy may correlate with each other quite well on a qualitative level.

6.4 NMR Spectroscopic Studies of Structure of Selector–Selectand Complexes in Solution

NMR spectroscopy has been well established as one of the major techniques suitable for studies of noncovalent intermolecular complexes in solution [225]. The

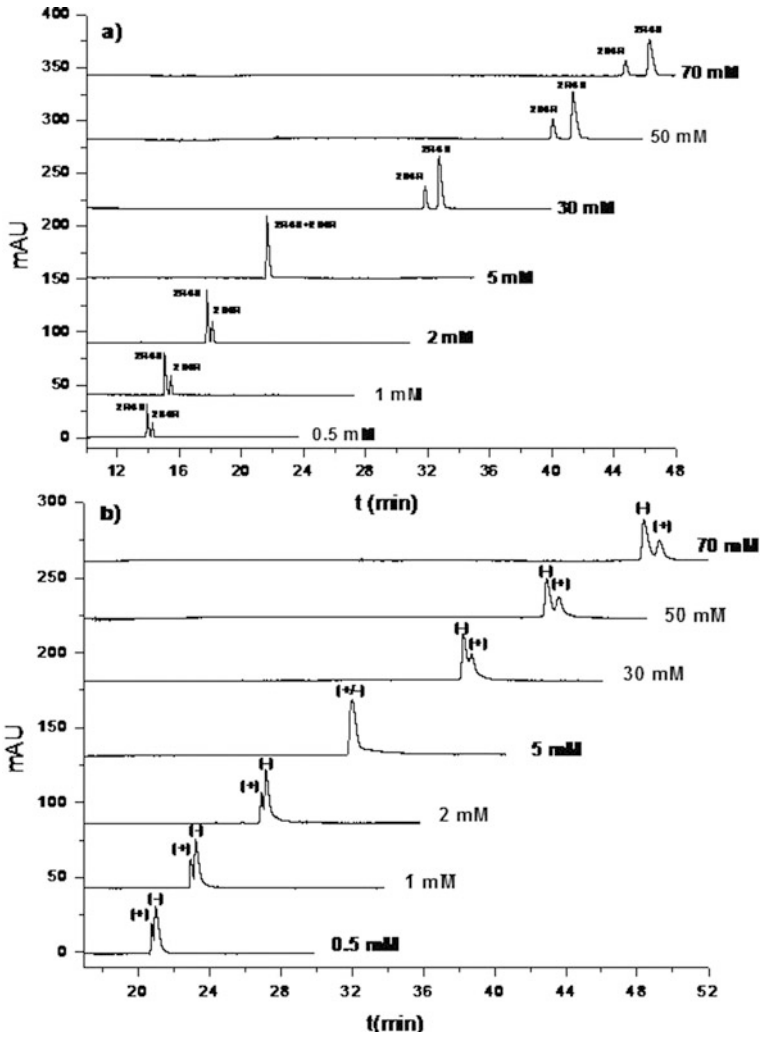


Fig. 15 Effect of the concentration of HP-β-CD on separation of enantiomers of ketoconazole (**a**) and terconazole (**b**) (reproduced with permission from [7])

chemical shift pattern, line-shape analysis, and NOE may be used in order to obtain information on the structure and the dynamics of the complexes. The data obtained using NOE are easier to interpret and seem to be more direct. Several studies illustrate the feasibility of this technique for structural elucidation of complexes relevant to chiral CE [132–137, 139, 141, 208, 213, 226]. It does not seem reasonable to perform an NOE experiment in a routine way for any selector–selectand complex because this experiment requires measurements with rather strong magnets and may be very expensive and time consuming. However, in certain cases, NOE studies

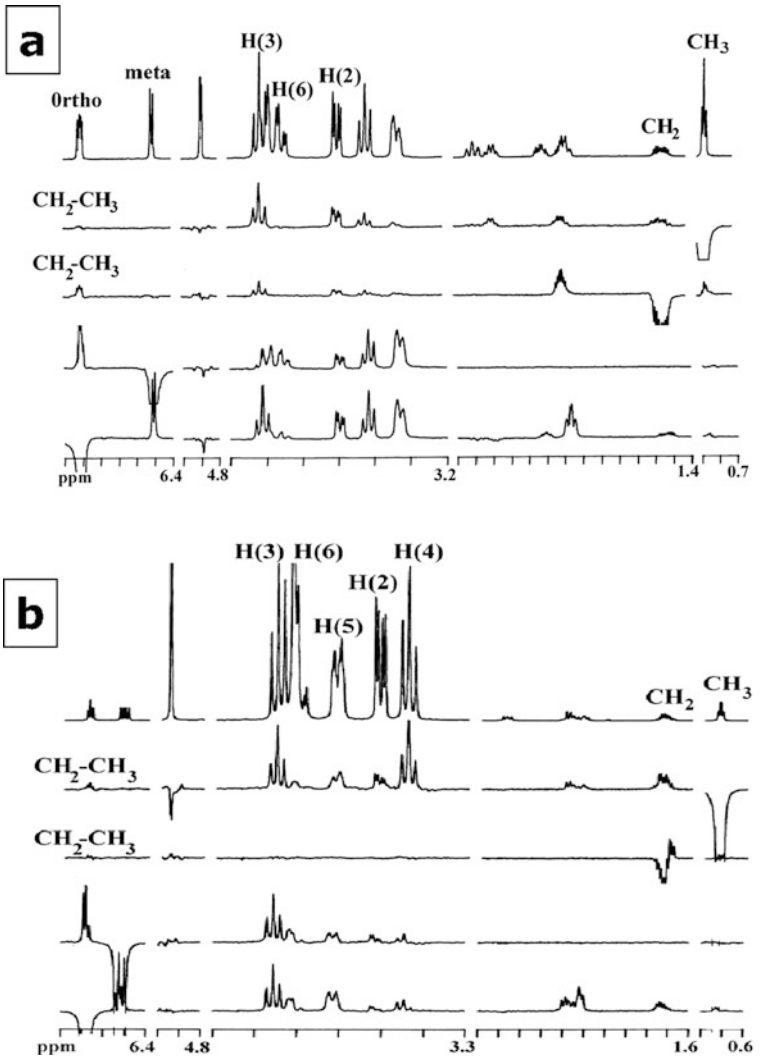


Fig. 16 1D-ROESY spectra of (±)-aminoglutethimide and two equivalents of β-CD (**a**) and γ-CD (**b**) (reproduced with permission from [137])

provide unique information which is impossible to obtain using any alternative technique. One example of NOE studies related to chiral CE is shown in Fig. 16 [137]. Rather strong "NOE-like" effects observed on the external CD protons in this experiment make it questionable whether the structure represented in Fig. 17a for AGT/β-CD complex is the only possible structural element of this complex or if the alternative structures are also present.

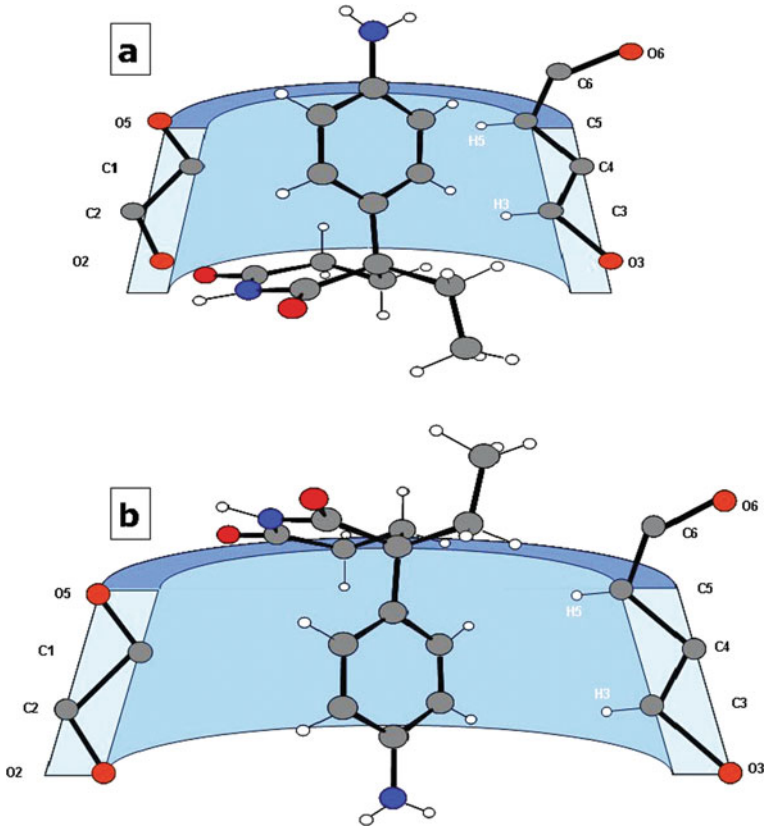


Fig. 17 Structure of aminoglutethimide complexes with β-CD (**a**) and γ-CD (**b**) derived based on the 1D-ROESY spectra depicted in Fig. 16 (reproduced with permission from [137])

In contrast to the AGT/β-CD complex, the NOE experiment in the case of (±)-AGT/γ-CD complex supports a complex formation from the narrower primary side of γ-CD with amino group ahead (Fig. 17b). The glutarimide ring is apparently less involved in the complex formation in this case. However, the involvement of the methyl group in complex formation by a still unknown mechanism cannot be completely excluded. The structure of the (±)-AGT/γ-CD complex depicted in Fig. 17b was derived by saturation of the aromatic protons of AGT. The structure was also supported by the data which were obtained when the γ-CD protons were saturated and the response was observed for the aromatic protons of AGT [137].

Thus, as illustrated above, one-dimensional rotating frame nuclear Overhauser and exchange spectroscopy (1D-ROESY) experiments may provide a reasonable explanation for significant qualitative and quantitative differences observed in selector–selectand interactions related to chiral CE. This information becomes even

more important when alternative techniques for structural elucidation fail. This was the case with the AGT/CD complexes which formed monocrystals, but of a twin-type, which were not suitable for X-ray crystallographic studies. Although 1D-ROESY seems to be a powerful technique for the structural investigation of non-covalent complexes in a liquid phase, the experimental data need to be interpreted very carefully. Thus, a detailed analysis of above-mentioned 1D-ROESY spectra of the AGT/CD complexes may indicate that the structures shown in Fig. 17 are just fragments of rather complex supramolecular aggregates. This seems even more likely when considering a certain tendency of AGT to form dimers in aqueous solution which has also been confirmed from the X-ray crystallographic data in the solid state.

A "confusing" example of 1D-ROESY studies is described in [208]. In this study, the structure of the complex between antihistaminic drug brompheniramine (BrPh) and β-CD and TM-β-CD was studied by 1D-ROESY experiments in solution. For the complexes of (+)-BrPh with both CDs, unambiguous confirmation was obtained indicating the inclusion of the 4-bromophenyl moiety of the analyte into the cavity of the CD. In addition, in the case of the (+)-BrPh complex with β-CD, a week but positive NOE effect was also observed for the protons of the maleate counteranion when saturating the CD protons H-3 and H-5 located inside the cavity. This observation may indicate the simultaneous inclusion of the 4-bromophenyl moiety and maleate counteranion into the cavity of β-CD, but this contradicts simple geometric considerations and the assumption that the stoichiometry of the complex is 1:1. Thus, the involvement of alternative techniques may become sometimes necessary for the unambiguous interpretation of 1D-ROESY data.

6.5 X-Ray Crystallographic Studies of the Structure of Selector–Selectand Complexes in the Solid State

X-ray crystallography has a long history as a powerful technique for structural investigation of CDs and their complexes in the solid state. The first experimental evidence of the inclusion complex formation ability of CDs in the solid state was obtained by Hybl et al. in 1965 using this technique [227]. X-ray studies of CD complexes have been summarized in several recent reviews [228, 229]. However, studies relevant to chiral CE have been scarcely published [208]. This may be due to the following reasons: (a) X-ray crystallography is a solid-state technique and a separation in CE is performed in solution. Therefore, these two techniques may not ideally correlate with each other. (b) Growing monocrystals of suitable size containing both counterparts (a selector and a selectand) requires experience. (c) High-quality X-ray crystallographic experiments on monocrystals is time consuming and rather expensive. (d) The structural generation from the experimental data point requires powerful computer software and is not always trivial. Despite the aforementioned, X-ray crystallography may appear sometimes very useful for structural studies related to enantioseparations in CE.

As mentioned above, the 1D-ROESY studies performed on the complex between (+)-BrPh and β-CD in solution did not allow explaining the NOE effect observed on the protons of the maleate counteranion [208]. X-ray crystallographic study performed on the monocrystals obtained from a 1:1 aqueous solution of (+)-BrPh maleate and β-CD (Fig. 18) provides a plausible explanation for the above-mentioned contradiction. In particular, as shown in Fig. 18, (+)-BrPh forms with β-CD, at least in the solid state, not a 1:1 complex but a complex with 1:2 stoichiometry. In this complex, the (+)-BrPh molecule is sandwiched between two molecules of β-CD. The 4-bromophenyl moiety of (+)-BrPh enters the cavity of one of the β-CD molecules, whereas the cavity of another β-CD molecule is occupied by the maleate counteranion. Thus, X-ray crystallography may provide useful information on the supramolecular structure of the selector–selectand complexes and in this way complement well 1D-ROESY data. However, the aforementioned possible differences between the structure of the complexes in solution and in the solid phase must be considered.

One additional application of X-ray crystallography in chiral CE-related studies may be the determination of the structure of various selectors and selectands. These data may appear very useful as thermodynamically most stable starting structures for further optimization based on the molecular mechanics and molecular dynamics calculations.

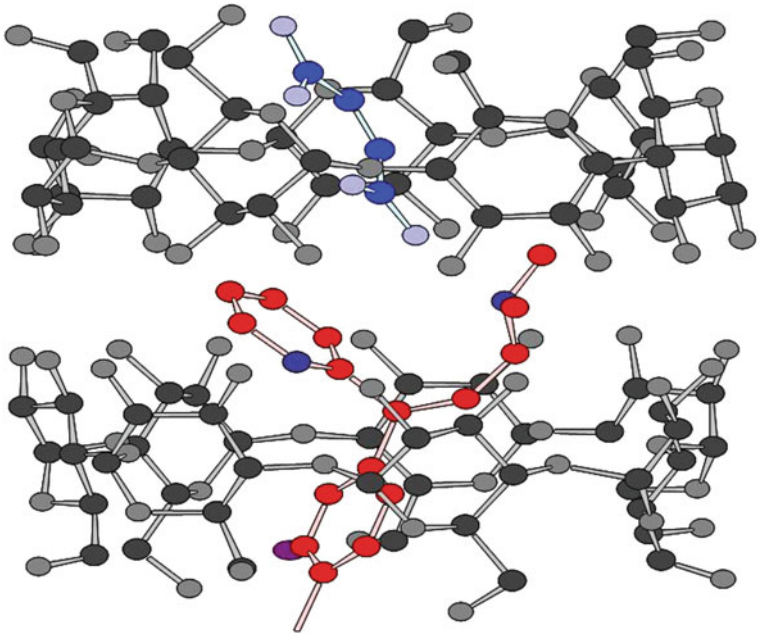


Fig. 18 Structure of (+)-bromophenyl maleate–β-CD complex in the solid state determined by X-ray crystallography (reproduced with permission from [208])

7 Molecular Modeling of Selector–Selectand Interactions in Chiral CE

In the ideal case, molecular modeling (MM) calculations may allow to compute the energy and structure of intermolecular complexes of biomedical, pharmaceutical, and chemical relevance in a reasonable time and rather precisely.

In the early 1990s, several studies on the computation of selector–selectand interactions in chiral CE [230–232] and other separation techniques were published [233–235]. This relates basically to the interactions between CDs and their chiral guests which seem to be caused by the fact that CDs are rather rigid molecules of medium size and therefore calculations for these molecules are easier, faster, and may be precise. In addition, many CDs are well studied by alternative techniques for structural elucidation. Among these, X-ray crystallographic data are of highest interest.

The major difference between the mathematical models of chiral CE and molecular modeling studies discussed in this chapter is that the former models describe the entire separation process and do not pay any significant attention to the selector–selectand interactions and/or to solvent molecules. In contrast, the molecular modeling techniques summarized in this section try to describe the selector–selectand interactions at the molecular level and do not estimate their role in the overall separation process.

A separation factor observed in any instrumental technique is defined by the difference between the free energy of formation of transient diastereomeric complexes between enantiomers and a chiral selector. Therefore, the exact calculation of the absolute energy values is not required in molecular modeling studies related to enantioseparations. This simplifies the calculations. On the other hand, due to extremely high efficiency of CE, this technique allows to observe enantioseparations even in those selector–selectand pairs where the above-mentioned difference between the free energy of formation of diastereomeric complexes is extremely small. The precise calculation of very small energy differences remains a challenging task even for very sophisticated state-of-the-art energy minimization softwares. Additional care must be taken in order to maximally approach a model to the real separation conditions. Thus, for instance, the molecular modeling calculations are often performed in vacuum without taking into account the effect of the medium. However, the aqueous medium commonly used in CE dramatically affects hydrophobic and hydrogen bonding interactions. Moreover, the ionic strength of the buffer plays a decisive role in electrostatic intermolecular interactions.

Another important point is a correct selection of the starting and the boundary conditions for energy minimization. Incorrectly defined conditions may totally confuse the calculations. For instance, when performing the molecular modeling calculations for the complex between TM-β-CD and (+)-BrPh in a neutral form, one may conclude that the complex formation with the alkylamino moiety included into the cavity of TM-β-CD would be energetically favorable [208]. The structure with the alkylamino moiety included into the cavity was also observed in X-ray experiment performed on the monocrystals

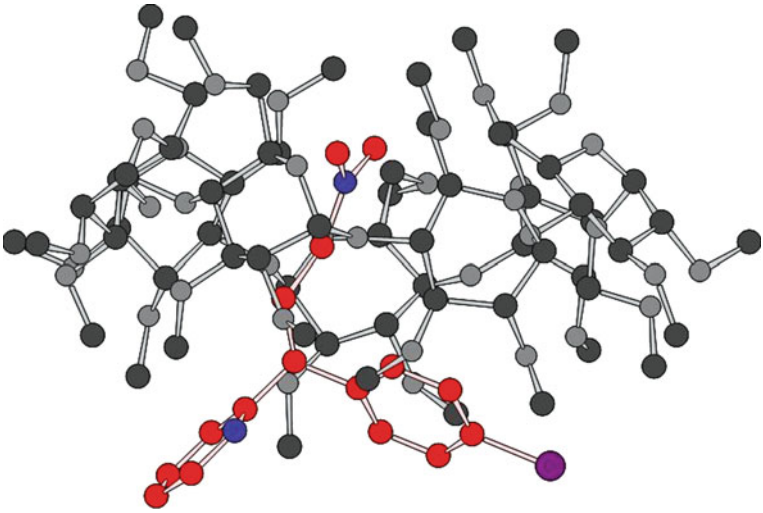


Fig. 19 Structure of (+)-bromophenyl maleate–TM-β-CD complex in the solid state determined by X-ray crystallography (reproduced with permission from [208])

obtained from the mixture of aqueous suspension of deprotonated (+)-BrPh as a free base and TM-β-CD (Fig. 19). These results are contradictory to the structure derived from the 1D-ROESY experiment in solution. The intermolecular NOE effects observed in this experiment clearly indicated the inclusion of the 4-bromophenyl moiety into the cavity of TM-β-CD (Fig. 20) [208]. Taking into

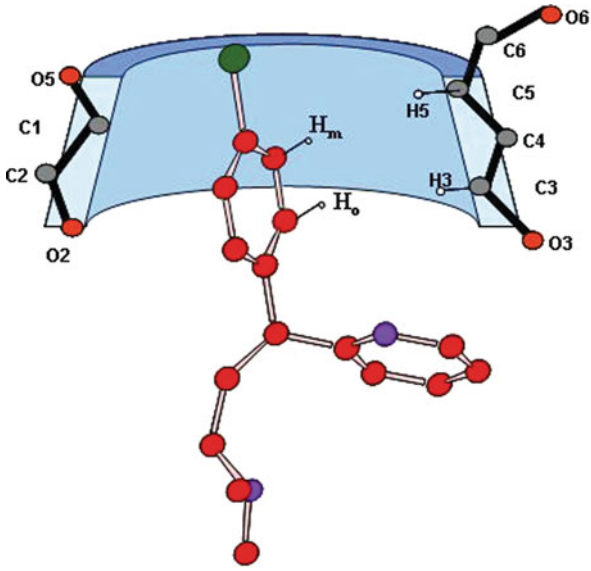


Fig. 20 Structure of (+)-bromophenyl maleate–β-CD complex in solution derived from 1D-ROESY experiment (reproduced with permission from [208])

consideration that the (+)-BrPh maleate, e.g., the protonated form of (+)-BrPh molecule, was applied for the 1D-ROESY studies in solutions, the force-field calculations were performed again for interactions of a single positively charged (+)-BrPh with TM-β-CD. The energy values obtained in this case clearly indicated that the complex formation with the 4-bromophenyl moiety of the (+)-BrPh molecule included into the cavity of TM-β-CD is energetically favorable, which is in agreement with the structure observed using 1D-ROESY studies in solution (Fig. 20) [208].

The critical remarks regarding the computation of cyclodextrin complexes and enantioselective recognition in these complexes have been published by Dodziuk and co-workers [236, 237]. In our opinion the most effective application of MM calculations in studies related to chiral CE seems to be the computation of intermolecular forces responsible for selector–selectand binding and enantioselective recognition based on the structure of complexes which are determined by using alternative experimental techniques. The determination of the binding pattern and structure of the intermolecular complexes based on energy minimization strategies shall not be a major application of calculation methods in studies related to chiral EKC. MM techniques under certain conditions can be used for the determination of the tentative structure of intermolecular complexes but these data need to be confirmed by alternative instrumental techniques.

References

1. Ettre LS (1994) New, unified nomenclature for chromatography. Chromatographia 38: 521–526
2. Chankvetadze B, Blaschke G (2001) Enantioseparations in capillary electromigration techniques: recent developments and future trends. J Chromatogr A 906:309–363
3. Chankvetadze B (1997) Separation selectivity in chiral capillary electrophoresis with charged selectors. J Chromatogr A 792:269–295
4. Rizzi AM, Kremser L (1999) pK(a) shift-associated effects in enantioseparations by cyclodextrin-mediated capillary zone electrophoresis. Electrophoresis 20:2715–2722
5. Süss F, Sänger-van de Griend K, Scriba KGE (2003) Migration order of dipeptide and tripeptide enantiomers in the presence of single isomer and randomly sulfated cyclodextrins as a function of pH. Electrophoresis 24:1069–1076
6. Chankvetadze B, Lindner W, Scriba G (2004) Enantiomer separations in capillary electrophoresis in the case of equal binding constants of the enantiomers with a chiral selector: commentary on the feasibility of the concept. Anal Chem 76:4256–4260
7. Lomsadze K, Martinez-Giron AB, Castro-Puyana M, Chankvetadze L, Crego A, Salgado A, Marina ML, Chankvetadze B (2009) About the role of enantioselective selector–selectand interactions and the mobilities of temporary diastereomeric associates in enantiomer separations using capillary electrophoresis. Electrophoresis 30:2803–2811
8. Terabe S, Ozaki H, Otsuka K, Ando T (1985) Electrokinetic chromatography with 2-*O*-carboxymethyl-β-cyclodextrin as a moving “stationary” phase. J Chromatogr 332:211–217
9. Pirkle W, Pochapsky T (1989) Considerations of chiral recognition relevant to the liquid chromatographic separation of enantiomers. Chem Rev 89:347–362
10. Zarbly E, Franco P, Lämmerhofer M, Lindner W (2000) Poster presentation P264 on HPCE, February, Saarbrücken, Germany

11. Chankvetadze B, Endresz G, Blaschke G (1994) About some aspects of the use of charged cyclodextrins for capillary electrophoretic enantioseparations. Electrophoresis 15:804–807
12. Chankvetadze B (1999) Recent trends in chiral separations using capillary electromigration techniques. TrAC (Trends Anal Chem) 18:485–498
13. Offord RE (1966) Electrophoretic mobilities of peptides on paper and their use in the determination of amide groups. Nature 211:591–593
14. Wren SAC, Rowe RC (1992) Theoretical aspects of chiral separation in capillary electrophoresis. I. Initial evaluation of a model. J Chromatogr 603:235–241
15. Chankvetadze B, Burjanadze N, Bergenthal D, Blaschke G (1999) Potential of flow-counterbalanced capillary electrophoresis for analytical and micropreparative separations. Electrophoresis 20:2680–2685
16. Giddings JC (1967) Physico-chemical basis of chromatography. J Chem Educ 44:704–709
17. Chankvetadze B, Schulte G, Blaschke G (1997) Nature and design of enantiomer migration order in chiral capillary electrophoresis. Enantiomer 2:157–178
18. Chankvetadze B (2002) Enantiomer migration order in chiral capillary electrophoresis. Electrophoresis 23:4022–4035
19. Chankvetadze B, Schulte G, Blaschke G (1996) Reversal of enantiomer elution order in capillary electrophoresis using charged and neutral cyclodextrins. J Chromatogr A 732: 183–186
20. Valtcheva L, Mohammed J, Pettersson G, Hjerten S (1993) Chiral separation of β-blockers by high-performance capillary electrophoresis based on non-immobilized cellulase as enantioselective protein. J Chromatogr 638:263–267
21. Tanaka Y, Terabe S (1995) Partial separation zone technique for the separation of enantiomers by affinity electrokinetic chromatography with proteins as chiral pseudo-stationary phases. J Chromatogr A 694:277–284
22. Fanali S, Desiderio C (1996) Use of vancomycin as chiral selector in capillary electrophoresis. Optimization and quantitation of loxiglumide enantiomers in pharmaceuticals. J High Resolut Chromatogr 19:322–326
23. Schulte G, Heitmeier S, Chankvetadze B, Blaschke G (1998) Chiral capillary electrophoresis–electrospray mass spectrometry coupling with charged cyclodextrin derivatives as chiral selectors. J Chromatogr A 800:77–82
24. Fanali S, Desiderio C, Schulte G, Heitmeier S, Chankvetadze B, Blaschke G (1998) Chiral capillary electrophoresis–electrospray mass spectrometry coupling using vancomycin as chiral selector. J Chromatogr A 800:69–76
25. Jäverfalk E, Amini A, Westerlund D, Andren PE (1998) Chiral separation of local anaesthetics by a capillary electrophoresis/partial filling technique coupled on-line to microelectrospray mass spectrometry. J Mass Spectrom 33:183–189
26. Amini A, Westerlund D (1998) Evaluation of association constants between drug enantiomers and human α1-acid glycoprotein by applying a partial-filling technique in affinity capillary electrophoresis. Anal Chem 70:1425–1430
27. Amini A, Merclin N, Bastami S, Westerlund D (1999) Determination of association constants between enantiomers of orciprenaline and methyl-β-cyclodextrin as chiral selector by capillary zone electrophoresis using a partial filling technique. Electrophoresis 20:180–188
28. Amini A, Westerlund D (1999) Principle and applications of the partial filling technique in capillary electrophoresis. Chromatographia 50:497–506
29. Culbertson CT, Jorgenson JW (1994) Flow counterbalanced capillary electrophoresis. Anal Chem 66:955–962
30. Culbertson CT, Jorgenson JW (1998) Separation of fluorescently derivatized deuterated isotopomers of phenylalanine using micellar electrokinetic chromatography and flow counterbalanced micellar electrokinetic chromatography. J Microcolumn Sep 11:175–183
31. Zhao J, Hooker T, Jorgenson JW (1999) Synchronous cyclic capillary electrophoresis using conventional capillaries: system design and preliminary results. J Microcolumn Sep 11: 431–437

32. Cheng YF, Wu S, Cheng DY, Dovichi NJ (1990) Interaction of capillary zone electrophoresis with a sheath flow cuvette detector. Anal Chem 62:496–503
33. Ross D, Locascio LE (2002) Microfluidic temperature gradient focusing. Anal Chem 74:2556–2564
34. Balss KM, Vreeland WN, Phinney KW, Ross D (2004) Simultaneous concentration and separation of enantiomers with chiral temperature gradient focusing. Anal Chem 76: 7243–7249
35. Balss KM, Vreeland WN, Howell PB, Henry AC, Ross D (2004) Micellar affinity gradient focusing: a new method for electrokinetic focusing. J Am Chem Soc 126:1936–1937
36. Koegler WS, Ivory CF (1996) Field gradient focusing: a novel method for protein separation. Biotechnol Prog 12:822–836
37. Koegler WS, Ivory CF (1996) Focusing proteins in an electric field gradient. J Chromatogr A 726:229–236
38. Ivory CF (2000) A brief review of alternative electrofocusing techniques. Sep Sci Technol 35:1777–1793
39. Huang Z, Ivory CF (1999) Digitally controlled electrophoretic focusing. Anal Chem 71:1628–1632
40. Zhao J, Jorgenson JW (1999) Application of synchronous cyclic capillary electrophoresis: isotopic and chiral separations. J Microcolumn Sep 11:439–449
41. Kimata K, Hosoya K, Tanaka N (1997) Direct chromatographic separation of racemates on the basis of isotopic chirality. Anal Chem 69:2610–2612
42. Terabe S (1989) Electrokinetic chromatography: an interface between electrophoresis and chromatography. Trends Anal Chem 8:129–134
43. Meyring M, Chankvetadze B, Blaschke G (1999) Enantioseparation of thalidomide and its metabolites using capillary electrophoresis with various cyclodextrins and their combinations as chiral buffer additives. Electrophoresis 20:2425
44. Mazzeo JR, Swartz ME, Grover ER (1995) A resolution equation for electrokinetic chromatography based on electrophoretic mobilities. Anal Chem 67:2966–2973
45. Fillet M, Hubert PH, Crommen J (2000) Enantiomeric separations of drugs using mixtures of charged and neutral cyclodextrins. J Chromatogr A 875:123–134
46. Lurie IS (1997) Separation selectivity in chiral and achiral capillary electrophoresis with mixed cyclodextrins. J Chromatogr A 792:297–307
47. Fillet M, Chankvetadze B, Crommen J, Blaschke G (1999) Designed combination of chiral selectors for improvement of enantioseparation selectivity in capillary electrophoresis. Electrophoresis 20:2691–2697
48. Chankvetadze B, Burjanadze N, Crommen J, Blaschke G (2001) Enantioseparation of warfarin using cyclodextrin type chiral selectors. Chromatographia 53:S296–S301
49. Fillet M, Bechet I, Schombug G, Hubbert P, Crommen J (1996) Enantiomeric separation of acidic drugs by capillary electrophoresis using a combination of charged and uncharged β-cyclodextrins as chiral selectors. J High Resolut Chromatogr 19:669–673
50. Lelievre F, Gareil P, Bahadd Y, Galons H (1997) Intrinsic selectivity in capillary electrophoresis for chiral separations with dual cyclodextrin systems. Anal Chem 69:393–401
51. Armstrong DW, Chang LW, Chang SSC (1998) Mechanism of capillary electrophoresis enantioseparations using a combination of an achiral crown ether plus cyclodextrins. J Chromatogr A 793:115–134
52. Jakubetz H, Juza M, Schurig V (1998) Dual chiral recognition system involving cyclodextrin derivatives in capillary electrophoresis II. Enhancement of enantioselectivity. Electrophoresis 19:738–744
53. Fanali S, Ossicini L, Foret F, Bocék P (1989) Resolution of optical isomers by capillary zone electrophoresis: study of enantiomeric and diastereoisomeric Co(III) complexes with ethylenediamine and amino acid ligands. J Microcolumn Sep 1:190–194
54. Lin M, Wu N, Barcer GE, Sun P, Huie CW, Hartwick RA (1993) Enantiomeric separation by cyclodextrin-modified micellar electrokinetic chromatography using bile salt. J Liq Chromatogr 16:3667–3674

55. Terabe S, Miyashita Y, Ishihama Y, Shibata O (1993) Cyclodextrin-modified micellar electrokinetic chromatography: separation of hydrophobic and enantiomeric compounds. J Chromatogr 636:47–55
56. Wang J, Warner IM (1995) Combined polymerized chiral micelle and γ-cyclodextrin for chiral separation in capillary electrophoresis. J Chromatogr A 711:297–304
57. Kuhn R, Wagner J, Walbroehl Y, Bereuter T (1994) Potential and limitations of an optically active crown ether for chiral separation in capillary zone electrophoresis. Electrophoresis 15:828–834
58. Kuhn R, Steinmetz C, Bereuter T, Haas P, Erni F (1994) Enantiomeric separations in capillary zone electrophoresis using a chiral crown ether. J Chromatogr A 666:367–373
59. Huang WX, Xu H, Fazio SD, Vivilechia RV (1997) Chiral separation of primary amino compounds using a non-chiral crown ether with β-cyclodextrin by capillary electrophoresis. J Chromatogr B 695:157–162
60. Huang WX, Fazio SD, Vivilechia RV (1997) Achievement of enantioselectivity of non-polar primary amines by a non-chiral crown ether. J Chromatogr A 781:129–137
61. Huang WX, Xu H, Fazio SD, Vivilechia RV (2000) Enhancement of chiral recognition by formation of a sandwiched complex in capillary electrophoresis. J Chromatogr A 875: 361–369
62. Ye B, Khaledi MG (1994) Poster No 113 presented at the 6th international symposium on high performance capillary electrophoresis, San Diego, CA, USA
63. Bjornsdottir I, Hansen SH, Terabe S (1996) Chiral separation in non-aqueous media by capillary electrophoresis using the ion-pair principle. J Chromatogr A 745:37–44
64. Piette V, Crommen J, Lämmerhoefer M, Lindner W (1999) Enantiomeric separation of N-protected amino acids by non-aqueous capillary electrophoresis using quinine or *tert*-butyl carbamoylated quinine as chiral additive. Chirality 11:622–630
65. Piette V, Fillet M, Lindner W, Crommen J (2000) Non-aqueous capillary electrophoretic enantioseparation of N-derivatized amino acids using cinchona alkaloids and derivatives as chiral counter-ions. J Chromatogr A 875:353–360
66. Vincent JB, Vigh G (1998) Nonaqueous capillary electrophoretic separation of enantiomers using the single-isomer heptakis(2,3-diacetyl-6-sulfato)-β-cyclodextrin as chiral resolving agent. J Chromatogr A 816:233–241
67. Tacker M, Glukhovskiy P, Cai H, Vigh G (1999) Nonaqueous capillary electrophoretic separation of basic enantiomers using heptakis(2,3-dimethyl-6-sulfato)-β-cyclodextrin. Electrophoresis 20:2794–2798
68. Valko IE, Siren H, Riekkola M-L (1998) Characteristics of electroosmotic flow in capillary electrophoresis in water and in organic solvents without added ionic species. J Microcolumn Sep 11:199–208
69. Mori Y, Ueno K, Umeda T (1997) Enantiomeric separations of primary amino compounds by nonaqueous capillary zone electrophoresis with a chiral crown ether. J Chromatogr A 757:328–332
70. Wang F, Khaledi M (1998) Nonaqueous capillary electrophoresis chiral separations with quaternary ammonium β-cyclodextrin. J Chromatogr A 817:121–128
71. Servais A-C, Rousseau A, Fillet M, Lomsadze K, Salgado A, Crommen J, Chankvetadze B (2010) Separation of propranolol enantiomers by capillary electrophoresis using sulfated β-cyclodextrin derivatives in aqueous and nonaqueous electrolytes: comparative CE and NMR studies. Electrophoresis 31:1467–1474
72. Hutt LD, Glavin DP, Bada JL, Mathies RA (1999) Microfabricated capillary electrophoresis amino acid chirality analyzer for extraterrestrial exploration. Anal Chem 71:4000–4006
73. Rodriguez I, Jin LJ, Li SFY (2000) High-speed chiral separations on microchip electrophoresis devices. Electrophoresis 21:211–219
74. Reetz MT, Kuhling KM, Deege A, Hinrichs H, Belder D (2000) Super-high-throughput screening of enantioselective catalysts by using capillary array electrophoresis. Angew Chem Int Ed Engl 39:3891–3893

75. Gao Y, Shen Z, Wang H, Dai Z, Lin B (2005) Chiral separations on multichannel microfluidic chips. Electrophoresis 26:4774–4779
76. Belder D, Deege A, Maass M, Ludwig M (2002) Design and performance of a microchip electrophoresis instrument with sensitive variable-wavelength fluorescence detection. Electrophoresis 23:2355–2361
77. Schwarz MA, Hauser PC (2001) Rapid chiral on-chip separation with simplified amperometric detection. J Chromatogr A 928:225–232
78. Olvecka E, Maser M, Kaniansky D, Johnk M, Stanislawski B (2001) Isotachophoresis separations of enantiomers on a planar chip with coupled separation channels. Electrophoresis 22:3347–3453
79. Ludwig M, Belder D (2003) Coated microfluidic devices for improved chiral separations in microchip electrophoresis. Electrophoresis 24:2481–2486
80. Piehl N, Ludwig M, Belder D (2004) Subsecond chiral separations on a microchip. Electrophoresis 25:3848–3852
81. Belder D, Ludwig M (2003) Microchip electrophoresis for chiral separations. Electrophoresis 24:2422–2430
82. Belder D, Ludwig M, Wang L-W, Reetz MT (2006) Enantioselective catalysis and analysis on a chip. Angew Chem Int Ed Engl 45:2463–2466
83. Cho SI, Lee K-N, Kim Y-K, Jang JH, Chung DS (2002) Chiral separation of gemifloxacin sodium-containing media using chiral crown ether as a chiral selector by capillary and microchip electrophoresis. Electrophoresis 23:972–979
84. Nagl S, Schulze P, Ludwig M, Belder D (2009) Progress in microchip enantioseparations. Electrophoresis 30:2765–2772
85. Glukhovskiy P, Vigh G (1999) Analytical- and preparative-scale isoelectric focusing separation of enantiomers. Anal Chem 71:3814–3820
86. Rizzi AM, Kremser L (1999) Enantioseparation of derivatized amino acids by capillary isoelectric focusing using cyclodextrin complexation. Electrophoresis 20:3410–3416
87. Stalcup AM, Gahm KH, Gratz SR, Sutton RC (1998) Application of classical gel electrophoresis to the chiral separation of milligram quantities of terbutaline. Anal Chem 70:144–148
88. Sutton RC, Gratz SR, Stalcup AM (1998) Use of capillary electrophoresis as a method development tool for classical gel electrophoresis. Analyst 123:1477–1480
89. Lanz M, Caslavska J, Thormann W (1998) Enantiomeric separation of methadone by cyclodextrin-based capillary and recycling isotachophoresis. Electrophoresis 19:1081–1090
90. Kaniansky D, Simunicova E, Ölvecka E, Ferancova A (1999) Separations of enantiomers by preparative capillary isotachophoresis. Electrophoresis 20:2786–2793
91. Thome B, Ivory CF (2002) Continuous fractionation of enantiomer pairs in free solution using an electrophoretic analog of simulated moving bed chromatography. J Chromatogr A 953:263–277
92. Thome B, Ivory CF (2003) Development of a segmented model for a continuous electrophoretic moving bed enantiomer separation. Biotechnol Prog 19:1703–1712
93. Glukhovskiy P, Vigh G (2000) Use of single-isomer, multiply charged chiral resolving agents for the continuous, preparative-scale electrophoretic separation of enantiomers based on the principle of equal-but-opposite analyte mobilities. Electrophoresis 21:2010–2015
94. Thome BM, Ivory CF (2007) Increasing the scale of true moving bed electrophoretic separations using filtration to reduce solvent volumetric flows between sections II and III. J Chromatogr A 1138:291–300
95. Schulte M, Strube J (2001) Preparative enantioseparation by simulated moving bed chromatography. J Chromatogr A 906:399–416
96. Nardi A, Eliseev A, Bocek P, Fanali S (1993) Use of charged and neutral cyclodextrins in capillary zone electrophoresis: enantiomeric resolution of some 2-hydroxy acids. J Chromatogr 638:247–253

97. Vincent JB, Sokolowski AD, Nguyen TV, Vigh G (1997) A family of single-isomer chiral resolving agents for capillary electrophoresis. 1. Heptakis(2,3-diacetyl-6-sulfato)-β-cyclodextrin. Anal Chem 69:4226–4233
98. Vincent JB, Kirby DM, Nguyen TV, Vigh G (1997) A family of single-isomer chiral resolving agents for capillary electrophoresis. 2. Hepta-6-sulfato-β-cyclodextrin. Anal Chem 69:4419–4428
99. Cai H, Nguyen TV, Vigh G (1998) A family of single-isomer chiral resolving agents for capillary electrophoresis. 3. Heptakis(2,3-dimethyl-6-sulfato)-β-cyclodextrin. Anal Chem 70:580–589
100. Maynard DK, Vigh G (2001) Heptakis(2-*O*-methyl-3,6,di-*O*-sulfo)-β-cyclodextrin: a single-isomer, 14-sulfated β-cyclodextrin for use as a chiral resolving agent in capillary electrophoresis. Electrophoresis 22:3152–3162
101. Busby BM, Vigh G (2005) Synthesis of heptakis(2-*O*-methyl-3-*O*-acetyl-6-*O*-sulfo)-cyclomaltoheptaose, a single-isomer, sulfated β-cyclodextrin carrying nonidentical substituents at all the C2, C3, and C6 positions and its use for the capillary electrophoretic separation of enantiomers in acidic aqueous and methanolic background electrolytes. Electrophoresis 26:1978–1987
102. Busby MB, Vigh G (2005) Synthesis of a single-isomer sulfated β-cyclodextrin carrying nonidentical substituents at all of the C2, C3, and C6 positions and its use for the electrophoretic separation of enantiomers in acidic aqueous and methanolic background electrolytes, Part 2: Heptakis(2-*O*-methyl-6-*O*-sulfo) cyclomaltoheptaose. Electrophoresis 26:3849–3860
103. Rousseau A, Chiap P, Oprean R, Crommen J, Fillet M, Servais A-C (2009) Effect of the nature of the single-isomer anionic CD and the BGE composition on the enantiomeric separation of b-blockers in NACE. Electrophoresis 30:2862–2868
104. Sanchez-Vindas S, Vigh G (2005) Non-aqueous capillary electrophoretic enantiomer separations using the tetrabutylammonium salt of heptakis(2,3-*O*-diacetyl-6-*O*-sulfo)-cyclomaltoheptaose, a single-isomer sulfated β-cyclodextrin highly-soluble in organic solvents. J Chromatogr A 1068:151–158
105. Chankvetadze B, Endresz G, Blaschke G (1996) Charged cyclodextrin derivatives as chiral selectors in capillary electrophoresis. Chem Soc Rev 25:141–153
106. O'Keeffe F, Shamsi SA, Darcy R, Schwinte P, Warner IM (1997) A persubstituted cationic β-cyclodextrin for chiral separations. Anal Chem 69:4773–4782
107. Hynes JL, Shamsi SA, O'Keeffe F, Darcy R, Warner IM (1998) Cationic β-cyclodextrin derivative for chiral separations. J Chromatogr A 803:261–271
108. Kano K, Kitae T, Takashima H (1996) Use of electrostatic interaction for chiral recognition. Enantioselective complexation of anionic binaphthyls with protonated amino-β-cyclodextrin. J Incl Phenom Mol Recognit Chem 25:243–248
109. Galaverna G, Corradini R, Dossena A, Marcelli R, Vecchio G (1997) Histamine-modified β-cyclodextrins for the enantiomeric separation of dansyl-amino acids in capillary electrophoresis. Electrophoresis 18:905–911
110. Galaverna G, Corradini R, Dossena A, Marcelli R (1999) Histamine-modified cationic β-cyclodextrins as chiral selectors for the enantiomeric separation of hydroxy acids and carboxylic acids by capillary electrophoresis. Electrophoresis 20:2619–2629
111. Nzeadibe K, Vigh G (2007) Synthesis of mono-6-deoxy-6-*N*,*N*,*N*′,*N*′,*N*′- pentamethylethylenediammonio-cyclomaltoheptaose, a single-isomer, monosubstituted, permanently dicationic β-CD and its use for enantiomer separations by CE. Electrophoresis 28: 2589–2605
112. Rousseau A, Chiap P, Ivanyi R, Crommen J, Fillet M, Servais A-C (2008) Validation of a nonaqueous capillary electrophoretic method for the enantiomeric purity determination of *R*-flurbiprofen using a single-isomer amino cyclodextrin derivative. J Chromatogr A 1204: 219–225

113. Rousseau A, Pedrini M, Chiap P, Ivanyi R, Crommen J, Fillet M, Servais A-C (2008) Determination of flurbiprofen enantiomers in plasma using a single-isomer amino cyclodextrin derivative in nonaqueous capillary electrophoresis. Electrophoresis 29:3641–3648
114. Chankvetadze B, Schulte G, Bergenthal D, Blaschke G (1998) Comparative capillary electrophoresis and NMR studies of enantioseparation of dimethindene with cyclodextrins. J Chromatogr A 798:315–323
115. Lelievre F, Gueit C, Gareil P, Bahaddi Y, Galons H (1997) Use of a zwitterionic cyclodextrin as a chiral agent for the separation of enantiomers by capillary electrophoresis. Electrophoresis 18:891–896
116. Tanaka Y, Terabe S (1997) Enantiomer separation of acidic racemates by capillary electrophoresis using cationic and amphoteric β-cyclodextrins as chiral selectors. J Chromatogr A 781:151–160
117. Chiari M, Desparti V, Gretich M, Crini G, Janus L, Morcellet M (1999) Vinylpyrrolidine-β-cyclodextrin copolymer: a novel chiral selector for capillary electrophoresis. Electrophoresis 20:2614–2618
118. Chankvetadze B, Saito M, Yashima E, Okamoto Y (1998) Enantioseparation of atropisomeric 1,1′-binaphthyl-2,2′-diyl-hydrogen phosphate in capillary electrophoresis using di- and oligosaccharides as chiral selectors. Chirality 10:134–139
119. Nishi H, Izumoto S, Nakamura K, Nakai H, Sato T (1996) Dextran and dextrin as chiral selectors in capillary zone electrophoresis. Chromatographia 42:617–630
120. Yarabe HH, Billiot E, Warner IM (2000) Enantiomeric separations by use of polymeric surfactant electrokinetic chromatography. J Chromatogr A 875:179–206
121. El Rassi Z (2000) Chiral glycosidic surfactants for enantiomeric separation in capillary electrophoresis. J Chromatogr A 875:207–233
122. Otsuka K, Terabe S (2000) Enantiomer separation of drugs by micellar electrokinetic chromatography using chiral surfactants. J Chromatogr A 875:163–178
123. Mechref Y, El Rassi Z (1997) Capillary electrophoresis of herbicides. II. Evaluation of alkylglucoside chiral surfactants in the enantiomeric separation of phenoxy acid herbicides. J Chromatogr A 757:263–273
124. Armstrong D, Nair UB (1997) Capillary electrophoretic enantioseparations using macrocyclic antibiotics as chiral selectors. Electrophoresis 18:2331–2342
125. Desiderio C, Fanali S (1998) Chiral analysis by capillary electrophoresis using antibiotics as chiral selector. J Chromatogr A 807:37–56
126. Haginaka J (2000) Enantiomer separation of drugs by capillary electrophoresis using proteins as chiral selectors. J Chromatogr A 875:235–254
127. Tanaka Y, Terabe S (1999) Studies on enantioselectivities of avidin, avidin–biotin complex and streptavidin by affinity capillary electrophoresis. Chromatographia 49:489–495
128. Jung G, Hofstetter H, Feiertag S, Stoll D, Hofstetter O, Wiesmüller K-H, Schurig V (1996) Cyclopeptidbibliotheken als neue chirale Selektoren für die Kapillar elektrophorese. Angew Chem 108:2261–2263
129. Chiari M, Desperati V, Manera E, Longhi R (1998) Combinatorial synthesis of highly selective cyclohexapeptides for separation of amino acid enantiomers by capillary electrophoresis. Anal Chem 70:4967–4973
130. Vegvari A, Schmid MG, Kilar F, Gübitz G (1998) Chiral separation of α-amino acids by ligand-exchange capillary electrophoresis using *N*-(2-hydroxy-octyl)-L-4-hydroxyproline as a selector. Electrophoresis 19:2109–2114
131. Sanchez Pena M, Zhang Y, Thibodeaux S, McLaughlin ML, Munoz de la Pena A, Warner IM (1996) Synthesis of a water-soluble chiral *N*-acylcalix(4)arene amino acid derivative. Tetrahedron Lett 37:584–587
132. Chankvetadze B, Burjanadze N, Pintore G, Bergenthal D, Bergander K, Mühlenbrock C, Breitkreutz J, Blaschke G (2000) Mechanistic study of opposite migration order of dimethindene enantiomers in capillary electrophoresis in the presence of native β-CD and heptakis-(2,3,6-tri-*O*-methyl)-β-CD. J Chromatogr A 875:455–470

133. Yamashoji Y, Ariga T, Asano S, Tanaka M (1992) Chiral recognition and enantiomeric separation of alanine β-naphthylamide by cyclodextrins. Anal Chim Acta 268:39–47
134. Chankvetadze B, Lomsadze K, Bergenthal D, Breitkreuz J, Bergander K, Blaschke G (2001) Mechanistic studies on the opposite migration order of clenbuterol enantiomers in capillary electrophoresis with β-CD and single-isomer heptakis(2,3-diacetyl-6-sulfo)-β-CD. Electrophoresis 22:3178–3184
135. Chankvetadze B, Burjanadze N, Maynard DM, Bergander K, Bergenthal D, Blaschke G (2002) Comparative enantioseparations with native β-cyclodextrin and heptakis-(2-*O*-methyl-3,6-di-*O*-sulfo)-β-cyclodextrin in capillary electrophoresis. Electrophoresis 23:3027–3034
136. Tanaka M, Asano S, Yoshinaga M, Kawaguchi Y, Tatsumi T, Shono T (1991) Separation of racemates by capillary zone electrophoresis based on complexation with cyclodextrins. Fresenius J Anal Chem 339:63–70
137. Chankvetadze B, Fillet M, Burjanadze N, Bergenthal D, Bergander K, Luftmann H, Crommen J, Blaschke G (2000) Enantioseparation of aminoglutethimide with cyclodextrins in capillary electrophoresis and studies of selector–selectand interactions using NMR spectroscopy and electrospray ionization mass spectrometry. Enantiomer 5:313–322
138. Chankvetadze B, Lomsadze K, Burjanadze N, Breitkreutz J, Pintore G, Chessa M, Bergenthal D, Bergander K, Blaschke G (2003) Comparative enantioseparations with native β-cyclodextrin, randomly acetylated β-cyclodextrin and heptakis-(2,3-di-*O*-acetyl)-β-cyclodextrin in capillary electrophoresis. Electrophoresis 24:1083–1091
139. Chankvetadze B (2004) Combined approach using capillary electrophoresis and NMR spectroscopy for an understanding of enantioselective recognition mechanisms by cyclodextrins. Rev Chem Soc 33:337–347
140. Chankvetadze B (2009) Separation of enantiomers with charged chiral selectors in CE. Electrophoresis 30:S211–S221
141. Chankvetadze B (1997) Capillary electrophoresis in chiral analysis. John Wiley & Sons, Chichester, p 555
142. Horimai T, Ohara M, Ichinose M (1997) Optical resolution of new quinolone drugs by capillary electrophoresis with ligand-exchange and host-guest interactions. J Chromatogr A 760:235–244
143. Zhu W, Vigh G (2000) Capillary electrophoretic separation of the enantiomers of weak acids in a high pH background electrolyte using the new, single-isomer, octakis(2,3-diacetyl-6-sulfato)-γ-cyclodextrin as chiral resolving agent. J Microcolumn Sep 12:167–171
144. Tamisier-Karolak SL, Stenger MA, Bommart A (1999) Enantioseparation of β-blockers with two chiral centers by capillary electrophoresis using sulfated β-cyclodextrins. Electrophoresis 20:2656–2663
145. Ishibushi K, Izumoto S, Nishi H, Sato T (1997) Enantiomer separation of denopamine by capillary electrophoresis with charged and uncharged cyclodextrins. Electrophoresis 18:1007–1012
146. Jakubetz H, Juza M, Schurig V (1997) Electrokinetic chromatography employing an anionic and a cationic β-cyclodextrin derivative. Electrophoresis 18:897–904
147. Katayama H, Ishihama Y, Asakawa N (1997) Migration order reversal of enantiomers in capillary electrophoretic separation. J Chromatogr A 764:151–156
148. Katayama H, Ishihama Y, Asakawa N (2000) Enantiomeric separation by capillary electrophoresis with an electroosmotic flow-controlled capillary. J Chromatogr A 875:315–322
149. Sabah S, Scriba G (1998) Electrophoretic stereoisomer separation of aspartyl dipeptides and tripeptides in untreated fused-silica and polyacrylamide-coated capillaries using charged cyclodextrins. J Chromatogr A 822:137–145
150. Schmitt T, Engelhardt H (1993) Derivatized cyclodextrins for the separation of enantiomers in capillary electrophoresis. J High Resolut Chromatogr 16:525–529
151. Scriba G (2002) Selected fundamental aspects of chiral electromigration techniques and their application to pharmaceutical and biomedical analysis. J Pharm Biomed Anal 27:373–399

152. Sabah S, Scriba GKE (1999) pH-Dependent reversal of the chiral recognition of tripeptide enantiomers by carboxymethyl-β-cyclodextrin. J Chromatogr A 833:261–266
153. Sabah S, Scriba GKE (2001) Separation of dipeptide and tripeptide enantiomers in capillary electrophoresis using carboxymethyl-β-cyclodextrin and succinyl-β-cyclodextrin: influence of the amino acid sequence, nature of the cyclodextrin and pH. Electrophoresis 22: 1385–1393
154. Süß F, Poppitz W, Saenger-van de Griend C, Scriba GKE (2001) Influence of the amino acid sequence and nature of the cyclodextrin on the separation of small peptide enantiomers by capillary electrophoresis using randomly substituted and single isomer sulfated and sulfonated cyclodextrins. Electrophoresis 22:2416–2423
155. Sabah S, Süß F, Scriba GKE (2001) pH-dependence of complexation constants and complex mobility in capillary electrophoresis separations of dipeptide enantiomers. Electrophoresis 22:3163–3170
156. Sidamonidze N, Süß F, Poppitz W, Scriba GKE (2001) Influence of the amino acid sequence and nature of the cyclodextrin on the separation of small peptide enantiomers by capillary electrophoresis using α-, β-, and γ-cyclodextrin and the corresponding hydroxypropyl derivatives. J Sep Sci 24:777–783
157. Süß F, Kahle C, Holzgrabe U, Scriba GKE (2002) Studies on the chiral recognition of peptide enantiomers by neutral and sulfated β-cyclodextrin and heptakis-(2,3-di-*O*-acetyl)β-cyclodextrin using capillary electrophoresis and nuclear magnetic resonance. Electrophoresis 23:1301–1307
158. Süß F, Poppitz W, Scriba GKE (2002) Separation of dipeptide and tripeptide enantiomers in capillary electrophoresis by the cationic cyclodextrin derivative 2-hydroxypropyltrimethylammonium-β-cyclodextrin and by neutral β-cyclodextrin derivatives at alkaline pH. J Sep Sci 25:1147–1154
159. Aturki Z, Fanali S (1994) Use of β-cyclodextrin polymer as a chiral selector in capillary electrophoresis. J Chromatogr A 680:137–146
160. Schulte G, Chankvetadze B, Blaschke G (1997) Enantioseparation in capillary electrophoresis using hydroxypropyl trimethylammonium salts of ß-CD as a chiral selector. J Chromatogr A 771:259–266
161. Fanali S (2000) Enantioselective determination by capillary electrophoresis with cyclodextrins as chiral selectors. J Chromatogr A 875:89–122
162. Fillet M, Hubert P, Crommen J (1998) Method development strategies for the enantioseparation of drugs by capillary electrophoresis using cyclodextrins as chiral additives. Electrophoresis 19:2834–2840
163. Roos N, Ganzler K, Szeman J, Fanali S (1997) Systematic approach to cost- and time-effective method development with a starter kit for chiral separations by capillary electrophoresis. J Chromatogr A 782:257–269
164. Plackett RC, Burman JB (1946) Biometrika 23:305–325
165. Carlson R (1992) Design and optimization of organic synthesis. Elsevier, Amsterdam
166. Box GEP (1952) Statistical design in the study of analytical methods. Analyst 77:879–889
167. Cochran WG, Cox GM (1957) Experimental designs. Wiley, New York
168. Small TS, Fell AF, Coleman MW, Berridge JC (1995) Central composite design for the rapid optimisation of ruggedness and chiral separation of amlodipine in capillary electrophoresis. Chirality 7:226–234
169. Wan H, Andersson PE, Engstrom P, Blomberg LG (1995) Direct and indirect chiral separation of amino acids by capillary electrophoresis. J Chromatogr A 704:179–193
170. Varesio E, Gauvrit JY, Longeray R, Lanteri P, Veuthey J-L (1997).Central composite design in the chiral analysis of amphetamines by capillary electrophoresis. Electrophoresis 18: 931–936
171. Rogan MM, Altria KD, Goodall DM (1994) Plackett–Burman experimental design in chiral analysis using capillary electrophoresis. Chromatographia 38:723–729
172. Boonkerd S, Detaevernier MR, Vander Heyden Y, Vindevogel J, Michotte Y (1996) Determination of the enantiomeric purity of dexfenfluramine by capillary electrophoresis:

use of a Plackett-Burman design for the optimization of the separation. J Chromatogr A 736:281–289
173. Fanali S, Furlanetto S, Aturki Z, Pinzauti S (1998) Experimental design methodologies to optimize the CE separation of epinephrine enantiomers. Chromatographia 48:395–400
174. Gotti R, Furlanetto S, Andrisano V, Cavrini V, Pinzauti S (2000) Design of experiments for capillary electrophoretic enantioresolution of salbutamol using dermatan sulfate. J Chromatogr A 875:411–422
175. Jimidar MI, Vennekens T, Van Ael W, Redlich D, De Smet M (2004) Optimization and validation of an enantioselective method for a chiral drug with eight stereo-isomers in capillary electrophoresis. Electrophoresis 25:2876–2884
176. Gong WJ, Zhang YP, Choi S-H, Zhang YJ, Lee KP (2006) Application of response surface methodologies in capillary electrophoresis. Microchim Acta 156:327–335
177. Zhang Y-J, Gong W-J, Zhang J-M, Zhang Y-P, Wang S-M, Wang L, Xue H-Y (2008) Optimization strategies using response surface methodologies in high performance liquid chromatography. J Liq Chromatogr Relat Technol 31:2893–2916
178. Borges KB, Pupo MZ, de Freitas LAP, Bonato PS (2009) Box–Behnken design for the optimization of an enantioselective method for the simultaneous analysis of propranolol and 4-hydroxypropranolol by CE. Electrophoresis 30:2874–2881
179. Wren SAC, Rowe RC (1992) Theoretical aspects of chiral separation in capillary electrophoresis: II. The role of organic solvent. J Chromatogr 609:363–367
180. Stepanova ND, Stepanov AV (1969) Zh Prikl Khimii (Russ J Appl Chem Engl Edn) 42:1576–1578
181. Penn SG, Goodall DM, Loran JS (1993) Differential binding of tioconazole enantiomers to hydroxypropyl-β-cyclodextrin studied by capillary electrophoresis. J Chromatogr 636: 149–152
182. Penn SG, Bergstrom ET, Goodall DM, Loran JS (1994) Capillary electrophoresis with chiral selectors: optimization of separation and determination of thermodynamic parameters for binding of tioconazole enantiomers to cyclodextrins. Anal Chem 66:2866–2873
183. Rogan MM, Altria KD, Goodall DM (1994) Enantiomeric separation of salbutamol and related impurities using capillary electrophoresis. Electrophoresis 15:808–817
184. Copper CL, Davis JB, Cole RO, Sepaniak M (1994) Separations of derivatized amino acid enantiomers by cyclodextrin-modified capillary electrophoresis: mechanistic and molecular modeling studies. Electrophoresis 15:785–792
185. Baumy P, Morin P, Dreux M, Viaud MC, Boye S, Guillaumet G (1995) Determination of β-cyclodextrin inclusion complex constants for 3,4-dihydro-2-*H*-1-benzopyran enantiomers by capillary electrophoresis. J Chromatogr A 707:311–326
186. Rawjee YY, Staerk DU, Vigh G (1993) Capillary electrophoretic chiral separations with cyclodextrin additives. I. Acids: chiral selectivity as a function of pH and the concentration of β-cyclodextrin for fenoprofen and ibuprofen. J Chromatogr 635:291–306
187. Rawjee YY, Williams RL, Vigh G (1994) Capillary electrophoretic chiral separations using cyclodextrin additives. III. Peak resolution surfaces for ibuprofen and homatropine as a function of the pH and the concentration of β-cyclodextrin. J Chromatogr 680:599–607
188. Rawjee YY, Williams RL, Buckingham LA, Vigh G (1994) Effects of pH and hydroxypropyl β-cyclodextrin concentration on peak resolution in the capillary electrophoretic separation of the enantiomers of weak bases. J Chromatogr A 688:273–282
189. Rawjee YY, Vigh G (1994) A peak resolution model for the capillary electrophoretic separation of the enantiomers of weak acids with hydroxypropyl β-cyclodextrin-containing background electrolytes. Anal Chem 66:619–627
190. Williams RL, Vigh G (1996) Maximization of separation efficiency in capillary electrophoretic chiral separations by means of mobility-matching background electrolytes. J Chromatogr A 730:273–278
191. Guttman A, Brunet S Cooke N (1996) Capillary electrophoresis separation of enantiomers by cyclodextrin array chiral analysis. LC-GC Int 9:88–95

192. Ingelse BA, Sarmani K, Reijenga JC, Kenndler E, Everaerts FM (1997) Chiral interactions in capillary zone electrophoresis: computer stimulation and comparison with experiment. Electrophoresis 18:938–942
193. Reijenga JC, Ingelse BA, Everaerts FM (1997) Training software for chiral separations in capillary electrophoresis. J Chromatogr A 772:195–202
194. Lelievre F, Gareil P, Bahadd Y, Galons H (1997) Intrinsic selectivity in capillary electrophoresis for chiral separations with dual cyclodextrin systems. Anal Chem 69: 393–401
195. Surapaneni S, Ruterbories K, Lindstrom T (1997) Chiral separation of neutral species by capillary electrophoresis evaluation of a theoretical model. J Chromatogr A 761:249–257
196. Abushoffa AM, Fillet M, Hubert PH, Crommen J (2002) Prediction of selectivity for enantiomeric separations of uncharged compounds by capillary electrophoresis involving dual cyclodextrin systems. J Chromatogr A 948:321–329
197. Abushoffa AM, Fillet M, Marini RD, Hubert PH, Crommen J (2003) Enantiomeric separation of aminoglutethimide by capillary electrophoresis using native cyclodextrins in single and dual systems. J Sep Sci 26:536–542
198. Mofaddel N, Krajian H, Villemin D, Desbene PL (2009) Enantioseparation of binaphthol and its monoderivatives by cyclodextrin-modified capillary zone electrophoresis: a mathematical approach. Talanta 78:631–637
199. Gibbs JW Jr (1881) Letter to American Academy of Arts and Science, January, 1881 (cited from Melander WR, Erard JF, Horvath CS (1983) J Chromatogr 282:211)
200. Chankvetadze B, Blaschke G (1999) Selector–selectand interactions in chiral capillary electrophoresis. Electrophoresis 20:2592–2604
201. Tiselius A (1930) The moving-boundary method of studying the electrophoresis of proteins. Nova Acta Reg Soc Sci Uppsala IV-7:1–107
202. Rundlett KL, Armstrong DW (1996) Examination of the origin, variation, and proper use of expressions for the estimation of association constants by capillary electrophoresis. J Chromatogr A 721:173–186
203. Rundlett KL, Armstrong DW (1997) Methods for the estimation of binding constants by capillary electrophoresis. Electrophoresis 18:2194–2202
204. Vespalec R, Bocek P (2000) Calculation of stability constants for the chiral selector-enantiomer interactions from electrophoretic mobilities. J Chromatogr A 875:431–445
205. Job P (1928) Application of the spectrographic and spectrophotometric method to the study of the hydrolysis of some alkaline salts. C R Acad Sci Paris 186:1546–1548
206. Branch SK, Holzgrabe U, Jefferies TM, Malwitz H, Matchet MW (1994) Chiral discrimination of phenethylamines with β-cyclodextrin and heptakis(2,3-di-*O*-acetyl)β-cyclodextrin by capillary electrophoresis and NMR spectroscopy. J Pharm Biomed Anal 12:1507–1517
207. Scott RL (1956) Recl Trav Chim Pays Bas 75:787–790
208. Chankvetadze B, Burjanadze N, Pintore G, Bergenthal D, Bergander K, Breitkreutz, Mühlenbrock C, Blaschke G (2000) Separation of brompheniramine enantiomers by capillary electrophoresis and study of chiral recognition mechanisms of cyclodextrins using NMR-spectroscopy, UV-spectrometry, ESI–MS and X-ray crystallography. J Chromatogr A 875:471–484
209. Chankvetadze B, Endresz G, Bergenthal D, Blaschke G (1996) Enantioseparation of mianserine analogues using capillary electrophoresis with neutral and charged cyclodextrin buffer modifiers, ^{13}C NMR study of the chiral recognition mechanism. J Chromatogr A 717:245–253
210. Endresz G, Chankvetadze B, Bergenthal D, Blaschke G (1996) Comparative capillary electrophoresis and nuclear magnetic resonance studies of the chiral recognition of racemic metomidate with cyclodextrin hosts. J Chromatogr A 732:133–142
211. Owens PK, Fell AF, Coleman MW, Kinns M, Berridge JC (1997) Use of 1H-NMR spectroscopy to determine the enantioselective mechanism of neutral and anionic cyclodextrins in capillary electrophoresis. J Pharm Biomed Anal 15:1603–1619

212. Owens PK, Fell AF, Coleman MW, Berridge JC (1998) Effect of charged and uncharged chiral additives on the resolution of amlodipine enantiomers in liquid chromatography and capillary electrophoresis. J Chromatogr A 797:187–195
213. Chankvetadze B, Pintore G, Bergenthal D, Burjanadze N, Strickmann D, Cerri R, Blaschke G (1998) Capillary electrophoresis, nuclear magnetic resonance and mass-spectrometric studies of opposite chiral recognition of chlorpheniramine enantiomers with various cyclodextrins. Electrophoresis 19:2101–2108
214. Chankvetadze B, Burjanadze N, Pintore G, Strickmann D, Bergenthal D, Blaschke G (1998) Chiral recognition of verapamil by cyclodextrins studied with capillary electrophoresis, NMR- and mass-spectrometry. Chirality 11:635–644
215. Chankvetadze B, Endresz G, Schulte G, Bergenthal D, Blaschke G (1996) Capillary electrophoresis and ^{1}H-NMR studies on chiral recognition of atropisomeric binaphthyl derivatives by cyclodextrin hosts. J Chromatogr A 732:143–150
216. Cescutti P, Carozzo D, Rizzo R (1996) Study of the inclusion complexes of aromatic molecules with cyclodextrins using ionspray mass spectrometry. Carbohydr Res 291: 105–115
217. Connors KA (1987) Binding constants. Wiley & Sons, New York
218. Rekharsky MV, Inoue Y (1998) Complexation thermodynamics of cyclodextrins. Chem Rev 98:1875–1918
219. Stefansson M, Novotny M (1993) Electrophoretic resolution of monosaccharide enantiomers in borate-oligosaccharide complexation media. J Am Chem Soc 115:11573–11580
220. Kano K, Tamiya Y, Hashimoto S (1992) Binding forces in complexation of p-alkylphenols with β-cyclodextrin and methylated β-cyclodextrins. J Incl Phenom Mol Recognit Chem 13:287–293
221. Benesi HA, Hildebrand JH (1949) A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. J Am Chem Soc 71:2703–2707
222. Foster R, Fyfe CA (1965) Interaction of electron acceptors with bases: Part 15. Determination of association constants of organic charge-transfer complexes by NMR spectroscopy. Trans Faraday Soc 61:1626–1631
223. Francotte E, Cherkaoui S, Faupel M (1993) Separation of the enantiomers of some racemic nonsteroidal aromatase inhibitors and barbiturates by capillary electrophoresis. Chirality 5:516–526
224. Castro-Puyana M, Crego AL, Marina ML, Garcia-Ruiz C (2007) Enantioselective separation of azole compounds by EKC. Reversal of migration order of enantiomers with CD concentration. Electrophoresis 28:2667–2674
225. Schneider HJ, Hacket F, Rüdiger V (1998) NMR Studies of cyclodextrins and cyclodextrin complexes. Chem Rev 98:1755–1786
226. Holzgrabe U, Mallwitz H, Branch SK, Jefferies TM, Wiese M (1997) Chiral discrimination by NMR spectroscopy of ephedrine and *N*-methylephedrine induced by β-cyclodextrin, heptakis(2,3-di-*O*-acetyl)β-cyclodextrin, and heptakis(6-*O*-acetyl)β-cyclodextrin. Chirality 9:211–219
227. Hybl A, Rundle RE, Williams DE (1965) The crystal and molecular structure of the cyclohexaamylose–potassium acetate complex. J Am Chem Soc 87:2779–2788
228. Saenger W, Jacob J, Gessler K, Steiner T, Hoffmenn D, Sanabe H, Koizumi K, Smith SM, Takaha T (1998) Structures of the common cyclodextrins and their larger analogues beyond the doughnut. Chem Rev 98:1787–1802
229. Harata K (1998) Structural aspects of stereodifferentiation in the solid state. Chem Rev 98:1803–1828
230. Kano K, Minami K, Horiguchi K, Ishihama T, Kodera M (1995) Ability of non-cyclic oligosaccharides to form molecular complexes and its use for chiral separation by capillary zone electrophoresis. J Chromatogr A 694:307–313
231. Copper CL, Davis JB, Sepaniak MJ (1995) Mechanisms of enantiomeric resolution in cyclodextrin-modified capillary electrophoretic separations of binaphthyl compounds. Chirality 7:401–408

232. Kano K, Tamiya Y, Otsuki C, Shimomura T, Ohno T, Hayashida O, Murakami Y (1993) Chiral recognition by cyclic oligosaccharides. Enantioselective complexation of binaphthyl derivatives with cyclodextrins. Supramol Chem 2:137–143
233. Lipkowitz KB (2000) Atomistic modeling of enantioselective binding. Acc Chem Res 33:555–562
234. Lipkowitz KB (1994) In: Subramanian G (ed) A practical approach in chiral separations by liquid chromatography. VCH, Weinheim, pp 19–55
235. Lipkowitz K (2001) Atomistic modeling of enantioselection in chromatography. J Chromatogr A 906:417–442
236. Dodziuk H, Lukin O, Nowińsk KS (2000) Molecular mechanics calculations of molecular and chiral recognition by cyclodextrins. Is it reliable? The selective complexation of decalins by β- cyclodextrin. J Mol Struct: THEOCHEM 503:221–230
237. Dodziuk H, Lukin O (2000) Modelling of molecular and chiral recognition by cyclodextrins. Is it reliable? Part 2. Molecular dynamics calculations in vacuum pertaining to the selective complexation of decalins by β-cyclodextrin. Pol J Chem 74:997–1001

Chiral Recognition Mechanism: Practical Considerations for Pharmaceutical Analysis of Chiral Compounds

Brian Lingfeng He

Contents

Abstract There are no particular differences between academic and industrial liquid chromatography chiral separations. In industry, throughput needs and time requirements force for a search for solutions, i.e., enantiomeric full separations, without time for additional investigations that could lead to an even better solution. The three-point interaction model is historically recalled and challenged. The

B.L. He (✉)
Bristol-Myers Squibb Research and Development, 1 Squibb drive, New Brunswick, NJ 08903, USA
e-mail: lingfeng_he@hotmail.com

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_6,

established mechanisms on the large variety of commercially available chiral stationary phases are rapidly described. The chapter finishes with examples taken from actual enantiomeric separations encountered in a pharmaceutical environment.

1 Introduction

Chirality is a pervasive theme in the living world. From the building blocks of life, such as amino acids, sugars, nucleosides, and nucleotides, to peptides, proteins, polysaccharides, and nucleic acids, chirality represents an intrinsic property of life. Consequently, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry and different responses are often observed when comparing the activities of a pair of enantiomers [1]. The first biological difference between enantiomers was reported by Louis Pasteur in 1858 that the dextro form of ammonium tartrate was more rapidly destroyed by *Penicillium glaucum* mold than was the levo isomer [2]. This discovery led Pasteur to eventually recognize the essential role that stereochemistry plays in the basic mechanisms of life. After 50 years, chirality entered mainstream pharmacological research due to the first pharmacological findings of the differential pressor effects of (−)- and (+)-epinephrine discovered by Abderhalde and Muller in 1908 [3]. With abundant evidences accumulated through extensive enantioselective pharmacokinetic and pharmacodynamic studies over the last century, it becomes evident that enantiomers of a chiral drug often demonstrate substantial differences in their pharmacokinetic, physiological, toxicological, and metabolic activities. Stereochemistry became a key issue in the development, approval, and clinical use of pharmaceuticals [4]. In 1992, US Food and Drug Administration (FDA) issued the first guideline for the development of stereoisomeric drugs [5], followed by other regulatory agencies worldwide [6]. In response to these regulatory requirements, control of stereochemistry quickly became an integral part of drug development in pharmaceutical industry. To date, many chiral drugs are only marketed as single enantiomer. In fact, the top five best-selling prescription medicines worldwide in 2005 were all single-enantiomer drugs with total sales topping $35 billions [7].

One of the driving forces behind these events has been the technological advances in the chromatographic separation of stereoisomeric compounds. In the past three decades, chiral chromatography has rapidly evolved from a scientific curiosity into a routine practice for the separation of stereoisomers. Chromatographic technologies such as high-performance liquid chromatography (HPLC) and super- or subcritical fluid chromatography (SFC) that employ chiral stationary phases (CSPs) are effective and reliable tools for accessing large quantities of enantiomers and determining stereoisomeric impurities of drug substances and drug products in pharmaceutical industry [8]. Since the introduction of the first HPLC CSP by Pirkle in 1981, hundreds of CSPs have become commercially available [9], and development of new chiral stationary phases remains an active field in academy and industry [10–12]. When the selection of an appropriate CSP for a separation is concerned, it would

be useful to understand how a CSP works, i.e., the mechanism governing a chiral recognition process. The chiral recognition mechanism on different CSPs has been the subject for numerous reviews and books, including this one [4, 13–20]. This chapter will first retrospect the evolution of chiral recognition model and then give an overview of chiral recognition mechanisms on some important CSPs. Finally, from the practical application point of view, this chapter will provide some perspectives on LC chiral column screening strategy and separation mode selection based on the consideration of chiral recognition mechanisms on several classes of CSPs that have been widely used in pharmaceutical industry.

2 Evolution of Chiral Recognition Model

2.1 Historical Development and the Three-Point Interaction Model

Understanding the chiral recognition mechanism behind stereoselectivity is extremely important for many fields, including proteins and biomimetic receptor design, drug discovery, and the development of more effective and versatile CSPs. The widely accepted model used to describe chiral recognition process is the three-point interaction (TPI) model or three-point rule whose origin can be traced back to as early as the work published by Easson and Stedman in 1933 [21]. As the pharmacological activity of a drug was concerned, they made the following statement:

> . . . a drug is attached to its specific receptor in the tissues in such a manner that a considerable proportion of the drug molecule is involved. If an asymmetric carbon atom is present, three of the groups linked to this atom may be concerned in the process. The exact nature of the attachment is immaterial to the main argument, but it is our view that this may be effected either by normal valencies or by adsorptive or other forces; or it may be of a loose type, somewhat analogous to the attachment of a glove to the hand, involving the contour of a large part of the molecule rather than points on its surface; or, finally, a combination of these possibilities may be involved.

In this classic paper, Easson and Stedman proposed that the enantiomers of a drug that demonstrated different pharmacological activities were due to their differential binding to a defined three-dimensional site on a receptor surface (Fig. 1). The receptor would distinguish between two enantiomers only if it possessed three nonequivalent binding sites. Discrimination occurred when one enantiomer simultaneously attached to all these sites of the receptor while the other one could not.

Inspired by Easson and Stedman's work, Dalgliesh established the "three-point attachment model" in 1952 to elucidate the chromatographic separation of D-/L-amino acids in cellulose paper chromatography [22]. The Dalgliesh's model was later improved by Lochmuller and Souter [23]. According to this three-point attachment model, it is necessary to have at least three attractive interactions, or two attractive and one repulsive (steric) interaction between the receptor and one of the

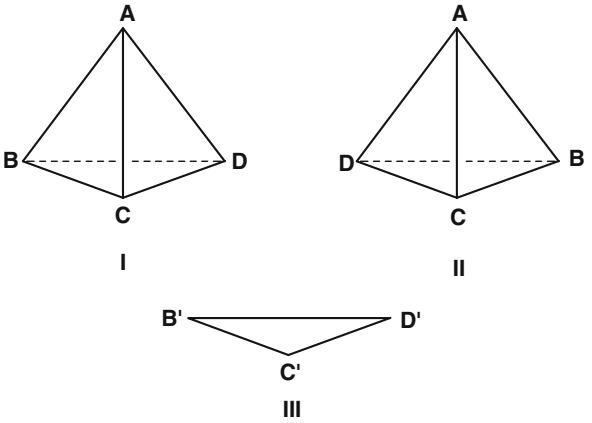


Fig. 1 The "three-point" interaction (TPI) model used by Easson and Stedman to explain stereochemical differences of chiral drug in pharmacological activities. I and II represent two enantiomers, and III depicts diagrammatically the surface of the specific receptor in the tissues. A, B, C, and D are the four groups linked to an asymmetric carbon of the chiral drug. The groups B, C, and D in the drug coincide with B′, C′, and D′, respectively, in the receptor (adapted from [21])

enantiomers to obtain enantioselectivity. At least one of these interactions must be stereospecifically related to the chiral centers of the receptor and the enantiomer.

2.2 Challenging the Three-Point Interaction Model

Davankov and other researchers made substantial contributions to impart the three-point interaction model with modern interpretation [24–26]. As pointed out by Davankov et al., it is required (but not necessarily sufficient) for the chiral selector to recognize the enantiomers to have at least three configuration-dependent active points, which are different in nature, on both chiral selector and enantiomer molecules. The active points on chiral selector must be complementary to and be able to simultaneously interact with those on enantiomer molecules. It is possible that two of the three required interactions can be repulsive if the third one is strong enough to promote the formation of diastereomeric associates between chiral selector and selectand [25]. Davankov et al. used the left- and right-hand model to vividly demonstrate that with the assistance of achiral surface, two-point or even one-point interaction is sufficient for chiral recognition. They treated these cases as expansion of TPI model rather than contradictions to it and asserted that the model is also applicable to CSPs based on proteins and polysaccharides. In some instances, achiral elements, such as solvent molecules and sorbent surfaces, may also participate in the chiral recognition process [24, 25].

The validity of the TPI model has been demonstrated in many stereoselective systems. In chromatography, this model is the basis of the reciprocity principle which Pirkle and his co-workers extensively utilized in the rational design of

CSPs [27]. In biological systems, the TPI model was first successfully applied by Ogston to explain the enzymatic formation of ketoglutarate from achiral citrate and decarboxylation of L-serine to glycine by enzymes [28]. Based on the same principle, Shallenberger et al. [29] established a similar model to explain how a sweet-taste receptor of the tongue distinguished D- and L-amino acids. In medicinal chemistry, the TPI model is a key element in structure–activity relationship studies [30].

However, it was reported that the TPI model did not always hold in some instances, and a number of alternative models were proposed to explain these observations, including two contact-point [31], extended three-point [32], four-location [33], and multi-site "stereocenter-recognition" (SR) models [34, 35]. Several limitations associated with the TPI model have been recognized by some researchers [31–39]. One of such limitations documented by Sundaresan and Abrol is that the chiral substrates originally used in the TPI model were enantiomers with a single chiral center; therefore, the requirements for chiral recognition of chiral molecules with multiple stereocenters may be different from what are specified in the TPI model [34, 35]. Based on the topological consideration of substrate, they proposed a general model, i.e., the SR model, to depict the enantioselective interactions between protein and substrate that have multiple stereocenters in enzymatic, neurological, and immunological systems. In this SR model, "interaction site" (a functional group attached to the chiral center of substrate) in the TPI model was replaced with "substrate location" (a functional group or groups) and "receptor site" that consists of specific functional groups or the contour of a large part of the receptor surface. According to the SR model, a substrate location may interact with multiple receptor sites, or multiple substrate location may interact with a single receptor site. The requirement for stereoselectivity to occur is that a minimum of N+2 substrate locations, where N is the number of stereocenters, must be involved in the interactions with receptor sites.

Another limitation recognized by Wainer et al. is that the TPI model does not take conformational mobility of the selector and the selectand into consideration, thus providing only a static and simplified view of chiral recognition. With this inadequacy of the TPI model in mind, Wainer et al. formulated a dynamic view of chiral recognition, i.e., the conformation-driven mechanism [37–39]. This model was derived from a series of experimental observations and molecular modeling of chromatographic enantioseparations on polysaccharide- and protein-based CSPs. According to this mechanism, each enantiomer of selectand interacts with the same sites on the chiral selector, and chiral recognition results from the conformational adjustments required for producing optimum interactions. The whole chiral recognition process consists of four interconnected steps that result in the differential stabilities of the obtained diastereomeric complexes: (1) the formation of the selector–selectand intermediate complex (tethering), a step driven by an initial attractive interaction. This step does not contribute to enantioselectivity. (2) The positioning of the selector–selectand to optimize interactions (conformational adjustments), a step allowing both selector and selectand molecules to adjust their conformation for the third step to take place. (3) The formation of

secondary interactions (activation of the diastereomeric complex), a step where secondary interactions help the two molecules take relative positions which determine the stability of final complexes. (4) Expression of the molecular fit (stabilizing and destabilizing interactions) [39]. Attractive interactions including electrostatic (or ionic), hydrogen-bonding, dipole–dipole, inclusion complexation, and π–π stacking interactions can stabilize the selector–selectand complex, while repulsive van der Waals interactions (or steric interaction) will have the opposite effect.

2.3 The Thermodynamics of a Chiral Separation

It should be borne in mind that chiral chromatography is a dynamic process of forming transient noncovalent diastereomeric complexes between chiral solutes and immobilized chiral selectors. Enantioselectivity (α) is a measurement of the thermodynamic stability ($\Delta\Delta G$) of the two diastereomeric enantiomer/chiral selector complexes. The $\Delta\Delta G$ parameter is the Gibbs free energy difference of the two complex selector–selectand formation. It includes both an enthalpic ($\Delta\Delta H$) and an entropic ($T\Delta\Delta S$) contributions (Eq. (1)):

$$\Delta\Delta G = \Delta\Delta H - T\Delta\Delta S = -RT \ \ln \ \alpha \tag{1}$$

Equation (1) derives from the classical Gibbs free energy equation for any chemical reaction:

$$\Delta G = \Delta H - T\Delta S \tag{2}$$

T_{iso} is a particular temperature at which the $\Delta\Delta G$ parameter cancels:

$$T_{\text{iso}} = \Delta\Delta H / \Delta\Delta S \tag{3}$$

The formation of complex results in a gain in enthalpy (binding) and a loss in entropy (higher order). Generally, enantioseparation is an enthalpy-driven process since the enthalpic contribution is due to attractive interactions that stabilize the diastereomeric complex. As the complex is more structured and involves more subtle interactions, the formation of complex could favor entropy change. This depends on temperature. At low temperature, the $T\Delta\Delta S$ term is lower than the $\Delta\Delta H$ term. At the T_{iso} temperature, both terms cancel (Eq. (3)) and there is no enantiomeric separation. At temperature higher than T_{iso}, the entropic term exceeds the enthalpic $\Delta\Delta H$ term. Now enantioseparation becomes entropy driven, which is seen by the reversal of the enantiomer elution order [25]. If the temperature is inappropriate, a successful chiral recognition does not necessarily result in a chiral discrimination of enantiomers in a chromatographic system. At temperatures below T_{iso}, enantioselectivity decreases with increasing temperature, which is the most common situation in practical chiral chromatography. It is the opposite at temperatures above T_{iso} where

enantioselectivity increases with increasing temperature. Usually T_{iso} is far beyond chromatographic operating temperatures [40]; however, such a specific temperature can be shifted to much lower temperatures (below room temperature) by changing mobile-phase composition [41].

3 Chiral Recognition Mechanism on Different CSPs

The evolution of the chiral recognition models reflects advances in understanding the enantioseparation mechanisms operating on various CSPs. According to the TPI model, it is important for chiral recognition to occur so that all interactions between the chiral selector and the solute take place around the stereocenters of both selector and solute, i.e., the interactions are configuration dependent. The interaction sites on chiral selector and selectand should be complementary to each other. The Wainer's conformation-driven chiral recognition mechanism points out that the formation of solute/CSP complexes is the key step in the enantioseparation and is always initiated by a variety of attractive interactions. Depending on CSPs, these initial attractive interactions can be quite different. For example, the driving force is chelating complexation for ligand-exchange CSPs, hydrogen-bonding and π–π interactions for Pirkle-type and polysaccharide-based CSPs, inclusion complexation interaction for cyclodextrin-based CSPs, and ionic interaction for macrocyclic glycopeptide and cinchona alkaloid CSPs. The detailed chiral recognition mechanism on a particular CSP is influenced by many factors, including the structure of the chiral solute, the mobile-phase composition, and temperature. It would be really important for understanding the chiral recognition mechanism on different CSPs to identify these key interaction elements. This section will provide a brief summary of the chiral recognition mechanisms on some important and commercially available CSPs under liquid chromatography (LC) and super- or sub-critical fluid chromatography (SFC) conditions.

3.1 Polysaccharide-Based CSPs

CSPs based on derivatized polysaccharides, i.e., tri-(phenylcarbamate) and tri-(benzoate) derivatives of amylose and cellulose, are well known for their extremely broad enantioselectivity and high separation efficiency [42–52]. They are the most useful CSPs at the moment [43]. The derivatized polysaccharide selectors can be coated [13, 43–49] or chemically immobilized on silica gel [50, 51]. The enantioseparation mechanisms on polysaccharide CSPs remain somewhat obscure at molecular level, due in part to the complexity of their structures that are not well defined. Many approaches involving chromatography, computation, and spectroscopy have greatly enhanced the knowledge and understanding of their chiral recognition process. X-ray studies revealed that triphenylcarbamate derivatives of cellulose and amylose have left-handed 3/2 and 4/1 helical structures, respectively. The polar carbamoyl

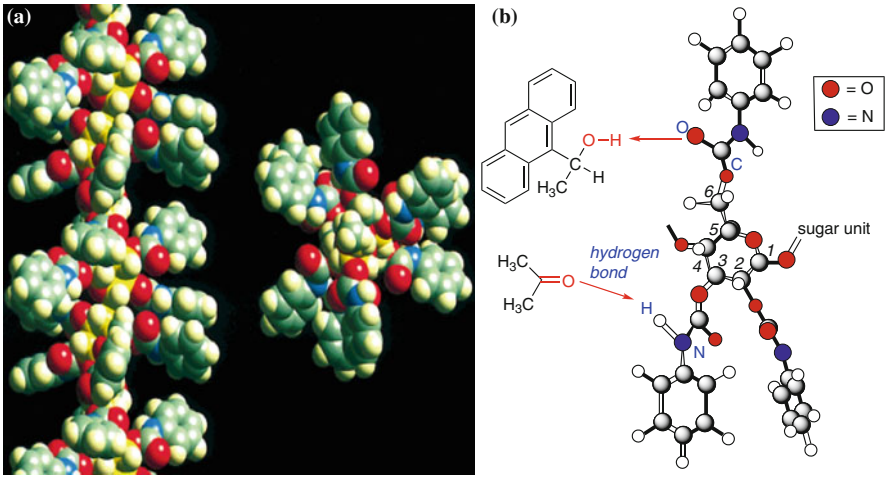


Fig. 2 (**a**) Optimized structure of cellulose triphenylcarbamate. (**b**) Possible interaction sites accounted for chiral recognition on cellulose triphenylcarbamate CSPs (adapted from [13])

groups preferably locate inside of and close to the chiral centers on the D-glucose backbone, while the hydrophobic aromatic groups locate outside of the polymer chain (Fig. 2) [13]. The H-bonding interactions between polar moieties (such as –OH, $-NH_2$, $-CONH_2$, $-O-$, $COO-$, $-COOH$) of chiral analytes and carbamate groups on the polysaccharide CSPs usually are the initial attractive interactions to facilitate the formation of a diastereomeric solute/CSP complex. They are essential for chiral discrimination since these H-bonding interactions are configuration dependant. Other intermolecular interactions such as $\pi-\pi$ interactions between phenyl groups on the CSP and aromatic groups of a solute, dipole–dipole interactions, and steric interactions due to the helical structure of CSPs all play important roles in the chiral recognition process. Also the side chains surrounding the helical polymer backbones can create nano-sized chiral cavities. The aromatic portion of chiral solutes can fit into these cavities and make additional contribution to the stability of the analyte–CSP complex [37, 52].

The chiral recognition ability of the polysaccharide stationary phases is significantly influenced by the substituents introduced to the phenyl group of the carbohydrate polymer since these substituents will change the acidity of the N–H group and the polarity of the C=O group on the carbamate linkage. The introduction of an electron-donating methyl group or an electron-withdrawing halogen at the *meta*- and/or *para*-position of the phenyl ring often improves the chiral recognition ability of the CSPs [45–49]. Consequently, different derivatized polysaccharide CSPs often show complementary enantioselectivity to each other. For analytes with ionizable functional groups, better separations are observed in their neutral form when H-bonding interactions between the neutral chiral analytes and polysaccharide CSPs are enhanced.

3.2 Macrocyclic Glycopeptide CSPs

Macrocyclic glycopeptide phases based on vancomycin (V), teicoplanin (T), and ristocetin (R) are the next most versatile class of CSPs after the polysaccharide CSPs introduced by Armstrong et al. [53–59]. Macrocyclic glycopeptides have been used to separate a wide variety of chiral molecules with diverse functionality, including derivatized and underivatized amino acids, peptides, hydroxy acids, amino esters, sulfoxides, imides, hydantoins, oxazolidinones, β-blockers, nonsteroidal anti-inflammatory drugs, and numerous other pharmaceutical and agrochemical compounds [53–64]. Like polysaccharides, macrocyclic glycopeptides are naturally occurring chiral molecules that consist of a peptide aglycone backbone to which are attached different sugar moieties (Fig. 3). The most popular polysaccharide CSPs were mainly coated onto silica gel particles. Only recently, bonded polysaccharide CSPs appeared on the market. The macrocyclic selectors were always chemically bonded to silica gel. The semi-rigid basket-shaped aglycone is the most essential part for chiral recognition, especially for the enantioseparation of chiral molecules with ionizable functional groups, while the sugar units may be involved in the resolution of the enantiomeric pairs of neutral compounds [57]. Also the sugar units are the major linkers that graft the native chiral selectors to silica gel surface.

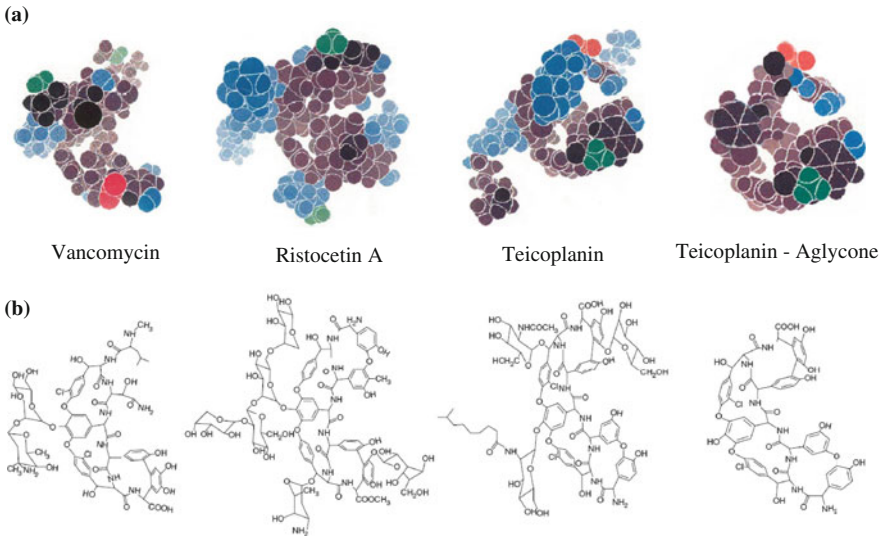


Fig. 3 Structures of the macrocyclic antibiotics vancomycin, teicoplanin, ristocetin A, and teicoplanin aglycone showing a profile view of the aglycone "basket" using (**a**) space-filling molecular models produced through energy minimization and (**b**) stick figures. The colored atoms in (**a**) denote the hydrophilic moieties, while the black portion designates the more hydrophobic regions. *Red* represents carboxylate groups, *green* ammonium groups, and *blue* hydroxyls. *Black* regions include the aromatic rings, connecting carbons, and amido linkages (adapted from [60])

Macrocyclic glycopeptide CSPs have a wide range of functional groups and characteristic shallow pocket conformation that enable them to interact with enantiomers through all possible intermolecular interactions. Depending on solute structure and mobile-phase conditions, ionic/electrostatic, H-bonding, and π–π interactions are probably the most dominant interactions between the solutes and CSPs, while dipole–dipole, hydrophobic, steric, and other interactions also play roles in the chiral recognition process [53–56].

Under certain circumstances, it is possible for enantiomers to form inclusion complexes with the aglycone shallow pockets of CSPs. A good example is the chiral separation of bupivacaine on Chirobiotic® V [40]. Under reversed-phase conditions within the temperature range of 5–45°C, the Van't Hoff plot of bupivacaine produced a positive change in both enthalpy (ΔH) and entropy (ΔS) with the $T\Delta S$ entropic terms of two enantiomers several times higher than the corresponding enthalpic terms. The entropy-driven selector–selectand interaction may indicate the formation of inclusion complexes between vancomycin and bupivacaine enantiomers [40]. For chiral molecules with ionizable functional groups, ionic interaction is believed to be responsible for the initial formation of analyte/CSP diastereomeric complex. The amine and carboxylic groups which are located at the mouth of the shallow pockets of the aglycone basket have been identified as the major binding sites for this class of CSPs [62, 63]. Complexation studies of vancomycin and teicoplanin with copper revealed that the chiral recognition ability of both CSPs was significantly deteriorated for acidic compounds and underivatized amino acids after the secondary amine on vancomycin and the primary amine on teicoplanin were blocked by copper through complexation.

The bonding procedure that is used to attach macrocyclic glycopeptides to silica gel can also modify the enantioselective properties of this class of chiral selectors. For example, vancomycin is an effective chiral selector in CE for the separation of anionic and cationic compounds [62, 64]; however, it showed much less enantioselectivity toward anionic compounds containing carboxylic group in HPLC [53, 54]. A possible reason is that both carboxylic and secondary amine groups of vancomycin aglycone remain free under CE conditions, while some of these groups may be partially hindered or consumed during the bonding process as a CSP in HPLC.

All three members of macrocyclic glycopeptides, V, R, and T, shown in Fig. 3 share a similar but not identical aglycone core. A distinct difference in their aglycone structures is that vancomycin has a carboxylic and a secondary amine groups, teicoplanin has a carboxylic and a primary amine groups, while ristocetin has one primary amine group but no free carboxylate group. The structural similarity and difference of macrocyclic glycopeptide CSPs gives rise to the operating principle of complimentary enantioseparation, which means that if only a partial enantioseparation is achieved on one CSP, then it is most likely that a baseline separation can be obtained on one of the related CSPs [61, 65]. In general and as a rule of thumb with pharmaceutical drugs, the vancomycin CSP does extremely well in separating basic compounds. The teicoplanin phase has high enantioselectivity for both basic and acidic compounds, while the ristocetin phase highly favors acidic compounds [53].

3.3 Cyclodextrin-Based CSPs

Cyclodextrins (CDs) make up a family of nonionic, homochiral, cyclic oligosaccharides composed of 6–12 D-(+)-glucopyranose units connected through α-1,4 linkages. The most commonly used α-, β-, γ-CDs contain six, seven, and eight glucopyranose units, respectively. CDs and their derivatives have been extensively employed in the resolution of enantiomers [66–85]. They predominate as CSPs in the applications of chiral GC [66] and in CE as chiral run buffer additives [67]. Covalently bonded CDs are important CSPs for HPLC [68–82] and SFC [83–85]. They have been successfully used to separate many pharmaceutical chiral compounds [69, 73]. The chiral recognition ability of CDs stems from their geometric structures and available functional groups.

β-CD is the most useful CSP in LC. Like other members of the CD family, β-CD has the toroidal shape of a hollow truncated cone in which seven primary hydroxyl groups on C_6 carbon are located at the smaller rim and fourteen secondary hydroxyl groups on the greater side of the cone with seven −OH on C_2 carbon pointing in a clockwise direction and the other seven −OH on C_3 carbon pointing counterclockwise (Fig. 4). Since the interior of the cavity is relatively hydrophobic compared to the hydrophilic hydroxyl-containing mouths, organic molecules tend to form inclusion complexes with CDs by fitting the hydrophobic moiety of the molecules into the cavity of CDs.

The hydroxyl groups, especially the secondary hydroxyl groups on the wide mouth, allow CDs to interact with analytes via hydrogen-bonding or dipole–dipole interactions. The existing interactions can be enhanced and new interactions can be introduced by chemically deriving hydroxyl groups of CDs with a variety of different groups, which in turn would alter the enantioselectivity of the bonded phases [75–80, 82, 84]. For example, an ionic interaction site can be introduced by incorporating a 3-methylimidazolium moiety into the structure of β-CD [84]. To promote π−π interaction, derivatized CD CSPs with aromatic substituents

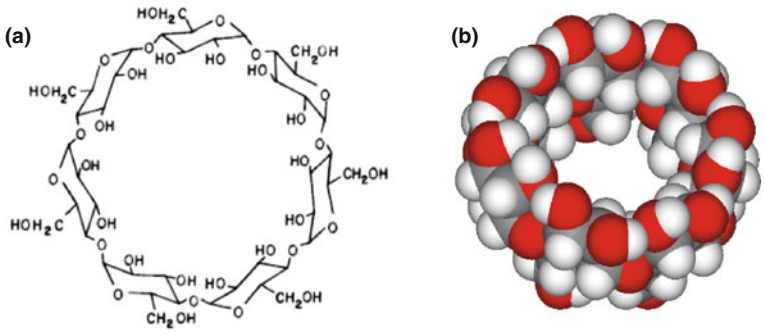


Fig. 4 The chemical structure (**a**) and space filling model (**b**) of β-cyclodextrin (carbon atoms are in *grey*, oxygen atoms are in *red*)

have been developed [76–78, 82]. The aromatic groups can be π-electron rich (i.e., π-basic), such as naphthylethyl in the case of 1-(1-naphthyl)ethyl carbamoylated β-CDs [76, 77] and dimethylphenyl in the case of 3,5-dimethylphenyl carbamoylated β-CD [82], or π-electron deficient (i.e., π-acidic), such as dinitrophenyl in the case of 2,6-dinitro-4-trifluoromethylphenyl ether-substituted β-cyclodextrin [78, 79]. Furthermore, carbamate linkage can provide additional sites for H-bonding and dipole–dipole interactions. After derivatization, steric repulsion at the mouth of the cavity would be increased for most included guest molecules since large number and greater size of substituents are attached to CDs. Recently, derivatized CD CSPs that carry ionic interaction site was also synthesized by incorporating 3-methylimidazolium moiety into the structure of β-CD [84].

Enantiomeric separations on CD-based CSPs have been achieved in reversed-phase, polar organic-phase, and normal-phase mode; however, the chiral recognition mechanisms under these mobile-phase conditions are quite different. The three-point interaction model was used to explain the observed enantioseparation quite well. In reversed-phase mode, inclusion complexation is thought to be the main factor of the chiral recognition and separation process for both native and derivatized CDs. A basic requirement for a molecule to form inclusion complex with CDs is to have aromatic or other hydrophobic groups (such as cyclohexyl). The strength of inclusion complex is determined by many factors including hydrophobic effect, hydrogen bonding between the guest molecule and CDs, release of high-energy water molecules from the CD cavity, decrease of strain energy of the CD macrocyclic ring system upon complexation, steric effects as well as the size and geometry of analytes relative to that of the CD cavity.

However, inclusion complexation does not necessarily lead to enantioselectivity due to the highly symmetrical geometry of the CD binding cavity [69, 73, 77, 80]. Consequently, other configuration-dependant interactions must take place in order to achieve chiral resolution. In the example of reversed-phase enantioseparation of propranolol on native β-CD CSP, naphthyl and hydroxyl groups of d- and l-propranolol are placed identically for optimal inclusion complex and hydrogen bonding, respectively. The important difference accounted for chiral discrimination originates from the hydrogen bonding between the secondary amine of propranolol and 2- and 3-hydroxyl groups of the β-CD selector (Fig. 5). In the d-propranolol complex, the nitrogen atom is positioned more favorably for hydrogen bonding. This implies that the D-isomer would preferentially interact with β-CD and thus be retained longer by the CSP. For derivatized CDs, π−π, dipolar, hydrogen-bonding interactions, and steric hindrance are also actively involved in the chiral recognition mechanism.

In polar organic mode, the mobile-phase system consisted mainly of an aprotic solvent such as acetonitrile in the presence of small portions of hydrogen-bonding modifiers (i.e., methanol, acetic acid, and triethylamine), which are used to regulate retention and enantioselectivity. Inclusion complexes are less likely to form in polar organic mode since the cyclodextrin cavity is occupied by acetonitrile, the dominant component of mobile phase. Instead, the hydrogen-bonding interactions between the analyte and the secondary hydroxyl groups at the mouth of the cyclodextrin

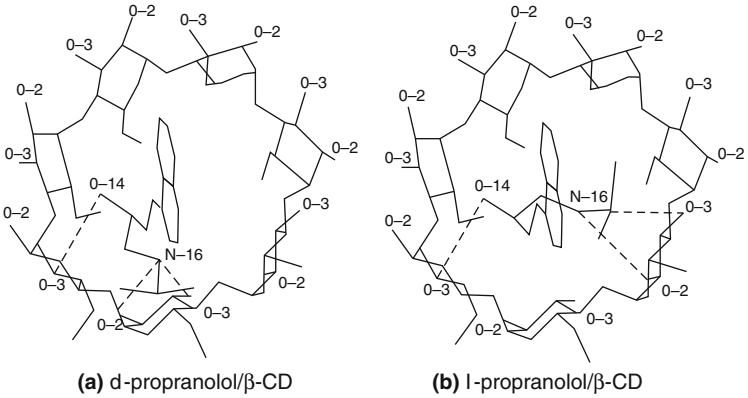


Fig. 5 Computer projection of inclusion complexes of (**a**) d-propranolol and (**b**) l-propranolol in β-cyclodextrin, from X-ray crystallographic data. The configurations shown represent the optimal orientation of each isomer on the basis of the highest degree of hydrogen bonding and complexation. The secondary amine moiety of analyte can potentially form hydrogen-bonds (*dotted lines*) with 2- and 3-hydroxyl groups at the rim of β-cyclodextrin with bond distances of 3.3 and 2.8 Å, respectively, for d-propranolol, and 3.8 and 4.5 Å, respectively, for l-propranolol (adapted from [69])

cavity are considered to be the most important interactions responsible for chiral recognition [73, 80]. π−π interactions (for derivatized CDs with aromatic groups), dipole stacking, and steric factors are also relevant. Among all CD-based CSPs, the bonded native β-CD CSP has the broadest enantioselectivity and usually requires a minimum of two hydrogen-bonding groups (such as a hydroxyl and a secondary amino group) for enantiomeric resolution with at least one of these groups in the vicinity of the stereogenic center. Derivatized CD CSPs including acetylated β-CD, 1-(1-naphthyl) ethyl carbamoylated β-CD and 2,6-dinitro-4-trifluoromethylphenyl ether-substituted β-cyclodextrin are also effective in polar organic mode.

In normal-phase mode, the apolar portion of the mobile phase (e.g., hexane or heptane) may occupy the CD cavity and hinder the formation of inclusion complexes. Consequently, chiral separation with native CDs is quite rare in this mode. In contrast, derivatized CDs, particularly 2,6-dinitro-4-trifluoromethylphenyl ether-substituted β-cyclodextrin [76, 77], (*R*)- and (*S*)-1-(1-naphthyl)ethyl carbamoylated β-CDs [78, 79], have shown good enantioselectivity under normal-phase conditions. It seems that both the CD cavity and substituent moieties may be involved in the chiral recognition process on these derivatized CD phases. The observation of experimental data suggests that the chiral recognition mechanism in normal-phase mode probably consists of two successive steps: (1) the solute first interacts with the aromatic substituents and residual hydroxyl groups of the derivatized CD through strong π−π, dipolar, or H-bonding interactions and (2) the hydrophobic portion of the solute fits into the CD cavity [76, 77, 81]. The CD cavity can have different degrees of contribution to the overall enantioselectivity depending on the structure of analytes. For (*R*)- and (*S*)-1-(1-naphthyl) ethyl carbamoylated β-CDs, it was suggested that the bonded moieties were entirely responsible for the observed

enantioselectivity with essentially no contribution from the CD. Indeed, reversals of the elution order were observed on (*R*)- and (*S*)-bonded CD CSPs [77]. Apparently, the incorporation of additional chiral centers into the CDs can be advantageous in controlling the elution order of enantiomers.

3.4 Protein-Based CSPs

Proteins are complex biopolymers with high molecular weight and consist of L-amino acids and sugars (in the case of glycoproteins). They play various important roles in biological systems and display highly stereo-specific affinity to small chiral molecules. This property has prompted numerous applications of proteins in the separation of enantiomers. A few classes of proteins including albumins, glycoproteins, and enzymes have been chemically bonded to silica gel and evaluated as HPLC CSPs [17]. Among them, CSPs based on bovine serum albumin (BSA) [86], human serum albumin (HSA) [87], α-acid glycoprotein (AGP) [88], ovomucoid (OVM) from chicken egg whites [89], avidin (AVI) [90], cellobiohydrolase I (CBH I) [91], and pepsin [92] are commercially available. Some important physical properties and applicability of these commercialized CSPs are given in Table 1.

Protein-based CSPs are almost exclusively used in reversed phase with aqueous mobile phases containing a phosphate buffer. Many of these CSPs, AGP in particular, show broad enantioselectivity for a wide range of acidic, basic, and neutral compounds including many chiral drugs [18, 93, 94]. The enantioselectivity and retention of a chiral separation on a protein-based CSP can be affected by numerous factors such as the mobile-phase pH, the nature and concentration of organic and/or charged modifier, the ionic strength, and temperature. Under reversed-phase conditions, a number of binding forces, i.e., ionic, hydrophobic, H-binding, $\pi-\pi$, may participate in the retention and chiral recognition of solutes, depending on its functional groups. AGP and CBH CSPs are extremely useful for the resolution of basic compounds implying that ionic interactions occur between the protonated amine group of the solute and some acidic residuals in the hydrophobic cavity of the proteins. Hydrophobic, H-bonding, or other interactions help to stabilize the analyte/protein complex and contribute to enantioselectivity. Also steric interactions can be important for protein-based CSPs considering the significant number of chiral cavities found in macromolecular proteins.

It is true that the unambiguous elucidation of chiral recognition mechanisms on various protein-based CSPs is challenging and often difficult since precise information about the tertiary and quaternary structures of proteins is not always available. Multiple stereo-specific sites may be involved in chiral recognition process. However, it is encouraging to see the progresses that have been made in this field in recent years [17, 95−102].

In general, two enantiomers interact with a protein CSP at the same binding site; however, a few exceptions were observed in cases where each enantiomer bound to different sites [97–99]. To understand the origin of enantioselectivity, it

Table 1 Physical properties of proteins and their applicability as HPLC CSPs (data from [17, 93])

Protein	Column trade name	Molecular mass	Isoelectric point	Applicability
α_1-Acid glycoprotein	CHIRAL-AGP[a]	~41,000	2.7	Acids, bases, and neutral compounds
Bovine serum albumin (BSA)	CHIRAL BSA[a] ULTRON ES-BSA[b] RESOLVOSIL BSA-7[c]	~66,000	4.7	Acids, zwitterionic and neutral compounds
Human serum albumin (HSA)	CHIRAL HSA[a]	~65,000	4.7	Acids, zwitterionic and neutral compounds
Cellobiohydrolysis I (CBH I)	CHIRAL-CBH[a]	~64,000	3.9	Acids, bases, and neutral compounds, but preferably bases
Ovomucoid (OVM)	ULTRON ES-OVM[b]	~28,000	4.1	Acids, bases, and neutral compounds
Avidin (AVI)	Bioptic AV-1[d]	~66,000	10.0	Acids, bases, and neutral compounds, but preferably acids
Pepsin	ULTRON ES-PEPSIN[b]	~34,600	<1	Bases and neutral compounds

[a]Regis Technologies, Inc., Morton Grove, IL 60053, USA
[b]Agilent Technologies, Santa Clara, CA 95051, USA and Shinwa Chemical Industries Ltd., Japan
[c]Macherey-Nagel, Düsseldorf, Germany
[d]GL Sciences, Torrance, CA 90503, USA

is necessary to clarify these stereo-specific binding sites on the protein CSP. As an example, HSA is one of the most studied proteins. HSA has two major enantioselective binding sites identified for drugs, i.e., warfarin–azapropazone (site I) and indole–benzodiazepine (sites II) sites. Both sites are located at hydrophobic cavities of subdomains IIa and IIIa of HSA, respectively (Fig. 6). Other minor sites were also proposed since the two major binding sites cannot satisfactorily explain the binding of some drugs. All these different stereoselective binding sites can be brought into operation under different mobile-phase conditions [17]. It was reported that (*S*)-warfarin caused an allosteric change in binding site II after it bound to site II. When a 40 μM concentration of (*S*)-warfarin was added to the mobile phase, HSA had much higher affinity for (*S*)-lorazepam hemisuccinate, resulting in a much higher enantioselectivity for (*R*)- and (*S*)-lorazepam [100].

In recent years, molecular modeling has become an increasingly important tool to gain information on intermolecular interaction between a chiral molecule and a CSP. A newly developed CSP based on immobilized penicillin G acylase (PGA) was found quite successful in the separation of acidic enantiomers [102].

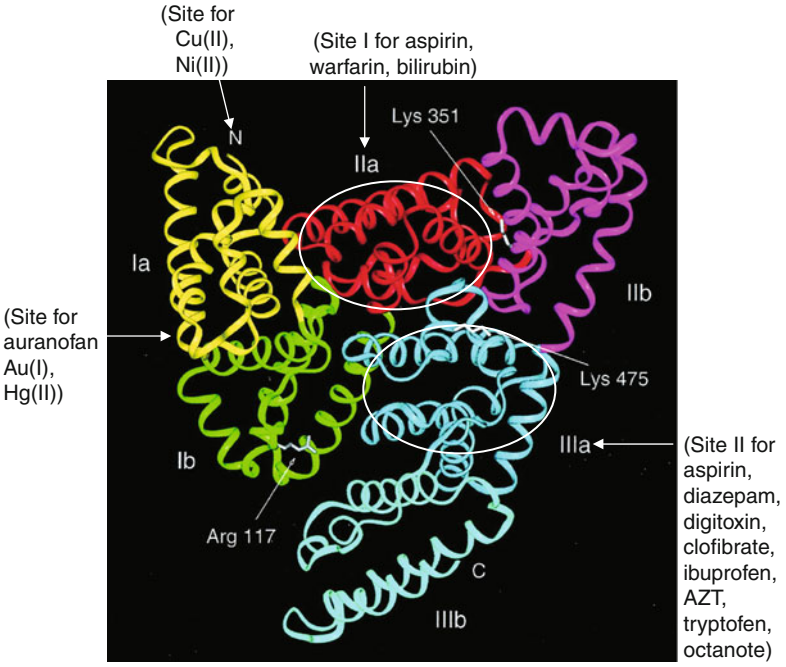


Fig. 6 Schematic drawing of the HSA molecule with identified binding sites for various ligands. Each subdomain is marked with a different color. Enantioselective binding sites I and II located at subdomains IIa and IIIa, respectively, are responsible for the chiral recognitions of many drugs (adapted from [95])

Chromatographic data suggest that the electrostatic interactions between compounds and basic residues of the enzyme might be the main binding force to form the stereospecific analyte/protein complex. Chiral recognition process takes place when the acid solute is attracted to the PGA hydrophobic cavity through ionic interactions between the solute carboxylic group and a positively charged group located at the entrance of the binding pocket of PGA. Next the solute hydrophobic moiety can be inserted into the PGA cavity [102]. Hydrophobic and steric interactions are considered to be important for observed enantioselectivity. By doing docking study, the binding mode of (*R*)- and (*S*)-2-(4-chlorophenyl)-2-phenoxyacetic acid inside the PGA cavity was established as shown in Fig. 7. It is clear from the figure that the (*R*)- and (*S*)-enantiomers have established interactions between their carboxylate group and the protein's cavities. However, the critical difference comes from a specific H bond between the phenoxyl ether oxygen of the solute and the ArgB263 side chain on protein (Fig. 7). The presence of this H bond makes the (*S*)-enantiomer/PGA more stable than the (*R*)-one and the (*S*)-enantiomer will be retained longer in chromatography. In this case, the molecular modeling result is in excellent agreement with chromatographic data, although not a single water molecule is shown.

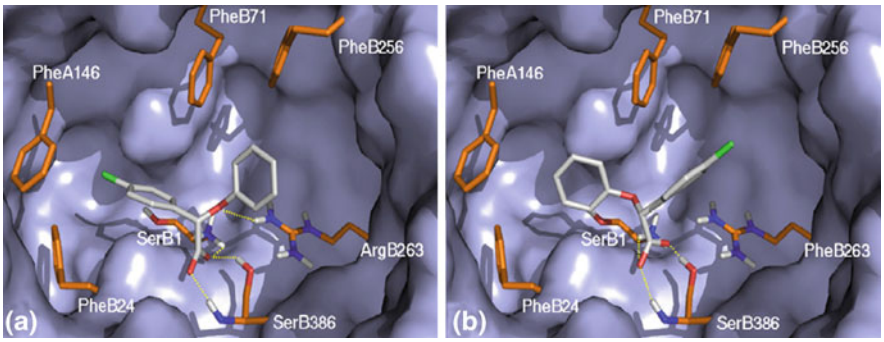


Fig. 7 Binding mode of (*S*)-2-(4-chlorophenyl)-2-phenoxyacetic acid (**a**) and (*R*)-2-(4-chlorophenyl)-2-phenoxyacetic acid (**b**) within the cavity of PGA CSP. Hydrogen bonds between analyte and protein are shown as *dashed yellow lines* (adapted from [102])

3.5 Pirkle-Type CSPs

Π-Complex CSPs, also called Pirkle-type CSPs, comprise a large collection of phases developed from low molecular mass chiral selectors, which were designed to contain only those interaction sites that are essential for the enantiomer differentiation [19, 103]. Pirkle and his co-workers have made significant contribution to the development and understanding of the chiral recognition mechanisms of π-complex-type CSPs [104–116]. The principle of reciprocity, a mechanistic rationale stemmed from the TPI model, has been long advocated by Pirkle and co-workers as the basis for the design of the chiral selectors and CSPs. It states that if a particular chiral selector has different affinities for the enantiomers of another substance, then a single enantiomer of the substance will necessarily have different affinities for the two enantiomeric forms of the initial selector [104]. This principle led to the development and commercialization of a series of useful CSPs for HPLC [105]. It is the π–π interaction that Pirkle-type CSPs intend to promote the most for the resolution of enantiomers. Hence, a prominent structural feature of these phases is the presence of π-acidic (π-electron acceptor) [106, 107], or π-basic (π-electron donator) [108], or both [104, 109] aromatic rings in the chiral selector. Another prevailing interaction among Pirkle-type CSPs is hydrogen bonding. Amido moieties from an amide, a carbamate, urea, or an amine group very close to the asymmetric center of the chiral selector are the most common sites for strong hydrogen binding. Other potential sites that may also be involved in H bonding include sulfinyl, phosphinyl, hydroxyl, ether oxygen, and even π-electron groups. Other interactions such as dipole–dipole and steric interactions may also play important roles in the chiral recognition on Pirkle-type CSPs depending on the analyte structure.

The success of the Whelk-O1® CSP is truly an excellent example of the rational design of CSP based on chiral recognition mechanistic considerations [104, 110]. The chiral selector of the Whelk-O1® CSP has a π-acidic (3,5-dinitrobenzamide) as well as a π-basic (naphthyl) group directly on (or near) the stereogenic centers

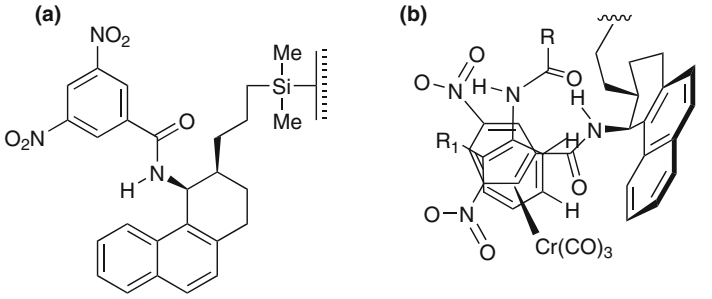


Fig. 8 The structure of (**a**) (*S*,*S*)-Whelk-O1® and the proposed chiral recognition model between (**b**) the CSP and the most retained enantiomer of amide-type planar tricarbonyl–chromium complexes (adapted from [111])

of the chiral selector (Fig. 8). The cyclohexyl ring contains two chiral centers: one has a dinitrobenzamide group with π-acidic properties and the other chiral center is attached to the silica backbone and allows increasing the conformational rigidity of selector controlling the orientation of the naphthyl group (Fig. 8a). Accordingly, the 3,5-dinitrobenzamide and naphthyl groups are perpendicular to each other and give a cleft shape to the chiral selector (Fig. 8b). Additionally, an R1 alkyl linkage in the *cis*-position of dinitrobenzamide (Fig. 8b) would hinder enantiomers to interact with the chiral selector from the back of the cleft, thus further improving enantioselectivity by enhancing the interactions inside the cleft between enantiomers and the chiral selector. On the basis of chromatographic and spectroscopic evidences, it was rationalized that the formation of diastereomeric solute/CSP complexes would be facilitated by three major attractive interactions, i.e., a hydrogen bond between the amide –NH of the chiral selector and the polar moiety of the analyte and simultaneous face-to-face and face-to-edge π−π interactions. As an example, a chiral recognition model proposed for the direct enantiomeric separation of amide-type planar tricarbonyl–chromium complexes on (*S*,*S*) Whelk-O1 CSP is shown in Fig. 8b. In this case, the hydrogen bond took place between the amide −N−H of the CSP and the carbonyl oxygen of the analyte, while the face-to-face and face-to-edge π−π interactions were provided by the aromatic portion of the analyte as it occupies the cleft and the 3,5-dinitrobenzamide and the naphthyl groups of the CSP, respectively [111].

3.6 Cinchona Alkaloid-Based CSPs

Quinine (QN) and quinidine (QD) are natural enantiomers belonging to the cinchona alkaloid family, an important subgroup of naturally occurring polycyclic β-carboline alkaloids. They are widely used as resolving agents for chiral acids via preferentially forming diastereomeric salts with one of the enantiomers [117]. From the reciprocity concept point of view, the logic path is that QN and QD are potential chiral selectors

for the resolution of the enantiomers of chiral acids with complementary interaction sites. Indeed, this has been the subject of many studies in the past few years, and the QN- and QD-based CSPs demonstrated high enantioselectivity for chiral aryl-, aryloxy-, arylthiocarboxylic acids, N-derivatized amino acids and peptides, and many other chiral acids containing sulfonic, phosphonic, and phosphoric acid groups [117–125]. Consequently, two weak anion-exchange HPLC CSPs (namely Chiralpak® QN-AX and QD-AX) derived from QN and QD, respectively, were developed and marketed by Lindner and his co-workers.

QN and QD consist of a planar quinoline and a rigid quinuclidine ring and are different in the configuration of C_8 and C_9 chiral centers, thus diastereomers to each other (Fig. 9a). Interestingly, QN and QD CSPs display "pseudo-enantiomeric" property in most cases. It means that an opposite elution order can be obtained when the same enantiomeric pair is separated on both CSPs. This property is likely due to

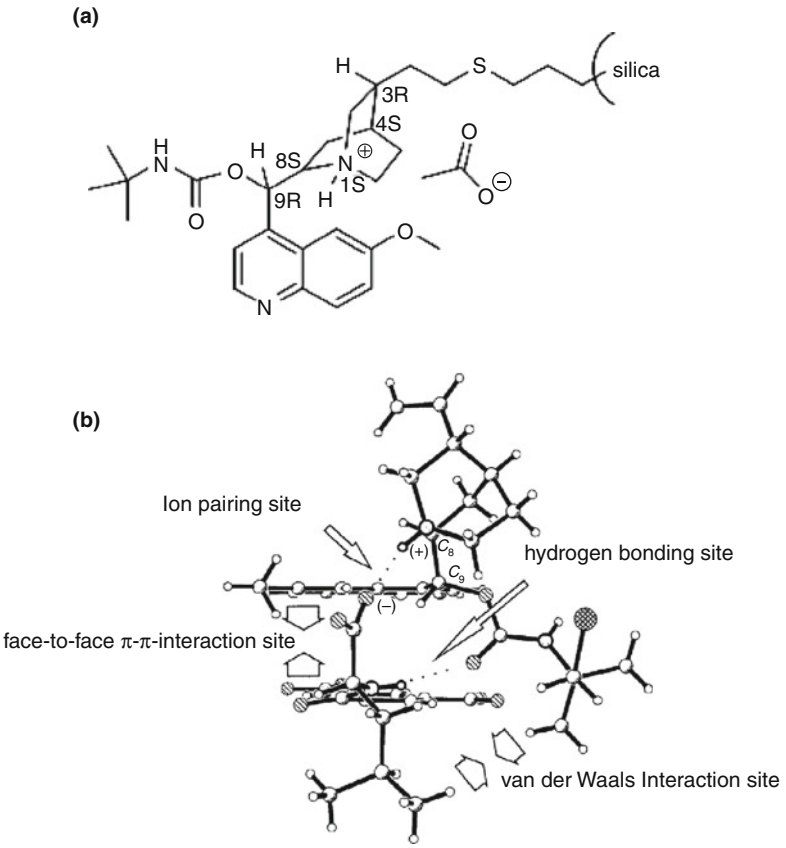


Fig. 9 Structure of (**a**) protonated *tert*-butylcarbamoylquinine CSP and (**b**) the proposed interaction model for the 1:1 diastereomeric complex form by DNB-(*S*)-leucine and β-chloro-*tert*-butyl carbamoyl quinine, an analogue of the chiral selector of *tert*-butylcarbamoylquinine CSP. The *arrows* point to the binding interactions contributing to the chiral recognition (adapted from [118])

the C_9 stereogenic center of the cinchona skeleton, which is the essential binding site for chiral recognition. Hence, the enantioselectivity is controlled by the chirality of the C_9 chiral center [117, 118]. The binding sites surrounding the C_9 stereogenic center include the basic bridge-headed nitrogen group of the quinuclidine ring for electrostatic interaction after protonation, the carbamate group for H-bonding and/or dipole–dipole interactions, and the π-basic quinoline ring for π−π interaction. Furthermore, the bulky quinuclidine group and the large planar quinoline ring are also available for steric interaction (attraction and/or repulsion). The long-range electrostatic/ionic interaction between the protonated tertiary amine of the chiral selector and the deprotonated carboxylic group of the selectand is believed to be responsible for the initial contact between analyte and CSPs [119]. This force may not contribute to chiral recognition; however, it would guide the solute to approach the CSPs in an energetically appropriate orientation and induce other stereospecific interactions such as H-bonding and/or dipole–dipole, π−π, and steric interactions to take place [117–119].

The importance of these stereospecific interactions was clearly shown in the enantioseparation of derivatized amino acids on quinine- and quinidine-based CSPs, where the N-protecting group and the bulkiness of the side chain of amino acids had significant effects on the enantioselectivity [119, 120]. The X-ray crystal structures of selector–selectand complexes provided important insights into the chiral recognition mechanism on cinchona alkaloid-based CSPs. As an example, in the X-structure shown in Fig. 9b, an energetically favored diastereomeric complex was formed by inserting DNB-(*S*)-leucine into the cleft of β-chloro-*tert*-butylcarbamoylquinine. The cleft configuration adopted by the chiral selector was defined by the bulky quinuclidine and quinoline groups. In this case, several simultaneous interactions lead to chiral recognition and discrimination. The docking ionic interaction between the deprotonated carboxylic group of the analyte and the protonated quinuclidine of the chiral selector is critical. The hydrogen bonding between amide moiety of the analyte and the carbamate group of the chiral selector, the face-to-face π−π interaction between the quinoline ring and the 3,5-dinitrobenzoyl group, and the steric interaction introduced by the side chain of the DNB-(*S*)-leucine complete the TPI rule [118, 119].

3.7 Chiral Crown Ether-Based CSPs

Chiral crown ethers are synthetic macrocyclic polyethers and were first introduced as CSPs for LC by Cram and co-workers in the late 1970s. In their pioneer works, bis-(1,1'-binaphthyl)-22-crown-6 was immobilized on silica gel [126] or polystyrene [127] to resolve α-amino acids and their derivatives. Since then, different chiral crown ether CSPs have been developed and successfully applied in the HPLC separation of enantiomers containing primary amine and secondary amine groups [20, 128–139]. Both dynamically coated [128, 129] and covalently bonded [130–132] chiral crown ether CSPs are commercially available.

Chemically bonded CSPs based on (18-crown-6) 2,3,11,12-tetracarboxylic acid ($18C_6TA$, Fig. 10) are highly effective in the resolution of natural and unnatural α-amino acids (except for proline) [131–133], primary and secondary amino alcohols [134–136], β-amino acids [137], aryl α-amino ketone [138], and α-amino acid derivatives [20, 131]. A number of pharmaceutically important compounds such as β-blockers [134, 135], fluoroquinolone antibacterial agents [139], amphetamine, phenylethanolamine, octopamine, and norepinephrine [132] were separated quite well on this crown ether CSP.

Depending on the available functional groups on both analyte and chiral selector, many interactions, including complexation, H-bonding, ionic, steric, π−π, and hydrophobic, may be involved in the chiral discrimination of enantiomers on chiral crown ether CSPs. The importance of these interactions varies with mobile-phase composition. In aqueous–organic mobile phase, it is believed that the tripodal complexation of the protonated ammonium ($R-NH^+$) inside the cavity of the polyether ring of the chiral selector mediated through three ^+N-HO hydrogen bonds is essential for chiral recognition [140, 141]. Consequently, enantiomers without a primary amine group are less likely to be resolved in acidic aqueous–organic mobile phase. Instead, polar organic mobile-phase and/or normal-phase modes are more suitable for the resolution of enantiomers with secondary amine or other chiral compounds with primary amine where H-bonding interaction is more important for chiral recognition [130, 134–136].

β-Blockers have the asymmetric center $R–NH–CH_2–CHOH–R'$ with a secondary amine. They were effectively separated on $18C_6TA$ crown CSP under moderately acidic polar organic mobile-phase conditions with acetic acid–triethylamine–ethanol–acetonitrile. Multiple hydrogen bonds were formed between analytes and the crown ether selector: the protonated secondary amine moiety of the analytes formed two hydrogen bonds with crown oxygens and the asymmetric hydroxyl group formed an additional hydrogen bond with carboxylate side chains of $18C_6TA$. Ionic interaction between the positively charged secondary ammonium and negatively charged carboxylate groups also made significant contribution to the retention and chiral recognition of β-blockers on $18C_6TA$ CSP [134–136]. In this instance, the enantioseparation is an entropically controlled process. However, when the

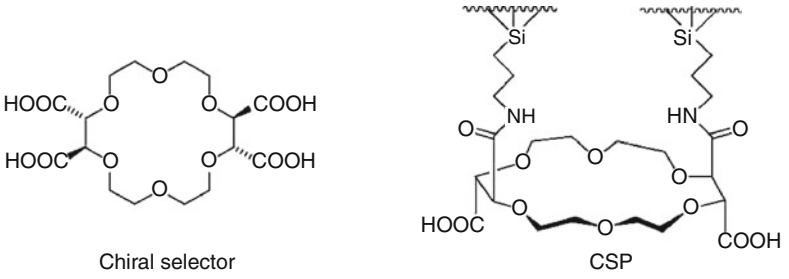


Fig. 10 Structure of CSP prepared from (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (adapted from [132])

secondary amine group was directly attached to the asymmetric center of amino alcohols, the enantioseparation became an enthalpically controlled process. Steric interactions played an important role as secondary interactions in the chiral discrimination of secondary amino alcohols [136]. Overall, it seems that a minimum of two H-bonding interaction sites around the solute chiral center is required for the analyte to be enantioselectively separated on $18C_6$TA crown CSP in nonaqueous mobile-phase conditions.

4 Practical Considerations of Chiral Recognition Mechanism in Pharmaceutical Analysis

4.1 Chiral Method Development in the Pharmaceutical Industry

Chiral chromatographic techniques that use chiral stationary phases (CSPs) in columns are often the preferred approaches to access large quantities of enantiomers and for the determination of enantiomeric impurity in drug substances and drug products in the pharmaceutical industry. The development of new CSPs has provided a great freedom of choice. Now, a chiral separation can be achieved on many different CSPs based on different chiral recognition mechanisms or in different separation modes on the same CSP. For example, the β-adrenergic blockers, an important class of chiral amino alcohol drugs, were directly resolved on a wide array of CSPs, including phases based on tris(3,5-dimethylphenylcarbamate) cellulose in normal-phase (NP) mode [70, 142, 143] and reversed-phase (RP) mode [70], macrocyclic glycopeptides in polar organic mode (POM) [53–55] and SFC [144], proteins in RP [145], β-cyclodextrins in RP [69] and POM [73], chiral crown ethers in POM [134], and Pirkle-type chiral selectors in NP [146].

4.1.1 Selecting a Chiral Column

When selecting a chiral column for analytical use, one must take a number of factors into consideration:

(i) In general, a suitable chiral column should give high enantioselectivity, excellent peak efficiency and sensitivity, a reasonable separation window, and the desired elution order (ideally the enantiomeric impurity elutes first). A column with an extremely large enantioselectivity may not be practically useful for chiral assays, since the second eluted enantiomer has too often a very poor peak efficiency resulting in broad peaks with low detection sensitivity. Also an enantioseparation with a short retention time for the first eluted enantiomer (i.e., close to the void volume) should be avoided for the purpose of quantitation of enantiomeric impurity even though the enantioselectivity is acceptable.
(ii) Chiral column selectivity for chemical impurities and diastereoisomers may be equally important. Often a high enantioselectivity is needed when a chiral

compound contains more than one stereogenic center (2^n rule for enantiomer number) and the sample is available in a complex matrix [147]. In this case, interferences with co-eluting impurities in the solute enantioseparation can be expected. An example is given in Fig. 11a: in the enantiomeric separation of 1-(2-naphthalenethioyl)-2-propanol on a Chiralcel® OD-H column, a chemical impurity was co-eluted with the *R*-enantiomer. This interfering peak must be resolved in order to accurately determine the enantiomeric purity and enantiomeric excess (ee) of the (*S*)-1-(2-naphthalenethioyl)-2-propanol [147]. Without sacrificing the short analysis time, a separation with improved enantioresolution and achiral selectivity was achieved on a Lux® Cellulose-1 column, which contains the same chiral selector as OD-H column but is prepared differently and available from a different vendor (Fig. 11b) [148]. Sometimes, an achiral impurity interfering with the chiral impurity can be circumvented by using highly selective MS detection [149].

(iii) The chiral separation should be compatible with detection method. For example, mass spectrometry detectors prefer a chiral separation in reversed phase or polar organic phase instead of in normal phase. Also, MS or light-scattering detectors allow only volatile salts as mobile-phase additives. Nonvolatile buffers, such as phosphate and perchlorate, should be replaced with more volatile salts such as ammonium acetate or ammonium formate.

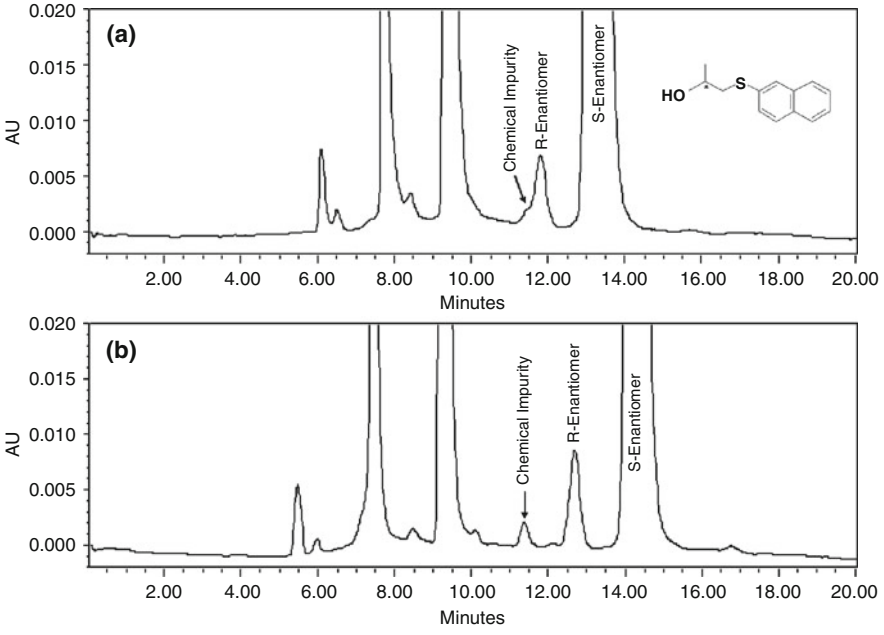


Fig. 11 Comparison of enantiomeric separation of 1-(2-naphthalenethioyl)-2-propanol on (**a**) Chiralcel® OD-H and (**b**) LUX® Cellulose-1 in the normal mobile phase (20/80 isopropanol/heptane). The sample was extracted from a reaction mixture. Please note that a chemical impurity co-eluted with (*R*)-enantiomer in (**a**) was well resolved in (**b**) (adapted from [148])

(iv) The physical and chemical properties of chiral analytes may be an important factor in column selection. For compounds that are poorly soluble (less than 10 μg/ml) or prone to hydrolysis in aqueous mobile phase, a chiral separation in waterless NP or POM mobile phases is sought [147].

(v) In pharmaceutical industry, the stages of pharmaceutical development should also be considered in chiral column selection and chiral method development [150]. In drug discovery and process, a high-throughput chiral method is required to accommodate huge sample volume and large numbers of novel chemical entities. Consequently, chiral SFC separation becomes advantageous due to its fast method development, high separation efficiency, and short analysis time [149, 151–153]. In early stage of pharmaceutical development, there is more flexibility in selecting chiral columns and separation technologies, and it is not unusual to adapt the method developed in drug discovery or process department. However, when a drug candidate moves to late stages of development, the sensitivity and the robustness of the chiral method become two major concerns in chiral column selection. At these stages, all the chiral methods related to the drug substance or chiral intermediates often need to be redeveloped. The selected chiral column must demonstrate excellent run-to-run and batch-to-batch reproducibility, which would facilitate the method transfer to worldwide manufacturing sites and quality control laboratories.

With the above-mentioned considerations related to chiral column selection in mind, it is very important to have an effective screening strategy to identify the appropriate chiral column.

As a matter of fact, selecting the proper chiral column is the most essential and challenging part of chiral method development. Although some progresses have been made in predicting enantioselectivity on a given CSP based on the molecular structure of a chiral compound [154] or the molecular structure similarity between an analyte and those already separated [155], there is still no way to foresee which chiral column is more suitable for the resolution of the enantiomers of a given compound. Indeed, the chiral recognition process is sensitive not only to the structures of both analyte and CSPs but also to the mobile-phase conditions. Currently, a practical approach that is widely utilized in pharmaceutical industry, particularly in drug discovery, is to screen a small set of selected CSPs which have demonstrated a broad enantioselectivity [149–153, 156–166]. The implementation of new technologies, such as automated systems [156, 157], multiple-column parallel screening stations [156, 157], mobile-phase gradient [152, 153, 158–161], and SFC [149, 151–153], has significantly accelerated the process of chiral column selection and makes it possible to develop and optimize chiral separation within a day [157].

4.1.2 The Domination of the Polysaccharide Chiral Stationary Phases

The most popular CSPs selected to be included in the reported chiral column screening schemes are four polysaccharide-based phases, including cellulose tris(3,5-dimethylphenylcarbamate), cellulose tris-4-methylbenzoate, amylose

tris(3,5-dimethylphenylcarbamate), and amylose tris(*S*)-α-methylbenzylcarbamate. Under SFC conditions, the success rate for these four columns could reach as high as or exceed 98% for SFC soluble enantiomers [153]. The success rate was close to 90% for these four columns under HPLC conditions [158, 159]. In recent years, new commercially available chloromethylphenylcarbamated and dichlorophenylcarbamated polysaccharide phases, and immobilized polysaccharide phases also demonstrated versatile applicability and complimentary enantioselectivity to the above-mentioned polysaccharide phases [45, 47]. Considering such a high success rate, it is not surprising that most reported chiral column screening systems are solely based on derivatized polysaccharide CSPs. It should be noted that the success of generic chiral screening approaches based on derivatized polysaccharide CSPs totally relies on the broad enantioselectivity of these CSPs. The screening can be implemented in a manner that does not require an analyst to have extensive experience and in-depth knowledge of chiral recognition mechanisms on CSP. In reality, the vast majority of pharmaceutical chiral compounds contain enough functional groups (i.e., aromatic ring, hydroxyl, carboxyl, amine, amide, carbamate) around their chiral center(s) that are crucial for chiral recognition. These important functional groups enable a polysaccharide CSP to interact with analytes on its complimentary binding sites through π−π, H-bonding, dipole–dipole, or steric interactions. Consequently, the generic screening approaches based on derivatized polysaccharide CSPs work well only for polysaccharide chiral column selection.

However, a question one may ask is: "Are the other types of CSPs worth of consideration?" A recent study based on a test set of 53 chiral pharmaceutical compounds revealed that four common polysaccharide-based CSPs demonstrated enantioselectivity for 87% of tested analytes, while three macrocyclic glycopeptide antibiotic phases (i.e., vancomycin, teicoplanin, and ristocetin) showed a lower 65% success rate. However, the combined success rate for two classes of CSPs was 96% [163]. Another study based on a different sample set showed that approximately 20% of over 100 racemates were not enantioseparated on any of the studied polysaccharide CSPs [167]. These results suggest that besides derivatized polysaccharide CSPs, other classes of CSPs such as macrocyclic glycopeptide, Pirkle-type, cyclodextrin, protein, or crown ether CSPs should be considered in the design of column screening strategy. A survey of 45 chiral methods used for Bristol-Meyer Squibb compounds in development stages reveals that polysaccharide CSPs were chosen for about 90% of the chiral separations (40 out of 45 methods), while applications of macrocyclic glycopeptide (2 methods) and Pirkle-type (3 methods) CSPs made up the rest 10%.

A knowledge-based chiral method development strategy can be very effective on chiral column selection. For example, a chiral screening method was used to monitor the enantiomeric purity of an atropisomeric drug candidate. It returned the Chiralcel OD-RH column in reversed phase with a baseline enantioseparation of both atropisomers (Fig. 12a). There was a need to redevelop the chiral method as the compound moved into development stages. Based on possible interaction sites around the chiral asymmetric axis of the compound (a primary amine for ionic and H-bonding interactions and an aromatic ring for π–π interaction), Chirobiotic V2 was tested since

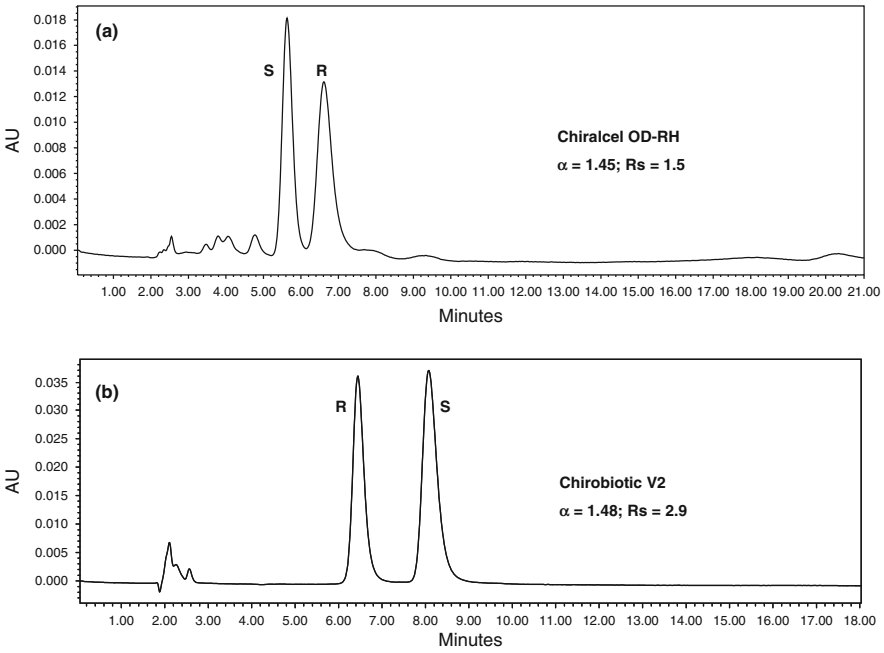


Fig. 12 Enantioseparation of atropisomers of a drug candidate containing a primary amine group obtained on (**a**) a Chiralcel OD-RH (150 × 4.6 mm i.d.) and (**b**) a Chirobiotic V2 (250 × 4.6 mm i.d.) column. Mobile phase: (**a**) 0.01 M Na_2HPO_4 (pH 7):MeCN:MeOH 55:15:30; (**b**) 95/5 (MeOH with 0.2% acetic acid and 0.1% TEA)/(0.1% TEAA in H_2O, pH 4.1). Note the opposite elution order observed on the two columns (adapted from [148])

vancomycin CSPs work really well for basic compounds in polar organic mode and the V2 CSP has enhanced enantioselectivity compared to Chirobiotic V [53]. A better enantioseparation was obtained in high organic–aqueous mobile phase with the undesired (*R*)-enantiomer eluting first as shown in Fig. 12b [148].

With challenging chiral analytes, i.e., molecule with multiple stereogenic centers, without functional groups on the chiral center, or possessing structural features preventing effective enantiorecognition by a CSP, it might be necessary to embrace a more comprehensive chiral method development strategy. First, a wider range of CSPs will be tested, but derivatization of targeted molecule and column coupling can also be envisaged. Recently the SFC coupling of two different chiral columns in series has been reported for the resolution of the four stereoisomers of pharmaceutical compounds containing two chiral centers [168, 169].

4.1.3 A Studied Case: The Anticancer Brivanib Alaninate

To illustrate the complexity of chiral method development, the case of the anticancer brivanib alaninate and its isomers is discussed [150, 166]. Several structural features of brivanib make its enantiomeric separation extremely difficult to achieve. First,

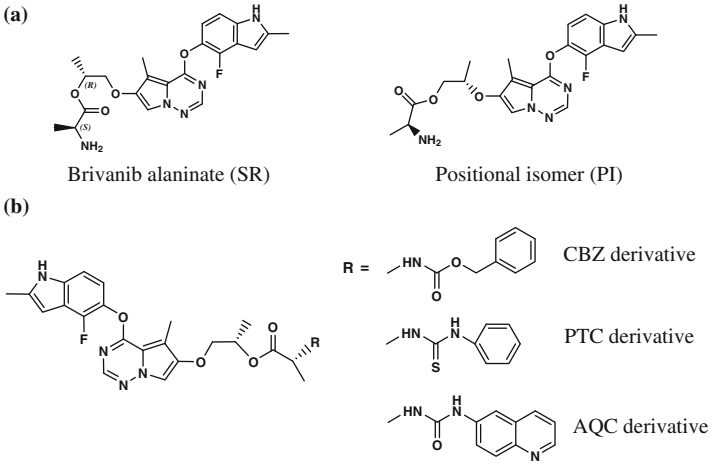


Fig. 13 The stereochemical structures of (**a**) brivanib alaninate and its isomers, and (**b**) derivatives of brivanib alaninate (adapted from [166])

brivanib possesses two stereogenic centers. Besides the three unwanted stereoisomers, a positional isomer may potentially be present in the drug substance of brivanib due to moderate regioselective control during the synthesis (Fig. 13a). To separate all five isomers of brivanib, the selected CSP must show a high enantio- and diastereomeric selectivity, and excellent peak efficiency. Second, the chiral center on the oxypropanyl moiety of the brivanib alaninate molecule lacks enough functional groups available for chiral recognition. This deficiency will prevent many CSPs from separating diastereomers. According to the TPI and SR models, a minimum of four interaction sites are required for brivanib and its isomers to be recognized by a CSP [34, 35]. Apparently, it is not easy for brivanib to meet this requirement. Third, the bulky indolyloxy pyrrolotriazinyl group, which is indirectly attached to the oxypropanyl stereogenic center via a long oxyethyl linkage, may contribute significantly to the analyte's retention, but not to its chiral recognition. Based on the considerations of brivanib's structure, three different amino derivatives were prepared: carbobenzoxy (CBZ), phenylthiocarbamide (PTC), and aminoquinilyl carbamide (AQC) derivatives as shown by Fig. 13b.

The comprehensive chiral HPLC method development approach designed for brivanib alaninate enantiomers and diastereoisomers separation included [150] the following:

A. Chiral column screening based on different chiral recognition mechanisms

   a) Polysaccharide-based phases (coated and immobilized)
   b) Chirobiotic phases – V/V2, T
   c) Chiral crown ether phases – Crownpak (coated) and ChiroSil (bonded)
   d) Cyclodextrin phases – Cyclobond 1 2000 RSP column

e) Amide-type phases – Sumichiral OA-4800 and OA-4900
f) Protein phases – AGP and CBH
g) Pirkle-type phases – Whelk-O1
h) Ruthenium complex phases – Ceramospher RU-1 and RU-2

B. Chiral ion-pair chromatography
C. Non-chiral derivatization
D. Two-dimensional (achiral + chiral tandem) separation

The CSPs with potential to show enantioselectivity were thoroughly investigated, and several CSPs including Chirobiotic V2, Sumichiral 4800, ChiroSil SCA (+)

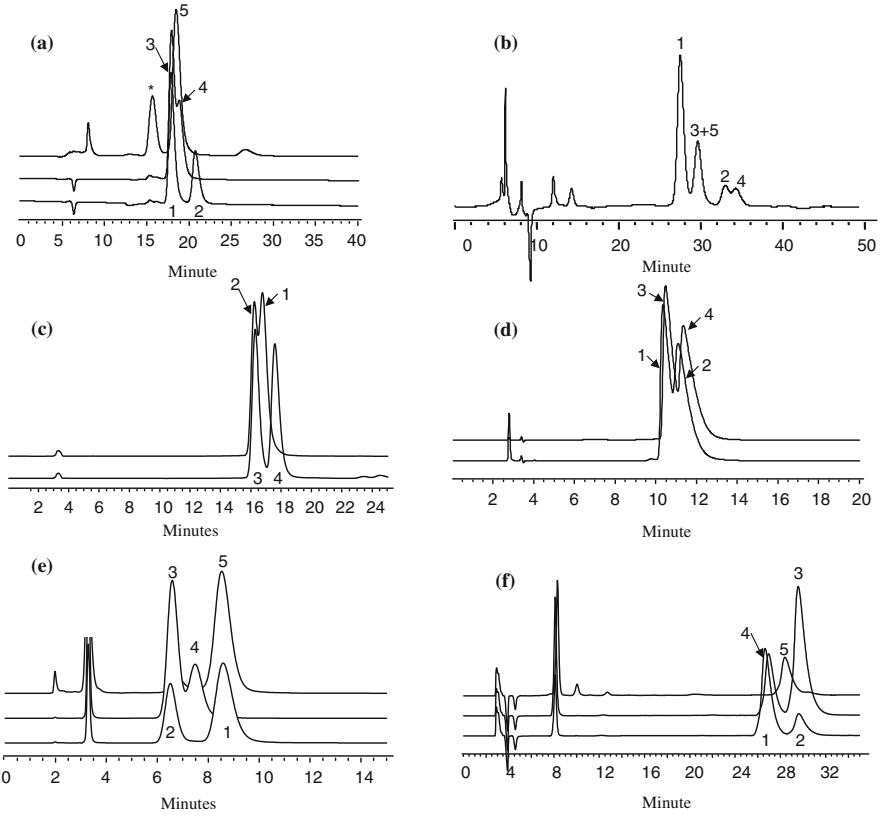


Fig. 14 Representative direct enantiomeric separations of brivanib and its isomers on different CSPs: (**a**) polysaccharide Chiralcel OD-H (coated cellulose carbamate), (**b**) Chiralpak IC (3,5-dichlorophenylcarbamate of cellulose, immobilized on 5-μm silica), (**c**) Chirobiotic V2 (vancomycin), (**d**) Cyclobond RSP (cyclodextrin), (**e**) ChiroSil SCA (–) (crown ether), and (**f**) Sumichiral OA-4800 (Pirkle) column. Peak identification: 1, (*SR*); 2, (*RS*); 3, (*RR*); 4, (*SS*); 5, positional isomer; *, chemical impurity (adapted from [150])

and RCA (–), Cyclobond I 2000 RSP, and derivatized polysaccharide phases gave fairly good enantioseparations (Fig. 14). As expected, all the columns showed poor selectivity for all five diastereomers. To enhance the isomeric interactions between brivanib and CSPs, new interaction sites for hydrogen-bonding, π–π, dipole–dipole, and steric interactions were introduced into brivanib structure via derivatizing the primary amine group on alanine moiety with achiral reagents. Consequently, the selectivity for enantiomers, diastereoisomers, and positional isomers was greatly enhanced on derivatized polysaccharide CSPs, and simultaneous separation of five CBZ-derivatized brivanib isomers was finally achieved on Chiralcel OJ-H in both RP and POM modes as shown in Fig. 15 [166].

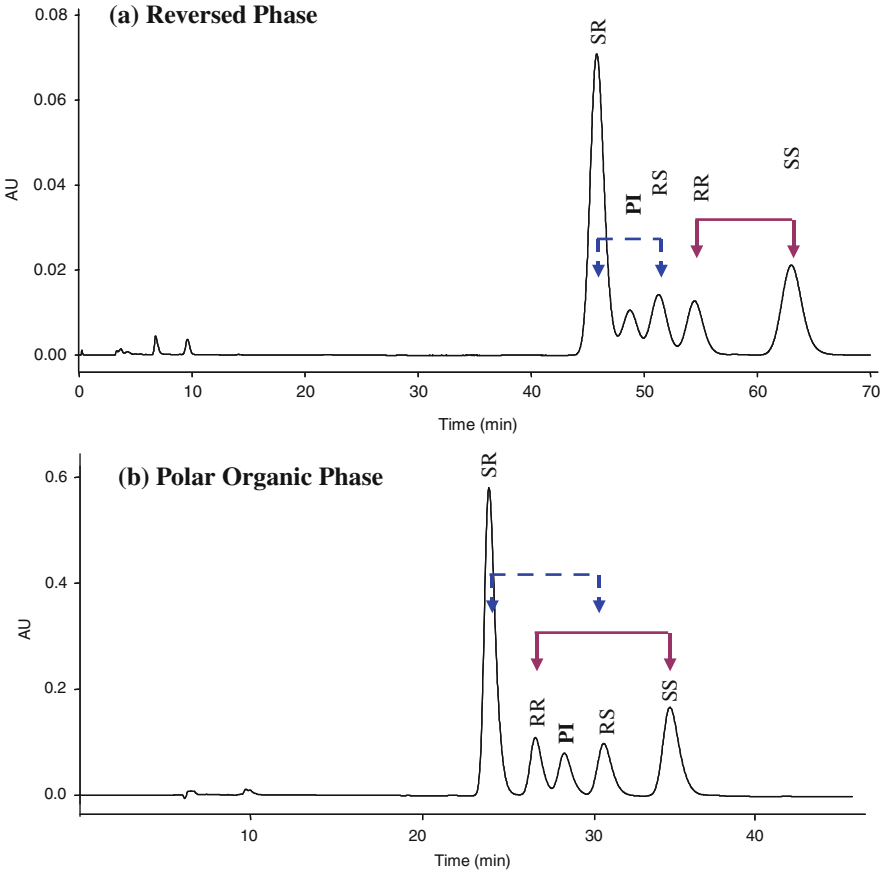


Fig. 15 Simultaneous separations of five CBZ-derivatized brivanib alaninate isomers on a 25-cm × 4.6-mm Chiralcel OJ-RH column in reversed phase (**a**) and an OJ-H column in polar organic phase (**b**). Mobile phase: (**a**) 50:50 20 mM NH_4OAc:ACN; (**b**) MeOH [166]. The *arrows* link the enantiomeric pairs (adapted from [166])

4.2 Chiral Separation in Different Separation Modes

There are many classes of CSPs applicable in different mobile-phase modes. In particular, CSPs based on derivatized polysaccharides, native and derivatized cyclodextrins, macrocyclic glycopeptides, and Pirkle-type chiral selectors operate quite well in four separation modes, i.e., RP, polar organic phase, NP, and super- or subcritical fluid chromatography (SFC) conditions. It is common that a chiral compound can be separated on the same CSP in more than one separation mode [58, 160, 166, 170–176]. For example, Nutlin-3, a small molecule antagonist of MDM2, has been baseline resolved from its enantiomer in all four mobile-phase conditions (Fig. 16) [170]. Multimodal enantioseparation on the same CSP would be greatly beneficial for chiral method development in pharmaceutical industry.

4.2.1 The Rebirth of Supercritical Fluid Chromatography (SFC)

In recent years, due to several admired features such as fast and high-efficient enantioseparation, short method development cycle resulting from fast column equilibrium and simple mobile-phase compositions, and great preparative potential, chiral SFC using the same packed columns as HPLC has gained increasing popularity as the preferred technology for high-throughput chiral analysis and accessing large quantities of pure enantiomers in drug discovery [149, 151–153]. However, chiral SFC is much less influential in pharmaceutical development. Instead, a chiral HPLC method is more commonly used for routine analysis of chiral samples due to its robustness, transferability, and wide instrument availability. Meanwhile, a reversed-phase or polar organic method is particularly favored for the analysis of biological samples when mass spectrometric detection is required. Apparently, it would be huge time and cost saving if a chiral separation could be directly transferred from SFC to HPLC or among three HPLC modes, i.e., reversed phase, polar organic phase, and normal phase on the same chiral column. Unfortunately, such a direct conversion is not always straightforward since the chiral recognition mechanisms might be different under different mobile-phase conditions. The feasibility is often related to the nature of CSP and solute.

4.2.2 SFC and Polysaccharide CSPs

Derivatized polysaccharide CSPs are operational, quite well, in all three HPLC separation modes as well as under super- or sub-critical fluid conditions [153, 160, 166, 170, 176–178]. A previous study based on a collection of more than 100 pharmaceutically important compounds with diverse structures clearly showed that polysaccharide CSPs generally had much higher success rate in resolving enantiomers under normal-phase and SFC conditions, followed by RP and polar organic modes (Fig. 17) [167]. This study also revealed that amylose tris(3,5-dimethylphenylcarbamate) AD phase was more effective than the other studied polysaccharide CSPs in polar organic mode and SFC, whereas cellulose tris(3,5-dimethylphenylcarbamate) phase is more applicable in reversed-phase mode. This observation is consistent with two other studies [159, 160].

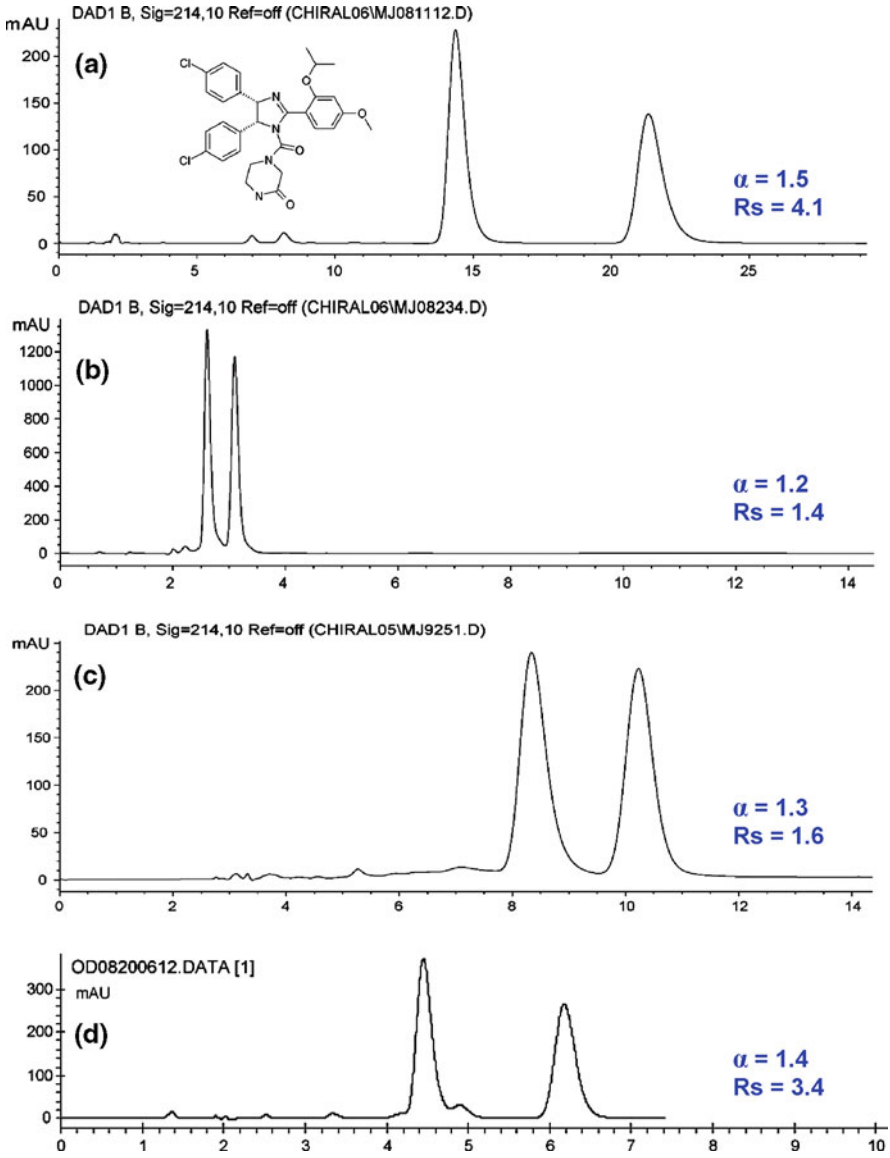


Fig. 16 Enantioseparation of a small molecule MDM2 antagonist Nutlin-3 and its enantiomer under (**a**) reversed phase, (**b**) polar organic, (**c**) normal phase, and (**d**) supercritical fluid chromatography conditions. Time in min (adapted from [170])

A variety of pharmaceutical drugs or intermediates, such as β-blockers, β-agonists, benzodiazepines, nonsteroidal anti-inflammatory drugs, and sulfoxides, can be enantioresolved on the same polysaccharide CSP in all HPLC separation modes and in SFC. A common structural feature of these compounds is that they all contain at least one aromatic group and multiple hydrogen-binding sites in

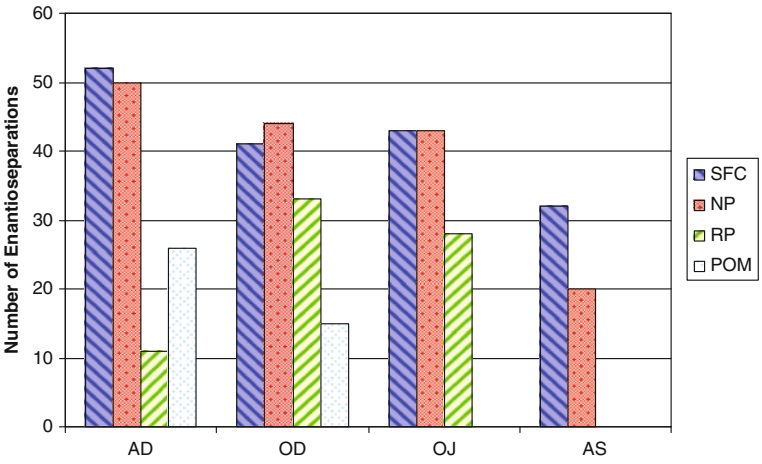


Fig. 17 Chiral screening results of a 148-enantiomer sample set on polysaccharide CSPs (AD, amylose tris(3,5-dimethylphenylcarbamate); OD, cellulose tris(3,5-dimethylphenylcarbamate); OJ, cellulose tri(4-methylphenylester); AS, amylose tris[(*S*)-1-phenylethylcarbamate]). The graph is derived from data listed in [167]

the proximity of their stereogenic centers. This clear dissymmetry allows a CSP to discriminate both enantiomers through different mechanisms promoted by different mobile-phase conditions. For example, 2-benzylsulfinylbenzamide, which contains an amide, a sulfinyl, and two phenyl moieties, is capable of interacting with polysaccharide CSPs through hydrogen-bonding, dipole–dipole, π–π, and steric interactions. The enantioseparation of this compound in polar organic mode was mainly mediated through hydrogen bonding. The importance of hydrogen-bonding interaction to enantioselectivity was evidenced when the mobile phase was changed from methanol to 2-propanol in which hydrogen-bonding interaction is more favored than hydrophobic interaction. An enantioselectivity as high as 111 was observed in the cellulose tris(3,5-dichlorophenylcarbamate) phase. In aqueous-alcohol mobile phase, H-bonding interaction was diminished with the increase of aqueous content. Consequently, the enantioselectivity was decreased. On the contrary, H-bonding interaction was further enhanced in hydrocarbon-alcohol mobile phase to such an extent that the second peak of 2-benzylsulfinylbenzamide was not eluted from cellulose tris(3-bromo-5-methylphenylcarbamate) column in 24 h with 30% *n*-hexane in the eluent [176].

A general trend of enantioseparation among the three HPLC separation modes is that the highest enantioselectivity was commonly observed under normal-phase conditions (an example is shown in Fig. 18), while the shortest retention was obtained in polar organic mode. This is not an absolute rule since a great number of exceptions have been noticed. In the case of Nutlin-3, the enantioselectivity and resolution were much higher in reversed-phase mode than in polar organic and normal-phase mode (Fig. 16). Another example is Naproxen, whose enantiomers were baseline resolved on a Chiralpak AD-RH column in RP mode, while no enantioselectivity

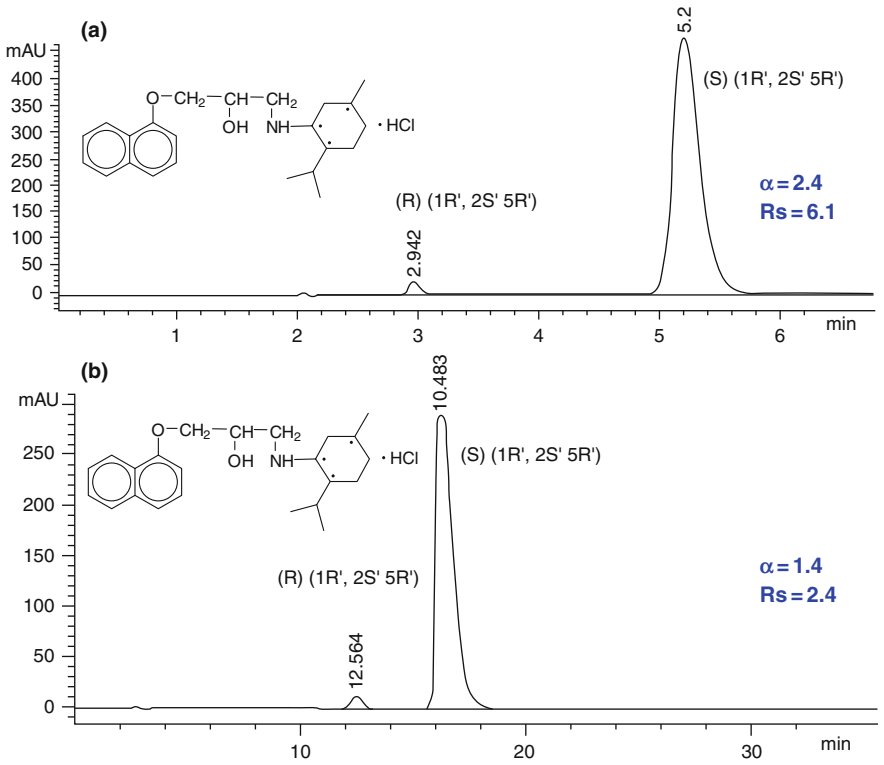


Fig. 18 Enantiomeric separation of a derivative of propranolol on a 25-cm × 4.6-mm cellulose tris(3,*S*-dimethylphenylcarbamate) column in (**a**) normal phase with hexane–alcohol and (**b**) reversed phase with aqueous $NaClO_4$–acetonitrile mobile phase (adapted from [70])

was obtained in both NP and SFC conditions [159, 177]. These exceptional examples imply that hydrophobic and $\pi-\pi$ interactions may contribute significantly to enantioselectivity since both types of interactions are promoted in RP mode but become less favorable in NP mode.

It appears from Fig. 17 that polysaccharide CSPs demonstrated comparable enantioselectivity under SFC and normal-phase conditions; however, it is not necessarily true that both technologies are interchangeable. In fact, a change from NPLC to SFC should change not only the physical (from hydrocarbon to carbon dioxide) but also the chemical properties of the mobile phase. Due to its high dipole moment, the carbon dioxide molecule may be actively involved in the chiral recognition process by participating in solute–mobile phase and stationary phase–mobile-phase interactions through solvation [175, 179]. It can be expected that SFC and LC may render unique enantioselectivity depending on the CSP and the analyte. Noticeable discrepancies between NPLC and SFC for polysaccharide CSPs have been well recognized in many studies [167, 171, 180–182].

A thermodynamic study of the optical isomers of diltiazem and its 3-hydroxy isomer on cellulose tris(4-chlorophenylcarbamate) CSP found that the enantioseparation of *trans*- and *cis*-isomers of diltiazem was entropy controlled and under supercritical conditions, it was enthalpy controlled. However, both enantioseparations were enthalpy controlled in normal-phase conditions. These results suggested that different chiral recognition mechanisms were involved in LC and SFC modes. The reason might be that the chiral active sites of the phenyl group of analytes and the phenylcarbamate group of the CSP have different solvation states in SFC and NPLC conditions [182]. The same conclusion was drawn from a systematic comparison of LC and SFC based on the enantioseparations of various pharmaceutical compounds on cellulose- and amylose-derived CSPs, i.e., Chiralcel OD and Chiralpak AD, respectively [171]. Chiral compounds with polar functional groups, such as primary or secondary hydroxyl and/or amine moieties, often exhibited marked discrepancy in enantioseparation on both CSPs under SFC and LC conditions, since the conformation of these analytes may change in the presence of carbon dioxide [183]. Another interesting observation of this study is that under supercritical fluid conditions, the pressure of carbon dioxide had remarkable influence on the enantioseparation of some solutes on the AD column, whereas there was slight effect on the OD column. These results indicated that the pressurized mobile phase may induce some changes in the helical structure of the AD CSP, which may help to explain some discrepancies in enantioselectivity observed between SFC and LC on AD column.

4.2.3 SFC and Cyclodextrin CSPs

As discussed in Sect. 3.3, the chiral recognition mechanisms in different HPLC modes on CD-based CSPs vary remarkably. Consequently, derivatized π-acidic and π-basic CD CSPs that are applicable to all three LC modes are able to resolve different classes of chiral compounds in reversed-phase and normal-phase modes. Underivatized CD CSPs are mainly used in reversed-phase and polar organic modes, but less likely in normal-phase mode. It is common for aromatic compounds with multiple H-bonding sites to be separated on CD CSPs in both RP and POM [73, 79, 82]. In these cases, the U-shaped retention behavior is typically observed, i.e., the analytes are more strongly retained under high aqueous content and high organic content mobile phases. An example is presented in Fig. 19 [78].

Although native CD CSPs are not effective in normal-phase mode, they were found quite useful in SFC. Enantioseparations of a few classes of compounds including 2-naphthoyl amide derivatives of 2-alkylamines, *tert*-phosphine oxides, α-methylene γ-lactone, and oxazepam have been reported on native β-CD CSP (Cyclobond I) with methanol as SFC mobile-phase additive [85, 172, 173]. Hydrogen bond between the secondary hydroxyl groups of CD and analytes was believed to be the initial driving force for the formation of CD CSP–solute complex. A two-step chiral recognition process was proposed for the observed enantioseparations: (i) the hydrophobic environment promoted the hydrogen-bonding interaction between solute and secondary hydroxyl groups at the mouth of CD ring and (ii) an

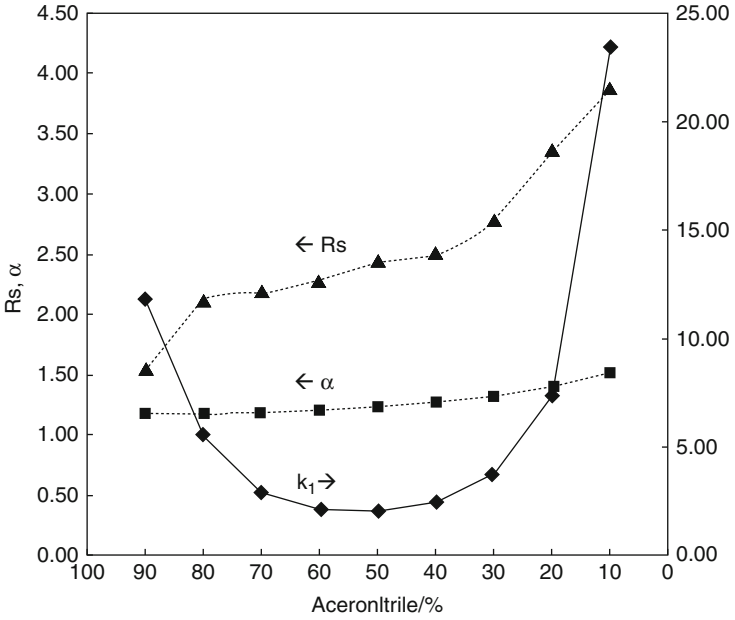


Fig. 19 The effect of organic content on the enantioseparation of nefopam hydrochloride on a dinitrophenyl (DNP)-substituted β-cyclodextrin (β-CD) CSP. The buffer is composed of 0.1% (v/v) triethylammonium acetate (TEAA) in water, pH 4.1 (adapted from [78])

inclusion complex was formed by tight fit of the aromatic moiety of solute to the CD cavity. Unlike hydrocarbon component in normal phase, it is much easier for carbon dioxide molecule in the CD cavity to be displaced by solute molecule due to its smaller size [85].

Derivatized CD CSPs such as (*R*)- and (*S*)-naphthylethylcarbamoylated-β-cyclodextrins (*R*-NEC-CD and *S*-NEC-CD) have been shown to be as effective in SFC as in three LC modes [82]. Since both SFC and NPLC share similar chiral recognition mechanisms on NEC-CD CSPs, the chiral separations obtained in NPLC could be easily reproduced in SFC with the same elution order of enantiomers. Comparable enantioselectivity was also observed for the majority of tested compound between SFC and the other two LC modes on NEC-CD CSPs. However, the marked enantioselectivity differences observed for cromakalim and tolperisone in the RPLC and SFC modes suggest that the chiral separations were obtained with completely different mechanisms in the two modes, SFC and RPLC and the NEC-CD CSP [83].

4.2.4 Macrocyclic Glycopeptide CSPs

Because of their diverse functionality, it is not surprising to see that CSPs based on macrocyclic glycopeptides, i.e., Chirobiotic phases, have a broad-spectrum applicability in all HPLC conditions, namely normal-phase, reversed-phase, polar organic,

and the specific polar ionic modes [54–61]. It is possible for a chiral compound, especially a neutral compound, to be enantioseparated in all four aforementioned LC modes [59]. This is illustrated with the enantioseparation of 5-methyl-5-phenylhydantoin as an example in Fig. 20. As for the other classes of CSPs, the chiral recognition mechanisms on Chirobiotic phases are different in each separation mode since different types of enantioselective interactions are promoted in the presence of different mobile-phase constituents. Additionally many compounds, particularly those possessing aromatic and polar groups, typically show the characteristic U-shaped retention behavior vs. mobile-phase composition. Obviously, enantioselective interactions are active part of the retention mechanism. The potential interactions that account for the discrimination of two enantiomers in each LC separation mode are listed in Table 2. The specific polar ionic mode with mobile phases usually consisting of polar organic solvents mixed with a small amount of acid and base or volatile salts is designed to enhance the electrostatic/ionic, hydrogen-bonding, and dipolar interactions between chiral solutes and Chirobiotic phases. Consequently, the polar ionic mode (PIM) is recommended to be the first choice for the enantioseparation of chiral compounds carrying ionizable functional groups. Compared to reversed-phase and normal-phase modes, the polar organic and ionic modes generally provide higher peak efficiency, shorter analysis time, and better solubility for pharmaceutical compounds and thus greater preparative potential (Fig. 20). The polar organic mode differs from the polar ionic mode in that it only consists of polar solvents without additives and is particularly used for neutral molecules. The organic solvents used in polar organic and ionic modes can be methanol, ethanol, acetonitrile, or their mixtures. Protic solvents generally give better enantioselectivity than do aprotic solvents for compounds carrying polar groups since hydrogen-bonding interaction is pervasive on macrocyclic glycopeptide phases.

In recent years, the application of macrocyclic glycopeptide CSPs has been expanded to SFC, and teicoplanin aglycone phase (Chirobiotic® TAG) was found to be the most effective closely followed by teicoplanin CSP (Chirobiotic® T) [144, 184]. Because of a large number of polar groups available on the Chirobiotic CSPs, it is often required to work with considerable amount of polar additives in the mobile

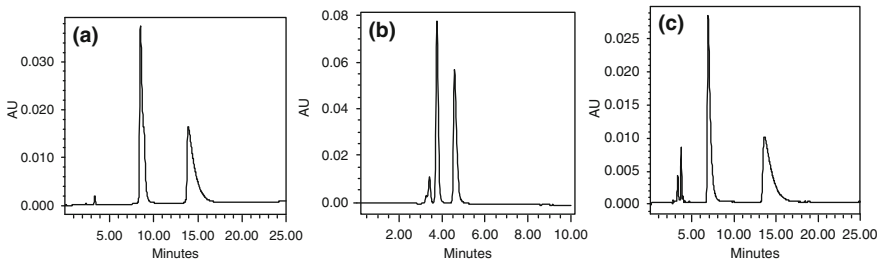


Fig. 20 Enantioseparation of 5-methyl-5-phenylhydantoin on Chirobiotic T in (**a**) reversed phase (mobile phase: 20/80 MeOH/10 mM NH_4OAc), (**b**) polar organic phase (mobile phase: MeOH) and (**c**) normal phase (mobile phase: 60/40 EtOH/heptane)

Table 2 Possible chiral interactions between glycopeptide CSP and solute (date from [189])

Separation mode	Interaction	Relative intensity
Polar ionic phase	Ionic interaction	Very strong (dominant)
	Hydrogen bonding	Moderate
	π–π interaction	Weak to moderate
	Dipole–dipole	Moderate
	Steric repulsive	Weak
Polar organic phase	Hydrogen bonding	Strong
	π–π interaction	Weak to moderate
	Dipole–dipole	Moderate
	Steric repulsive	Weak
Reversed phase	Hydrophobic interaction	Strong (dominant)
	Ionic interaction	Strong
	π–π interaction	Strong
	Hydrogen bonding	Weak
	Dipole–dipole	Weak
	Steric repulsive	Weak
Normal phase	Hydrogen bonding	Strong
	π–π interaction	Strong
	Dipole–dipole	Strong
	Steric repulsive	Weak

phase in order to elute the polar compounds. The chiral recognition mechanisms for sulfoxides were similar under both normal-phase (hexane–alcohol) and SFC (carbon dioxide–methanol) conditions, whereas the enantioselectivity was higher in LC than in SFC. Like in LC conditions, ionic interaction between analytes and Chirobiotic CSPs still played a key role in the chiral recognition mechanism of chiral compounds with ionizable groups in SFC. This finding was evidenced by the fact that acidic analytes were better resolved in basic SFC conditions [144].

4.2.5 SFC and Pirkle-Type CSPs

As discussed in Sect. 3.5, the interactions involved in the chiral recognition on Pirkle-type CSPs are mainly attractive forces, such as π–π, hydrogen-bonding, and dipole–dipole interactions. Although bonded Pirkle-type CSPs have been used in reversed phase and polar nonaqueous mobile phase, most of the applications were found in normal-phase mode. With the introduction of SFC for the resolution of enantiomers [185], bonded Pirkle-type CSPs were among the most studied CSPs in the early application of chiral SFC [172, 175, 181, 186]. Comparable enantioselectivity and the same elution order of enantiomers were usually observed for the enantioseparations of many compounds. Accordingly, similar chiral recognition mechanisms were believed to operate in both LC and SFC conditions [186]. However, when the enantioseparations of π-acidic compounds on the π-acidic

CSPs were concerned, drastic different enantioselectivities were obtained for some tested analytes. An example can be the separation of *N*-DNB-tyrosine *n*-butylamide (π-acidic) on a π-acidic Pirkle-type CSP. No enantioseparation was achieved in SFC, while baseline separation was observed in NPLC. It is interesting that the elution order of enantiomers was reversed by changing the polar component of the mobile phase from ethanol to chloroform [187]. This result was explained assuming that the solute had several interaction sites for the same type of interactions (two amides for dipole stacking and hydrogen-bonding interactions) and there were no dominant interactions that could guide the formation of analyte/CSP complex through one mechanism, regardless of the nature of the mobile phase. Regarding the interactions in different mobile phases, ethanol favors dipole–dipole interaction, while chloroform or methylene chloride promotes hydrogen bonding. It is reasonable that changing the polar solvent from ethanol to chloroform actually induced different chiral recognition mechanisms in normal-phase separations. In subcritical carbon dioxide–ethanol mobile phase, both dipole–dipole and hydrogen-bonding interactions might be equally important for chiral recognition. When two different chiral recognition mechanisms competed with each other, no enantioseparation was observed in SFC [187].

Significant discrepancies in enantioselectivity between SFC and NPLC were also reported for β-blockers and a series of propranolol analogues [179, 183]. Better enantioselectivity and resolution were found in SFC than in NPLC, as shown in the example of propranolol in Fig. 21. Chromatographic and NMR results suggested

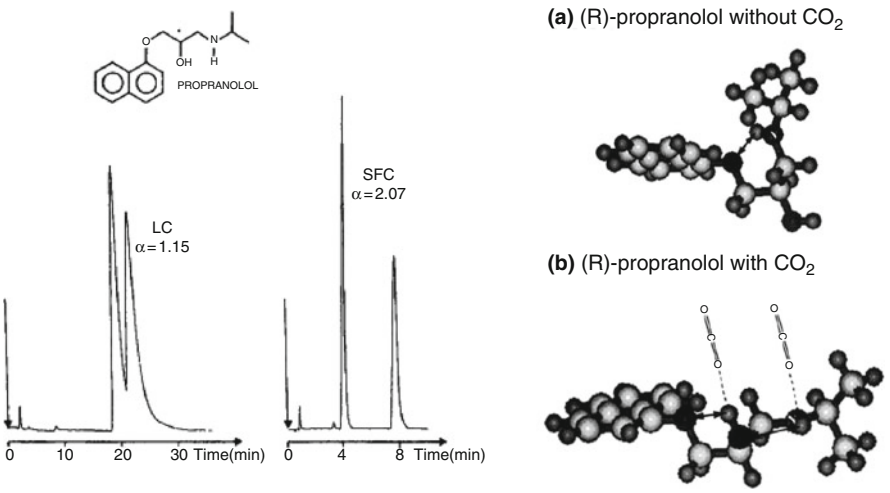


Fig. 21 LC and SFC enantioseparations of propranolol on a derivatized tyrosine Pirkle CSP. Mobile-phase conditions: LC, 95:5 hexane/(ethanol containing 1% *n*-propylamine) (v/v); SFC, 90/10 carbon dioxide/(methanol containing 1% *n*-propylamine) (v/v) [179]. Molecular modeling results are presented for (**a**) optimized structure of (*R*)-propranolol without CO_2 and (**b**) optimized structure of (*R*)-propranolol with CO_2. The intramolecular hydrogen bonding is denoted by an *arrow* (adapted from [183])

that super- or subcritical carbon dioxide may serve as an in situ derivatizing agent for the basic site of analyte molecule and form a transient complex with the analyte [179]. Molecular modeling study of propranolol further revealed that carbon dioxide changed the conformation of propranolol molecule through hydrogen bonding by assisting the analyte to adapt to a geometric structure that facilitated chiral discrimination [183]. These results may also help to explain that verapamil was better enantioselectively resolved on Whelk-O1 CSP in SFC than in NPLC [188].

5 Summary and Outlook

It is clear that each enantioseparation is unique in the way that the CSP interacts with both enantiomers through different chiral recognition mechanisms. The availability of a large number of different types of CSPs allows for the development of more robust and sensitive chiral methods or the achievement of difficult enantioseparations. Chromatographic approaches assisted with spectroscopic and molecular modeling tools have been effective ways to understand the chiral recognition mechanisms on different CSPs. The enriched knowledge in turn will help to design more effective and versatile CSPs, accelerate method development process, and better predict the enantioseparation of a given compound. Researchers should make full use of chiral SFC as a fast method to identify appropriate CSPs for normal-phase LC separations and to meet the demanding analytical and preparative needs in pharmaceutical industry. It must be borne in mind that it is possible that enantioselectivity discrepancies occur between SFC and normal-phase LC depending on the CSP and the analyte structure. Meanwhile, there is a need to develop new mechanically strong CSPs to keep up with the fast growth of ultra-high-pressure liquid chromatography (UHPLC) and understand the effects of ultra-high column pressure on the chiral recognition mechanism on each CSP.

References

1. Maier NM, Franco P, Lindner W (2001) Separation of enantiomers: needs, challenges, perspectives. J Chromatogr A 906:3–33
2. Pasteur L (1901) On the asymmetry of naturally occurring organic compounds. In: Richardson GM (ed) The foundations of stereo chemistry: memoirs by Pasteur, Van't Hoff, Le Bel and Wislicenus. American Book Co., New York
3. Casey AF (1970) Stereochemistry and biological activity. In: Burger A (ed) Medicinal chemistry, 3rd edn. Wiley-Interscience, New York
4. Aboul-Enein H, Wainer IW (1997) The impact of stereochemistry on drug development and use. John Wiley & Sons, New York
5. Food and Drug Administration (1992) FDA's policy statement for the development of new stereoisomeric drugs. 57 Fed. Reg. 22, 249
6. Committee for Proprietary Medical Products (1993) Working parties on quality, safety and efficacy of medical products. Note for guidance: investigation of chiral active substances. III/3501/91

7. Herper M, Kang P (2006) The world's ten best-selling drugs. http://www.forbes.com/2006/03/21/pfizer-merck-amgen-cx_mh_pk_0321topdrugs.html. Accessed 26 Oct 2009
8. Majors RE (1999) From nanograms to tons: chiral stationary phases in the pharmaceutical industry. LC-GC 17:400–405
9. Armstrong DW, Zhang B (2001) Chiral stationary phases for HPLC. Anal Chem 73: 557A–561A
10. Wang CL, Armstrong DW, Risley DS (2007) Empirical observations and mechanistic insights on the first boron-containing chiral selector for LC and supercritical fluid chromatography. Anal Chem 79:8125–8135
11. Hoffmann CV, Pell R, Lammerhofer M, Lindner W (2008) Synergistic effects on enantioselectivity of zwitterionic chiral stationary phases for separations of chiral acids, bases, and amino acids by HPLC. Anal Chem 80:8780–8789
12. Massolini G, Temporini C, Calleri E (2008) Penicillin G acylase as chiral selector in LC and CE: exploring the origins of enantioselectivity. J Chromatogr B 875:20–29
13. Okamoto Y, Yashima E (2001) Polysaccharide derivatives for chromatographic separation of enantiomers. Angew Chem Int Ed 37:1020–1043
14. Berthod A (2009) Chiral recognition mechanisms with macrocyclic glycopeptide selectors. Chirality 21:167–175
15. Armstrong DW (1984) Chiral stationary phases for high performance liquid chromatographic separation of enantiomers: a mini-review. J Liq Chromatogr 7:353–376
16. Kano K (1997) Mechanisms for chiral recognition by cyclodextrins. J Phys Org Chem 10:286–291
17. Haginaka J (2001) Protein-based chiral stationary phases for high-performance liquid chromatography enantioseparations. J Chromatogr A 906:253–273
18. Wainer IW (1993) HPLC chiral stationary phases for the stereochemical resolution of enantiomeric compounds: The current state of the art. In: Wainer IW (ed) Drug stereochemistry: analytical methods and pharmacology, 2nd edn. Marcel Dekker, New York
19. Macaudiere P, Lienne M, Tambute A, Caude M (1989) Pirkle type and related chiral stationary phases for enantiomeric resolution. In: Krstulovic AM (ed) Chiral separations by HPLC. Ellis Horwood Ltd, New York
20. Hyun MH (2006) Preparation and application of HPLC chiral stationary phases based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid. J Sep Sci 29:750–761
21. Easson EH, Stedman E (1933) CLXX. Studies on the relationship between chemical constitution and physiological action. V. Molecular dissymmetry and physiological action. Biochem J 27:1257–1266
22. Dalgliesh CE (1952) The optical resolution of aromatic amino-acids on paper chromatograms. J Chem Soc 137:3940–3942
23. Lochmuller CH, Souter RW (1975) Chromatographic resolution of enantiomers: selective review. J Chromatogr 113:283–302
24. Davankov VA, Meyer VR, Rais M (1990) A vivid model illustrating chiral recognition induced by achiral structures. Chirality 2:208–210
25. Davankov VA (1997) The nature of chiral recognition: is it a three-point interaction? Chirality 9:99–102
26. Meyer VR, Rais M (1989) A vivid model of chiral recognition. Chirality 1:167–169
27. Pirkle WH, Welch CJ, Lamm B (1992) Design, synthesis, and evaluation of an improved enantioselective naproxen selector. J Org Chem 57:3854–3860
28. Ogston AG (1948) Interpretation of experiments on metabolic process using isotopic trace elements. Nature 162:963
29. Shallenberger RS, Acree RE (1967) Molecular theory of sweet taste. Nature 216:480–482
30. Burger A (1970) Relation of chemical structure and biological activity. In: Burger A (ed) Medicinal chemistry, 3rd edn. Wiley-Interscience, New York
31. Garten S, Biedermann PU, Agranat I, Topiol S (2005) Two contact-point chiral distinction: model CHFClBr dimers. Chirality 17:S159–S170

32. Kafri R, Lancet D (2004) Probability rule for chiral recognition. Chirality 16:369–378
33. Mesecar AD, Koshland DE Jr (2000) A new model for protein stereospecificity. Nature 403:614–615
34. Sundaresan V, Abrol R (2002) Towards a general model for protein–substrate stereoselectivity. Protein Sci 11:1330–1339
35. Sundaresan V, Abrol R (2005) Biological chiral recognition: the substrate's perspective. Chirality 17:S30–S39
36. Bentley R (1983) Three-point attachment: past, present, but no future. Trans N Y Acad Sci Ser II 41:5–24
37. Wainer IW, Alembik MC (1986) Resolution of enantiomeric amides on cellulose based chiral stationary phases: steric and electronic effects. J Chromatogr 358:85–93
38. Booth TD, Wainer IW (1996) Investigation of the enantioselective separations of a-alkyl arylcarboxylic acids on an amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase using quantitative structure–enantioselective retention relationships (QSERR): identification of a conformationally driven chiral recognition mechanism. J Chromatogr A 737:157–169
39. Jozwiak K, Moaddel R, Ravichandran S, Plazinska A, Kozal J, Patel S, Yamaguchi R, Wainer IW (2008) Exploring enantiospecific ligand–protein interactions using cellular membrane affinity chromatography: chiral recognition as a dynamic process. J Chromatogr B 875: 200–207
40. Berthod A, He BL, Beesley TE (2004) Temperature and enantioseparation by macrocyclic glycopeptide chiral stationary phases. J Chromatogr A 1060:205–214
41. Stringham RW, Blackwell JA (1997) Factor that control successful entropically driven chiral separations in SFC and HPLC. Anal Chem 69:1414–1420
42. Yashima E, Okamoto Y (1997) Chiral recognition mechanism of polysaccharides chiral stationary phases. In: Aboul-Enein HY, Wainer IW (eds) The impact of stereochemistry on drug development and use. Wiley, New York
43. Yashima E (2001) Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation. J Chromatogr A 906:105–125
44. Tachibana K, Ohnishi A (2001) Reversed-phase liquid chromatographic separation of enantiomers on polysaccharide type chiral stationary phases. J Chromatogr A 906:127–154
45. Chankvetadze B, Yashima E, Okamoto Y (1994) Chloromethylphenylcarbamate derivatives of cellulose as chiral stationary phases for high-performance liquid chromatography. J Chromatogr A 670:39–49
46. Chankvetadze B, Chankvetadze L, Sidamonide S, Kasashima E, Yashima E, Okamoto Y (1997) 3-Fluoro-, 3-chloro- and 3-bromo-5-methylphenylcarbamates of cellulose and amylose as chiral stationary phases for high performance liquid chromatographic enantioseparation. J Chromatogr A 787:67–77
47. Chankvetadze B, Yashima E, Okamoto Y (1995) Dimethyl-, dichloro- and chloromethylphenylcarbamates of amylose as chiral stationary phases for high-performance liquid chromatography. J Chromatogr A 694:101–109
48. Okamoto Y, Kawashima M, Hatada K (1986) Chromatographic resolution. XI. Controlled chiral recognition of cellulose triphenylcarbamate derivatives supported on silica gel. J Chromatogr 363:173–186
49. Okamoto Y, Aburatani R, Fukumoto T, Hatada K (1987) Chromatographic resolution. XVII. Useful chiral stationary phases for HPLC. Amylose tris(3,5-dimethylphenyl carbamate) and tris(3,5-dichlorophenylcarbamate) supported on silica gel. Chem Lett 9: 1857–1860
50. Thunberg L, Hashemi J, Andersson S (2008) Comparative study of coated and immobilized polysaccharide-based chiral stationary phases and their applicability in the resolution of enantiomers. J Chromatogr B 875:72–80
51. Franco P, Zhang T (2008) Common approaches for efficient method development with immobilised polysaccharide-derived chiral stationary phases. J Chromatogr B 875:48–56

52. Kasat RB, Wee SY, Loh JX, Wang NL, Franses EI (2008) Effect of the solute molecular structure on its enantioresolution on cellulose tris(3,5-dimethylphenylcarbamate). J Chromatogr B 875:81–92
53. Supelco/Astec (2004) Chirobiotic handbook: a guide to using macrocyclic glycopeptide bonded phases for chiral LC separations, 5th edn. Supelco/Sigma-Aldrich, Bellefonte, PA
54. Armstrong DW, Tang Y, Chen S, Zhou Y, Bagwill C, Chen JR (1994) Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography. Anal Chem 66:1473–1484
55. Armstrong DW, Liu Y, Ekborgott KH (1995) A covalently bonded teicoplanin chiral stationary phase for HPLC enantioseparations. Chirality 7:474–497
56. Ekborg-Ott KH, Liu Y, Armstrong DW (1998) Highly enantioselective HPLC separations using the covalently bonded macrocyclic antibiotic, ristocetin A, chiral stationary phase. Chirality 10:434–483
57. Berthod A, Chen X, Kullman JP, Armstrong DW, Gasparrini F, D'Acquarica I, Villani C, Carotti A (2000) Role of the carbohydrate moieties in chiral recognition on teicoplanin-based LC stationary phases. Anal Chem 72:1767–1780
58. Sun P, Krishnan A, Yadav A, MacDonnell FM, Armstrong DW (2008) Enantioseparations of chiral ruthenium(II) polypyridyl complexes using HPLC with macrocyclic glycopeptide chiral stationary phases (CSPs). J Mol Struct 890:75–80
59. Berthod A, Xiao TL, Liu Y, Jenks WS, Armstrong DW (2002) Separation of chiral sulfoxides by liquid chromatography using macrocyclic glycopeptide chiral stationary phases. J Chromatogr A 955:53–69
60. Xiao TL, Rozhkov RV, Larock RC, Armstrong DW (2003) Separation of the enantiomers of substituted dihydrofurocoumarins by HPLC using macrocyclic glycopeptide chiral stationary phases. Anal Bioanal Chem 377:639–654
61. Chen S, Liu Y, Armstrong DW, Borrell JI, Martinez-Teipel B, Matallana JL (1995) Enantioresolution of substituted 2-methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles on macrocyclic antibiotic and cyclodextrin stationary phases. J Liq Chromatogr Relat Technol 18:1495–1507
62. Nair UB, Chang SSC, Armstrong DW, Rawjee YY, Eggleston DS, Mcardlez JV (1996) Elucidation of vancomycin's enantioselective binding site using its copper complex. Chirality 8:590–595
63. Berthod A, Valleix A, Tizon V, Leonce E, Caussignac C, Armstrong DW (2001) Retention and selectivity of teicoplanin stationary phases after copper complexation and isotopic exchange. Anal Chem 73:5499–5508
64. Armstrong DW, Rundlett KL, Chen JR (1994) Evaluation of the macrocyclic antibiotic vancomycin as a chiral selector for capillary electrophoresis. Chirality 6:496–509
65. Gasper MP, Berthod A, Nair UB, Armstrong DW (1996) Comparison and modeling study of vancomycin, ristocetin A, and teicoplanin for CE enantioseparations. Anal Chem 68: 2501–2514
66. He L, Beesley TE (2005) Applications of enantiomeric gas chromatography: a review. J Liq Chromatogr Relat Technol 28:1075–1114
67. Ward TJ (1994) Chiral media for capillary electrophoresis. Anal Chem 66:632A–640A
68. Armstrong DW, DeMond W (1984) Cyclodextrin bonded phases for the liquid chromatographic separation of optical, geometrical, and structural isomers. J Chromtogr Sci 22:411–415
69. Armstrong DW, Ward TJ, Armstrong RD, Beesley TE (1986) Separation of drug stereoisomers by the formation of β-cyclodextrin inclusion complexes. Science 232:1132–1135
70. Facklam C, Modler A (1994) Separation of some enantiomers and diastereomers of propranolol derivatives by high-performance liquid chromatography. J Chromatogr A 664: 203–211
71. Bressolle F, Audran M, Pham TN, Vallon JJ (1996) Cyclodextrins and enantiomeric separations of drugs by liquid chromatography and capillary electrophoresis: basic principles and new developments. J Chromatogr B 687:303–336

72. Hinze WL, Riehl TE, Armstrong DW, Demond W, Alak A, Ward T (1985) Liquid chromatographic separation of enantiomers using a chiral beta-cyclodextrin-bonded stationary phase and conventional aqueous-organic mobile phases. Anal Chem 57:237–242
73. Chang SC, Reid GL, Chen S, Chang CD, Armstrong DW (1993) Evaluation of a new polar-organic high-performance liquid chromatographic mobile phase for cyclodextrin-bonded chiral stationary phases. Trends Anal Chem 12:144–153
74. Lee SH, Berthod A, Armstrong DW (1992) Systematic study on the resolution of derivatized amino acids enantiomers on different cyclodextrin-bonded stationary phases. J Chromatogr 603:83–93
75. Stalcup AM, Chang SC, Armstrong DW, Pitha J (1990) (*S*)-2-Hydroxypropyl-β-cyclodextrin, a new stationary phase for reversed-phase liquid chromatography. J Chromatogr 513:181–194
76. Armstrong DW, Stalcup AM, Hilton ML, Duncan JD, Faulkner JR, Chang SC (1990) Derivatized cyclodextrins for normal-phase liquid chromatographic separation of enantiomers. Anal Chem 62:1610–1615
77. Stalcup AM, Chang SC, Armstrong DW (1991) Effect of the configuration of the substituents of derivatized beta-cyclodextrin bonded phases on enantioselectivity in normal-phase liquid chromatography. J Chromatogr 540:113–128
78. Zhong Q, He L, Beesley TE, Trahanovsky WS, Sun P, Wang C, Armstrong DW (2006) Development of dinitrophenylated cyclodextrin derivatives for enhanced enantiomeric separations by high-performance liquid chromatography. J Chromatogr A 1115: 19–45
79. Zhong Q, He L, Beesley TE, Trahanovsky WS, Sun P, Wang C, Armstrong DW (2006) Optimization of 2,6-dinitro-4-trifluoromethylphenyl ether substituted cyclodextrin bonded stationary phases for HPLC separation of enantiomers. Chromatographia 64:147–155
80. Armstrong DW, Chang LW, Chang SC, Wang X, Ibrahim H, Reid GR, Beesley TE (1997) Comparison of the selectivity and retention of beta-cyclodextrin vs. heptakis-2,3-*O*-dimethyl-beta-cyclodextrin LC stationary phases for structural and geometric isomers. J Liq Chromatogr Relat Technol 20:3279–3295
81. Macaudiere P, Caude M, Rosset R, Tambute A (1988) Chiral resolution of a series of 3-thienylcyclohexylglycolic acids by liquid or subcritical fluid chromatography. A mechanistic study. J Chromatogr 450:255–269
82. Ilisz I, Berkecz R, Forro E, Fulop F, Armstrong DW, Peter A (2009) The Role of π-acidic and π-basic chiral stationary phases in the high-performance liquid chromatographic enantioseparation of unusual β-amino acids. Chirality 21:339–348
83. Williams KL, Sander LC, Wise SA (1996) Comparison of liquid and supercritical fluid chromatography using naphthylethylcarbamoylated-β-cyclodextrin chiral stationary phases. J Chromatogr A 746:91–101
84. Wang RQ, Ong TT, Ng SC (2008) Synthesis of cationic β-cyclodextrin derivatives and their applications as chiral stationary phases for high-performance liquid chromatography and supercritical fluid chromatography. J Chromatogr A 1203:185–192
85. Macaudiere P, Caude M, Rosset R, Tambute A (1987) Chiral resolution of racemic amides and phosphine oxides on a β-cyclodextrin-bonded stationary phases by super critical fluid chromatography. J Chromatogr 405:135–143
86. Allenmark S, Bomgren B, Boren H (1983) Direct liquid chromatographic separation of enantiomers on immobilized protein stationary phases. III. Optical resolution of a series of *N*-aroyl D,L-amino acids by high-performance liquid chromatography on bovine serum albumin covalently bound to silica. J Chromatogr 264:63–68
87. Domenici E, Bertucci C, Salvadori P, Felix G, Cahagne I, Montellier S, Wainer IW (1990) Synthesis and chromatographic properties of an HPLC chiral stationary phase based upon human serum albumin. Chromatographia 29:170–176
88. Hermansson J (1983) Direct liquid chromatographic resolution of racemic drugs using alpha1-acid glycoprotein as the chiral stationary phase. J Chromatogr 269:71–80

89. Miwa T, Ichikawa M, Tsuno M, Hattori T, Miyakawa T, Kayano M, Miyake Y (1987) Direct liquid chromatographic resolution of racemic compounds. Use of ovomucoid as a column ligand. Chem Pharm Bull 35:682–686
90. Miwa T, Miyakawa T, Miyake Y (1988) Characteristics of an avidin-conjugated column in direct liquid chromatographic resolution of racemic compounds. J Chromatogr 457:227–233
91. Erlandsson P, Marle I, Hansson L, Isaksson R, Petterson C, Petterson G (1990) Immobilized cellulase (CBH I) as a chiral stationary phase for direct resolution of enantiomers. J Am Chem Soc 112:4573–4574
92. Haginaka J, Miyano Y, Saizen Y, Seyama C, Murashima T (1995) Separation of enantiomers on a pepsin-bonded column. J Chromatogr A 708:161–168
93. ChromTech user's guide: separation of chiral compounds on Chiral-AGP, Chiral-CBH, Chiral-HSA, 2nd edn. ChromTech, Congleton
94. Vandenbosch C, Massart D, Lindner W (1992) Evaluation of six chiral stationary phases in LC for their selectivity towards drug enantiomers. J Pharm Biomed Anal 10:895–908
95. Sugio S, Kashima A, Mochizuki S, Noda M, Kobayashi K (1999) Crystal structure of human serum albumin at 2.5 Å resolution. Protein Eng 12:439–446
96. Hage DS (2001) Chromatographic and electrophoretic studies of protein binding to chiral solutes. J Chromatogr A 906:459–481
97. Yang Y, Hage DS (1993) Characterization of the binding and chiral separation of D- and L-tryptophan on a high-performance immobilized human serum albumin column. J Chromatogr A 645:241–250
98. Yang Y, Hage DS (1996) Role of binding capacity versus binding strength in the separation of chiral compounds on protein-based high-performance liquid chromatography columns. Interactions of D- and L-tryptophan with human serum albumin. J Chromatogr A 725: 273–285
99. Yang Y, Hage DS (1997) Effect of mobile phase composition on the binding kinetics of chiral solutes on a protein-based high-performance liquid chromatography column: interactions of D- and L-tryptophan with immobilized human serum albumin. J Chromatogr A 766: 15–25
100. Domenici E, Bertucci C, Salvadori P, Wainer IW (1991) Use of a human serum albumin-based high-performance liquid chromatography chiral stationary phase for the investigation of protein binding: detection of the allosteric interaction between warfarin and benzodiazepine binding sites. J Pharm Sci 80:164–166
101. Haginaka J (2008) Recent progresses in protein-based chiral stationary phases for enantioseparations in liquid chromatography. J Chromatogr B 875:12–19
102. Massolini G, Temporini C, Calleri E (2008) Penicillin G acylase as chiral selector in LC and CE: exploring the origins of enantioselectivity. J Chromatogr B 875:20–29
103. Gasparrinia F, Misitia D, Villani C (2001) High-performance liquid chromatography chiral stationary phases based on low-molecular-mass selectors. J Chromatogr A 906:35–50
104. Pirkle WH, Welch CJ, Lamm B (1992) Design, synthesis, and evaluation of an improved enantioselective naproxen selector. J Org Chem 57:3854–3860
105. Regis Technologies chiral application guide VI. Regis Technologies, Morton Grove, IL
106. Pirkle WH, House DW (1979) Chiral high-pressure liquid chromatographic stationary phases. 1. Separation of the enantiomers of sulfoxides, amines, amino acids, alcohols, hydroxy acids, lactones, and mercaptans. J Org Chem 44:1957–1960
107. Pirkle WH, Burke JA (1991) Chiral stationary phase designed for β-blockers. J Chromatogr A 557:173–185
108. Pirkle WH, Murray PG (1993) Chiral stationary phase design: use of intercalative effects to enhance enantioselectivity. J Chromatogr 641:11–19
109. Pirkle WH, Gan KZ (1997) Facile and predictable means of separating the enantiomers of 5-arylhydantoins. J Chromatogr A 790:65–71
110. Pirkle WH, Welch CJ, Wilson S (1994) Assignment of absolute configuration to an improved enantioselective naproxen selector. Chirality 6:615–622

111. Villani C, Pirkle WH (1995) Direct high-performance liquid chromatographic resolution of planar chiral tricarbonyl (η^6-arene)–chromium(0) complexes. J Chromatogr A 693:63–68
112. Pirkle WH, Pochapsky TC (1987) Chiral molecular recognition in small bimolecular systems: a spectroscopic investigation into the nature of diastereomeric complexes. J Am Chem Soc 109:5975–5982
113. Pirkle WH, Welch CJ (1994) An investigation into the role of salvation in a well characterized chiral recognition system. J Liq Chromatogr 14:2027–2042
114. Pirkle WH, Welch CJ, Hyun MH (1992) Concerning the role of face-to-edge π–π interactions in chiral recognition. J Chromatogr 607:126–130
115. Pirkle WH, Murry PG, Burke JA (1993) Use of homologous series of analytes as mechanistic probes to investigate the origins of enantioselectivity two chiral stationary phases. J Chromatogr 641:21–29
116. Pirkle WH, Koscho ME (1997) Predictable chromatographic separations of enantiomers: acids and their derivatives. J Chromatogr A 761:65–70
117. Lammerhofer M, Lindner W (1996) Quinine and quinidine derivatives as chiral selectors. I. Brush type chiral stationary phases for HPLC based on cinchonan carbamates and their application as chiral anion exchangers. J Chromatogr A 741:33–48
118. Maier NM, Nicoletti L, Lammerhofer M, Lindner W (1999) Enantioselective anion exchangers based on cinchona alkaloid-derived carbamates: influence of C8/C9 stereochemistry on chiral recognition. Chirality 11:522–528
119. Czerwenka C, Lammerhofer M, Maier NM (2002) Direct high-performance liquid chromatographic separation of peptide enantiomers: study on chiral recognition by systematic evaluation of the influence of structural features of the chiral selectors on enantioselectivity. Anal Chem 74:5658–5666
120. Czerwenka C, Lammerhofer M, Lindner W (2003) Structure–enantioselectivity relationships for the study of chiral recognition in peptide enantiomer separation on cinchona alkaloid-based chiral stationary phases by HPLC: influence of the N-terminal protecting group. J Sep Sci 26:1499–1508
121. Oberleitner WR, Maier NM, Lindner W (2002) Enantioseparation of various amino acid derivatives on a quinine based chiral anion-exchange selector at variable temperature conditions. Influence of structural parameters of the analytes on the apparent retention and enantioseparation characteristics. J Chromatogr A 960:97–108
122. Czerwenka C, Lammerhofer M, Lindner W (2003) Micro-HPLC and standard-size HPLC for the separation of peptide stereoisomers employing an ion-exchange principle. J Pharm Biomed Anal 30:1789–1800
123. Krawinkler KH, Gavioli E, Maier NM, Lindner W (2003) Evaluation of different surface-anchoring strategies for cinchona carbamate type selectors. Chromatographia 58: 555–564
124. Pietle V, Lammerhofer M, Bischoff K, Lindner W (1997) High-performance liquid chromatographic enantioseparation of N-protected α-amino acids using nonporous silica modified by a quinine carbamate as chiral stationary phase. Chirality 9:157–161
125. Hoffmann CV, Reischl R, Maier NM, Lammerhofer M, Lindner W (2009) Stationary phase-related investigations of quinine-based zwitterionic chiral stationary phases operated in anion-, cation-, and zwitterion-exchange modes. J Chromatogr A 1216:1147–1156
126. Sousa LR, Sogah GDY, Hoffman DH, Cram DJ (1978) Host–guest complexation. 12. Total optical resolution of amine and amino ester salts by chromatography. J Am Chem Soc 100:4569–4576
127. Sogah GDY, Cram DJ (1979) Host–guest complexation. 14. Host covalently bound to polystyrene resin for chromatographic resolution of enantiomers of amino acid and ester salts. J Am Chem Soc 101:3035–3042
128. Shinbo T, Yamaguchi T, Nishimura K, Sugiura M (1987) Chromatographic separation of racemic amino acids by use of chiral crown ether-coated reversed-phase packings. J Chromatogr 405:145–153

129. Shinbo T, Yamaguchi T, Yanagishita H, Kitamoto D, Sakaki K, Sugiura M (1992) Improved crown ether-based chiral stationary phase. J Chromatogr 625:101–108
130. Jin YZ, Hirose K, Nakamura T, Nishioka R, Nishioka R, Ueshige T, Tobe Y (2006) Preparation and evaluation of a chiral stationary phase covalently bound with a chiral pseudo-18-crown-6 ether having a phenolic hydroxy group for enantiomer separation of amino compounds. J Chromatogr A 1129:201–207
131. Hyun MH, Jin JS, Lee W (1998) Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether. J Chromatogr A 822:155–161
132. Hyun MH, Jin JS, Koo HJ, Lee W (1999) Liquid chromatographic resolution of racemic amines and amino alcohols on a chiral stationary phase derived from crown ether. J Chromatogr A 837:75–82
133. Machida Y, Nishi H, Nakamura K, Nakai H, Sato T (1998) Enantiomer separation of amino compounds by a novel chiral stationary phase derived from crown ether. J Chromatogr A 805:85–92
134. Steffeck RJ, Zelechonok Y, Gahm KH (2002) Enantioselective separation of racemic secondary amines on a chiral crown ether-based liquid chromatography stationary phase. J Chromatogr A 947:301–305
135. Zhang D, Li F, Kim DH, Choi HJ, Hyun MH (2005) Resolution of β-blockers on a chiral stationary phase based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid: unusual temperature effect. J Chromatogr A 1083:89–95
136. Choi HJ, Park YJ, Hyun MH (2007) Liquid chromatographic resolution of secondary amino alcohols on a chiral stationary phase based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid. Dependence of temperature effect on analyte structure. J Chromatogr A 1164:235–239
137. Hyun MH, Cho YJ, Jin JS (2002) Liquid chromatographic direct resolution of β-amino acids on a chiral crown ether stationary phase. J Sep Sci 25:648–652
138. Hyun MH, Tan G, Cho YJ (2004) Liquid chromatographic resolution of aryl-amino ketones on chiral stationary phases based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid. J Liq Chromatogr Relat Technol 27:1671–1680
139. Hyun MH, Han SC, Jin JS, Lee W (2000) Separation of the stereoisomers of racemic fluoroquinolone antibacterial agents on a crown-ether-based chiral HPLC stationary phase. Chromatographia 52:473–476
140. Cram DJ (1988) The design of molecular hosts, guests, and their complexes. J Incl Phenom 6:397–413
141. Gerbaux P, De Winter J, Cornil D, Ravicini K, Pesesse G, Cornil J, Flammang R (2008) Noncovalent interactions between (18-crown-6)-tetracarboxylic acid and amino acids: electrospray–ionization mass spectrometry investigation of the chiral-recognition processes. Chem Eur J 14:11039–11049
142. Krstulovic AM, Fouchet MH, Burke JT, Gillet G, Durand A (1988) Direct enantiomeric separation of betaxolol with applications to analysis of bulk drug and biological samples. J Chromatogr 452:477–483
143. Ye YK, Stringham RW (2006) The effect of acidic and basic additives on the enantioseparation of basic drugs using polysaccharide-based chiral stationary phases. Chirality 18:519–530
144. Liu Y, Berthod A, Mitchell CR, Xiao TL, Zhang B, Armstrong DW (2002) Super/subcritical fluid chromatography chiral separations with macrocyclic glycopeptide stationary phases. J Chromatogr A 978:185–204
145. Karlsson A, Aspegren A (2000) Enantiomeric separation of amino alcohols on protein phases using statistical experimental design: a comparative study. J Chromatogr A 866:15–23
146. Aboul-Enein HY, Serignese V (1995) Direct separation of albuterol enantiomers in biological fluids and pharmaceutical formulations using α1-acid glycoprotein and Pirkle urea type columns. Chirality 7:158–162

147. Aubry A, Sebastian DS, Williams R, Boucher RJ (2001) Column selection and method development for the determination of the enantiomeric purity of investigational non-nucleoside reverse transcriptase inhibitors. Chirality 13:193–198
148. He L, Shi Y et al Unpublished results
149. Zhao Y, Woo G, Thomas S, Semin D, Sandra P (2003) Rapid method development for chiral separation in drug discovery using sample pooling and supercritical fluid chromatography–mass spectrometry. J Chromatogr A 1003:157–166
150. He BL, Shi Y (2008) Comprehensive chiral HPLC method development for pharmaceutical compounds during late stage development. Am Pharm Rev 11:47–52
151. Laskar DB, Zeng L, Xu R, Kassel DB (2008) Parallel SFC/MS-MUX screening to assess enantiomeric purity. Chirality 20:885–895
152. White C (2005) Integration of supercritical fluid chromatography into drug discovery as a routine support tool: Part I. Fast chiral screening and purification. J Chromatogr A 1074: 163–173
153. Maftouh M, Granier-Loyaux C, Chavana E, Marinia J, Pradinesa A, Heydenb YV, Picarda C (2005) Screening approach for chiral separation of pharmaceuticals: Part III. Supercritical fluid chromatography for analysis and purification in drug discovery. J Chromatogr A 1088:67–81
154. Berthod A, Chang SC, Armstrong DW (1992) Empirical procedure that uses molecular structure to predict enantioselectivity of chiral stationary phases. Anal Chem 64:395–404
155. Roussel C, Pierrot-Sanders J, Heitmann I, Piras P (2001) CHIRBASE: database current status and derived research applications using molecular similarity, decision tree and 3D "enantiophore" search. In: Subramanian G (ed) Chiral separation techniques: a practical approach, 2nd edn. Wiley-VCH Verlag GmbH, Weinheim (Federal Republic of Germany)
156. Wetli HA, Francotte E (2007) Automated screening platform with isochronal-parallel analysis and conditioning for rapid method development of chiral separations. J Sep Sci 30:1255–1261
157. Villeneuve MS, Anderegg RJ (1998) Analytical supercritical fluid chromatography using fully automated column and modifier selection valves for the rapid development of chiral separations. J Chromatogr A 826:217–225
158. Perrin C, Vu VA, Matthijs N, Maftouh M, Massart DL, Vander Heyden Y (2002) Screening approach for chiral separation of pharmaceuticals Part I. Normal-phase liquid chromatography. J Chromatogr A 947:69–83
159. Perrin C, Matthijs N, Mangelings D, Maftouh M, Massart DL, Vander Heyden Y (2002) Screening approach for chiral separation of pharmaceuticals: Part II. Reversed-phase liquid chromatography. J Chromatogr A 966:119–134
160. Matthijs N, Maftouh M, Vander Heyden Y (2006) Screening approach for chiral separation of pharmaceuticals: IV. Polar organic solvent chromatography. J Chromatogr A 1111:48–61
161. Puente M, White CT, Rivera-Sagredo A, Reilly J, Burton K, Harvey G (2003) Impact of normal-phase gradient elution in chiral chromatography: a novel, robust, efficient and rapid chiral screening procedure. J Chromatogr A 983:101–114
162. Zhang Y, Watts W, Nogle L (2004) Rapid method development for chiral separation in drug discovery using multi-column parallel screening and circular dichroism signal pooling. J Chromatogr A 1049:75–84
163. Andersson ME, Aslan D, Clarke A, Roeraade J, Hagman G (2003) Evaluation of generic chiral liquid chromatography screens for pharmaceutical analysis. J Chromatogr A 1005: 83–101
164. De la Puente ML (2004) Highly sensitive and rapid normal-phase chiral screen using high-performance liquid chromatography–atmospheric pressure ionization tandem mass spectrometry (HPLC/MS). J Chromatogr A 1055:55–62
165. Matthijs N, Perrin C, Maftouh M (2004) Definition and system implementation of strategies for method development of chiral separations in normal- or reversed-phase liquid chromatography using polysaccharide-based stationary phases. J Chromatogr A 1041:119–133

166. He BL, Shi Y, Kleintop B, Raglione T (2008) Direct and indirect separations of five isomers of brivanib alaninate using chiral high-performance liquid chromatography. J Chromatogr B 875:122–135
167. Borman P, Boughtflower B, Cattanach K, Crane K, Freebraine K, Jonas G, Mutton I, Patel A, Sanders M, Thompson D (2003) Comparative performances of selected chiral HPLC, SFC, and CE systems with a chemically diverse sample set. Chirality 15:S1–S12
168. Welch CJ, Biba M, Gouker JR, Kath G, Augustine P, Hosek P (2007) Solving multicomponent chiral separation challenges using a new SFC tandem column screening tool. Chirality 19:184–189
169. Barnhart WW, Gahm KH, Thomas S, Notari S, Semin D, Cheetham J (2005) Supercritical fluid chromatography tandem-column method development in pharmaceutical sciences for a mixture of four stereoisomers. J Sep Sci 28:619–626
170. Wang Z, Jonca M, Lambros T, Ferguson S, Goodnow R (2007) Exploration of liquid and supercritical fluid chromatographic chiral separation and purification of Butlin-3 – a small molecule antagonist of MDM2. J Pharm Biomed Anal 45:720–729
171. Bargmann-Leyder N, Tambute A, Caude M (1995) A comparison of LC and SFC for cellulose- and amylose-derived chiral stationary phases. Chirality 7:311–325
172. Macaudibre P, Caude M, Rosset R, Tambut A (1989) CO_2 supercritical fluid chromatography with chiral stationary phases: a promising coupling for the resolution of various racemates. J Chromatogr Sci 27:383–394
173. Macaudibre P, Caude M, Rosset R, Tambut A (1989) Chiral resolution in SFC: mechanisms and applications with various chiral stationary phases. J Chromatogr Sci 27:583–591
174. Macaudibre P, Lienne M, Caude M, Rosset R, Tambut A (1989) Resolution of π-acid racemates on π-acid chiral stationary phases in normal-phase liquid and subcritical fluid chromatographic modes. J Chromatogr 467:357–372
175. Anton K, Eppinger J, Frederiksen L, Francotte E, Berger TA, Wilson WH (1994) Chiral separations by packed-column super- and subcritical fluid chromatography. J Chromatogr A 666:395–401
176. Chankvetadze B, Yamamoto C, Okamoto Y (2001) Enantioseparation of selected chiral sulfoxides using polysaccharide-type chiral stationary phases and polar organic, polar aqueous–organic and normal-phase eluents. J Chromatogr A 922:127–137
177. Matthijs N, Maftouh M, Vander Heyden Y (2006) Chiral separation strategy in polar organic solvent chromatography and performance comparison with normal-phase liquid and supercritical-fluid chromatography. J Sep Sci 29:1353–1362
178. Cass QB, Batigalhia F (2003) Enantiomeric resolution of a series of chiral sulfoxides by high-performance liquid chromatography on polysaccharide-based columns with multimodal elution. J Chromatogr A 987:445–452
179. Siret L, Bargrmann N, Tambute A, Caude M (1992) Direct enantiomeric separation of β-blockers on ChyRoSine-A by supercritical fluid chromatography: supercritical carbon dioxide as transient in situ derivatizing agent. Chirality 4:252–262
180. Toribio L, Del Nozal MJ, Bernal JL, Alonso C, Jimenez JJ (2005) Comparative study of the enantioselective separation of several antiulcer drugs by high-performance liquid chromatography and supercritical fluid chromatography. J Chromatogr A 1091: 118–123
181. Yaku K, Aoe K, Nishimura N, Sato T, Morishita F (1997) Chiral resolution of four optical isomers of diltiazem hydrochloride on Chiralcel columns by packed-column supercritical fluid chromatography. J Chromatogr A 785:185–193
182. Yaku K, Aoe K, Nishimura N, Morishita F (1999) Thermodynamic study and separation mechanism of diltiazem optical isomers in packed-column supercritical fluid chromatography. J Chromatogr A 848:337–345
183. Bargmann-Leyder N, Sella C, Bauer D, Tambute A, Caude M (1995) Supercritical fluid chromatographic separation of beta-blockers on Chyrosine-A: investigation of the chiral recognition mechanism using molecular modeling. Anal Chem 67:952–958

184. Medvedovici A, Sandra P, Tortblo L, David F (1997) Chiral packed column subcritical fluid chromatography on polysaccharide and macrocyclic antibiotic chiral stationary phases. J Chromatogr A 785:159–171
185. Mourier PA, Eliot E, Caude MH, Rosset RH, Tambute AG (1985) Supercritical and subcritical fluid chromatography on a chiral stationary phase for the resolution of phosphine oxide enantiomers. Anal Chem 57:2819–2823
186. MaCaudiere P, Tambute A, Caude M, Rosset R, Alembik MA, Wainer IW (1986) Resolution of enantiomeric amides on a Pirkle-type chiral stationary phase, a comparison of subcritical fluid and liquid chromatographic approaches. J Chromatogr 371:177–193
187. MaCaudiere P, Lienne M, Caude M, Rosset R, Tambute A (1989) Resolution of π-acid racemates on π-acid chiral stationary phases in normal-phase liquid and subcritical fluid chromatographic modes: a unique reversal of elution order on changing the nature of the achiral modifier. J Chromatogr 467:357–372
188. Blum AM, Lynam KG, Nicolas EC (1994) Use of a new Pirkle-type chiral stationary phase in analytical and preparative subcritical fluid chromatography of pharmaceutical compounds. Chirality 6:302–313
189. Xiao TL, Gasper MP, Armstrong DW (2007) Macrocyclic antibiotics. In: Wilson ID, Cooke M, Poole CF (eds) Encyclopedia of separation science, vol 10. Elsevier Science Ltd. doi:10.1016/B978-012226770-3/10684-3

Chiral Recognition with Macrocyclic Glycopeptides: Mechanisms and Applications

Alain Berthod, Hai Xiao Qiu, Sergey M. Staroverov, Mikhail A. Kuznestov, and Daniel W. Armstrong

Contents

Abstract The macrocyclic glycopeptide chiral selectors are natural molecules produced by bacterial fermentation. Purified and bonded to silica particles, they make very useful chiral stationary phases (CSP) with a broad spectrum of applicability in enantiomeric separation. The macrocyclic glycopeptide CSPs are multimodal, the same column being able to work in normal phase mode with apolar mobile phase, in reversed-phase mode, or in polar ionic mode with 100% alcoholic mobile phase

A. Berthod (✉)
Laboratoire des Sciences Analytiques, Université de Lyon, CNRS UMR 5180,
Bat. CPE, 69622, Villeurbanne, France
e-mail: berthod@univ-lyon1.fr

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_7,

of adjusted pH. The role of the carbohydrate units is described as well as the critical charge–charge docking interaction responsible for the amino acid enantiomer recognition. The complimentary phenomenon is also exposed.

1 Introduction

The origin of the use of macrocyclic glycopeptide antibiotics as chiral selector is somewhat linked to their mechanism of interaction with amino acids. Knowing that these antibiotics inhibited the development of Gram-positive bacteria by binding to the D-Ala-D-Ala terminal group of a protein essential to cell wall building, Armstrong thought that these multifunctional molecules were potentially powerful chiral selectors for the D-alanine amino acid and patented his idea [1]. He found a way to bind different macrocyclic glycopeptides to chromatographic silica particles preserving their enantioselectivity toward amino acids and a wide variety of other enantiomers [2]. These chiral stationary phases (CSP) are marketed today under the trade name Chirobiotic® by Astec, a company of the Sigma-Aldrich group [3].

The commercial macrocyclic glycopeptide CSPs were found very rugged and able to work in different chromatographic modes: normal phase as well as reversed phase or polar organic modes, maintaining their separation properties. With protein and coated carbohydrate CSPs conformational changes were induced by switching chromatographic modes reducing or eliminating enantioselectivity. With macrocyclic glycopeptide CSPs, changes in chromatographic modes may also induce minor selector conformation changes, but these changes have been shown to be reversible [4]. This property associated to the complex structure of the selectors allows for many different selector–selectand interactions and explains their versatility. If the macrocyclic glycopeptide CSPs are not able to discriminate a variety of neutral enantiomers as large as that of the carbohydrate-based CSPs, especially in the normal phase and supercritical fluid modes, they do have a broad applicability when used in different chromatographic modes. These qualities definitively make them needed chiral columns in any chromatographic lab working in chiral separations [3–7].

This chapter will focus on chiral recognition mechanisms and molecular interactions. The commercialized macrocyclic glycopeptides: ristocetin, R, vancomycin, V, teicoplanin, T and its aglycon form, teicoplanin aglycon, TAG will be the main subject of the study since a large database is available in the literature [3–7]. However, non-commercialized avoparcin, balhimycin, and the recently introduced eremomycin macrocyclic glycopeptides [8] are also included in the study for more information on possible mechanisms.

The most distinctive feature of this class of chiral selectors is their ionic character. Without exception, all macrocyclic glycopeptide chiral selectors are ionizable. All of them bear primary or secondary amines that are positively charged at neutral and acidic pH values. Also, all of them but ristocetin have a carboxylic acid bearing a negative charge at neutral and basic pH. The net charge of the chiral selector is adjustable changing the mobile phase pH. The polar ionic mode (PIM)

of elution uses anhydrous polar mobile phases (methanol and/or acetonitrile) containing selected small amount of acids and/or bases. This allows for a fine tuning of the charges on the chiral selector that has a significant effect on both enantiomer retention and separation.

2 Structures of the Macrocyclic Glycopeptide Chiral Selectors

2.1 Physicochemical Properties

The physicochemical properties of the major macrocyclic glycopeptides are listed in Table 1. The corresponding structures are given in Fig. 1. The molecular weights range from 1196 for the artificially obtained teicoplanin aglycon (TAG) to 2066 for ristocetin A that bears six sugar units. Ristocetin is also the only member of this family without chlorine atom. The listed pK_a values correspond to the carboxylic and phenol groups producing negative charges and primary and secondary amine groups producing positive charges. They were calculated imputing the structural formulas of the macrocycles in the H++ software (Table 1, footnote 3). These pK_a are valid in pure water only. The listed values for the number of stereogenic centers and chemical groups include the attached sugar moieties.

2.2 A Common Macrocyclic "Basket" Skeleton

All seven macrocyclic glycopeptides share the same three macrocycle structure as evidenced in Fig. 1 using for reference the TAG structure as the simplest of the family. Teicoplanin and its aglycon have four macrocycles, each one made by two aromatic rings and a peptide sequence. The first macrocycle is made by Rings #1 and #2 linked by an ether bond and two amide groups (Fig. 1). The second and third macrocycles are produced, respectively, by Rings #2 and #3, an ether bond and two amide groups and by the two directly connected Rings #4 and #5 and two amide groups. The fourth macrocycle is produced by Rings #6 and #7, an ether bond and two amide groups (Fig. 1). These macrocycles form a three-dimensional structure recalling somewhat that of a basket or half-helical twist.

The three first macrocycles with the five aromatic rings numbered from 1 to 5 in Fig. 1 form a common skeleton found in all macrocyclic glycopeptide antibiotics with exactly the same stereochemical arrangement often called the "aglycon basket." The selectors differ by the chemical groups attached to the common skeleton. The teicoplanin and ristocetin selectors have a fourth macrocycle made by two more aromatic rings (numbered 6 and 7 in Fig. 1) and bearing a primary amine in alpha-position of Ring #7. Avoparcin has also two extra aromatic rings but they are not interlinked. These rings do not form a fourth macrocycle rigidly linked to the three macrocycle basket. Balhimycin, eremomycin, and vancomycin have only the five aromatic rings of the common three macrocycle basket skeleton. Also all four selectors with three macrocycles bear a secondary amine on the peptide chain.

Table 1 Physicochemical properties of macrocyclic glycopeptide chiral selectors[a]

Macrocycle (trade code)[b]	Avoparcin β	Balhimycin	Eremomycin	Ristocetin *A* (*R*)	Teicoplanin $A_{2\text{-}2}$ (*T*)	Teicoplanin aglycon (TAG)	Vancomycin (*V*)
Formula	$C_{88}H_{114}N_9O_{36}Cl_2$	$C_{66}H_{74}N_9O_{24}Cl_2$	$C_{73}H_{89}N_{10}O_{26}Cl$	$C_{95}H_{110}N_8O_{44}$	$C_{88}H_{97}N_9O_{33}Cl_2$	$C_{58}H_{43}N_7O_{18}Cl_2$	$C_{66}H_{75}N_9O_{24}Cl_2$
Molecular weight	1943	1447	1556	2066	1878	1196	1449
pK_a[c]	3; 7.3; 8; 8.2; 8.5; 10.8;11.9;12.5	2.9; 7; 8.1; 9.2; 10.4; 11.5	2.9; 7.1; 8.3; 9.2; 10.1; 10.9; 12.0	7.0; 8.3; 9.2; 9.9, 10.1; 10.9	2.8; 7.4; 8.5; 8.9; 9.3; 11.0	2.7; 7.3; 7.8; 8.3; 8.9; 9.2; 9.8; 11.1	2.9; 6.8; 7.7; 8.5; 10.4; 11.7
Isoelectric point	8.1	7.5	8.5	7.5	6.5	6.2	7.2
Stereogenic centers	32	16	22	38	23	8	18
Macrocycles	3	3	3	4	4	4	3
OH groups (phenols)	16 (4)	7 (3)	9 (3)	21 (4)	14 (4)	7 (6)	9 (3)
–NH_2 groups	2	1	2	2	1	1	1
Second amines	1	1	1	0	0	0	1
–COOH groups	1	1	1	0	1	1	1
Aromatic rings	7	5	5	7	7	7	5
Amide linkages	6	7	7	6	8	6	7
Sugar moieties	5	2	3	6	3	0	2
Produced by	*Spectromyces candidus*	*Amycolatopsis* sp.	*Actinomycete INA-238*	*Nocardia lurida*	*Actinoplanes teicomyceticus*	Chemical hydrolysis of *T*	*Spectromyces orientalis*

[a]Data from [3, 4, 8, 9, 12]

[b]Commercial name: Chirobiotic® from Astec, Supelco Sigma-Aldrich, Bellefonte, PA.

[c]Calculated pK_a values using H++ (http://biophysics.cs.vt.edu/H++/index.php).

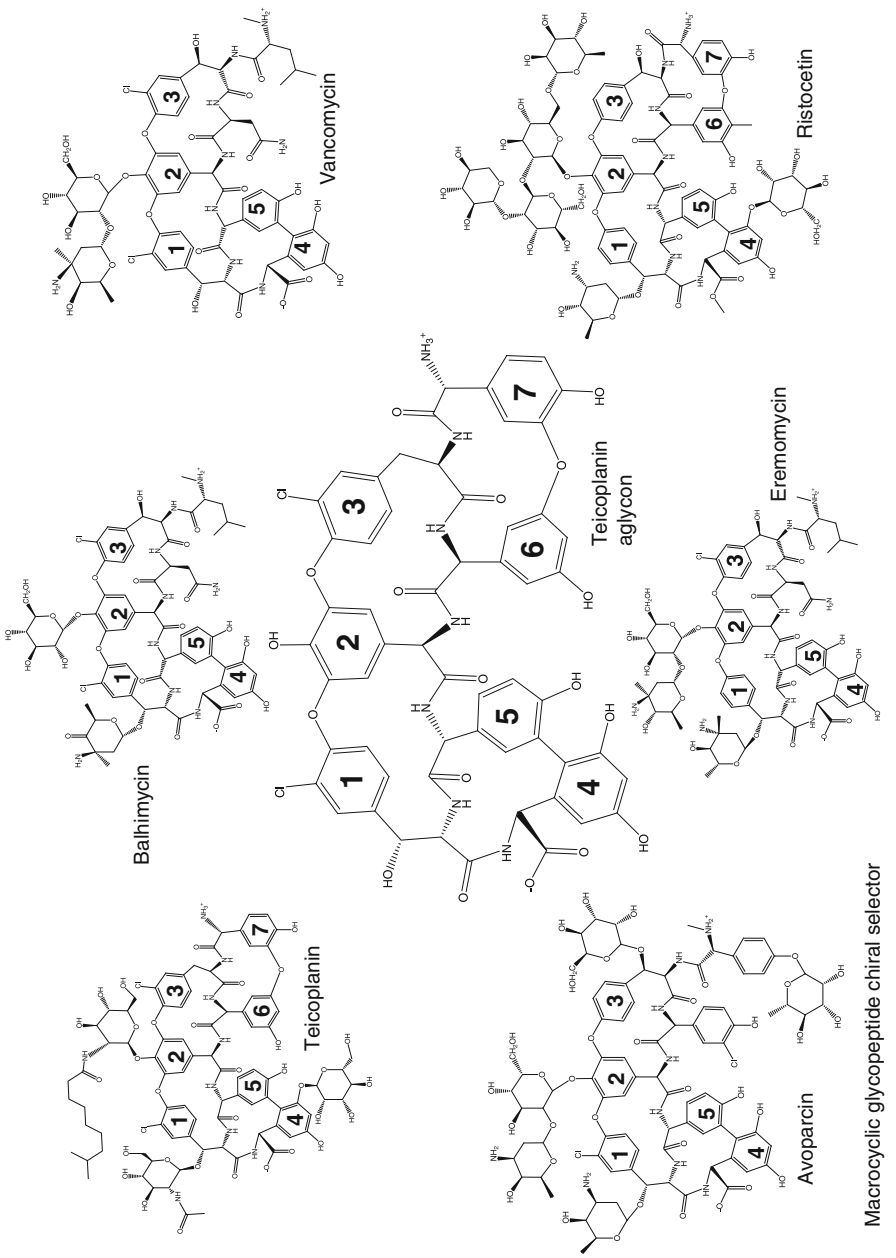


Fig. 1 Molecular structures of seven macrocyclic glycopeptides showing the common arrangement of five aromatic rings

2.3 Three-Dimensional Structures

The Fig. 1 structures were entered in a three-dimensional software (CS Chem3D Ultra, Cambridge Soft, Cambridge, MA 02140, USA) providing different tools to estimate the most probable three-dimensional spatial molecular arrangement minimizing the molecule energy. Figure 2 shows the teicoplanin aglycon three-dimensional molecules. The aromatic rings were numbered following Fig. 1. The polar groups were brightly colored. The anionic carboxylate group is red colored and bear the minus sign. The cationic primary ammonium $-NH_3^+$ groups is colored in bright green with a plus sign. All polar hydroxyl groups are yellow colored. The less polar atoms are dark colored. Figure 2 clearly shows the basket-like molecular arrangement of the aglycon core of teicoplanin.

Figure 3 shows a three-dimensional picture of the six macrocyclic glycopeptide containing carbohydrate units in their molecules. The color coding used for the TAG selector was also used for all six other selectors. The molecules were roughly oriented so that they match the Fig. 1 planar structure. The anionic carboxylate group (bright red color) was placed in the lower left side view. The primary or secondary amine on the peptide chain (bright green color) was placed in the lower right side of the view.

Figure 3 shows some similarities between selectors: eremomycin, balhimycin, and vancomycin, all three have close structures. It also shows the critical importance of the associated carbohydrate units: ristocetin and teicoplanin both share a

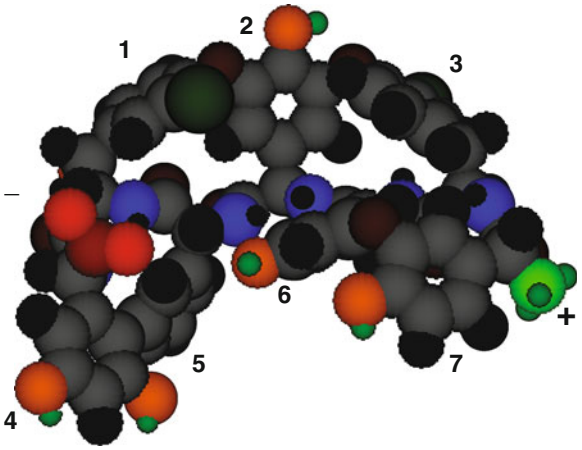


Fig. 2 Three-dimensional representation of the Teicoplanin aglycon chiral selector. The numbers correspond to the aromatic ring numbering shown in Fig. 1. The carboxylate anionic group is shown in bright *red*. The primary ammonium positive group is shown in *green*. The polar hydroxyl groups, either alcohols or phenols, are *yellow* colored. Less polar nitrogen atoms (amide) are in *dark blue*, less polar oxygen atoms (amide) are in *dark red*, and chlorine atoms are in *dark green*

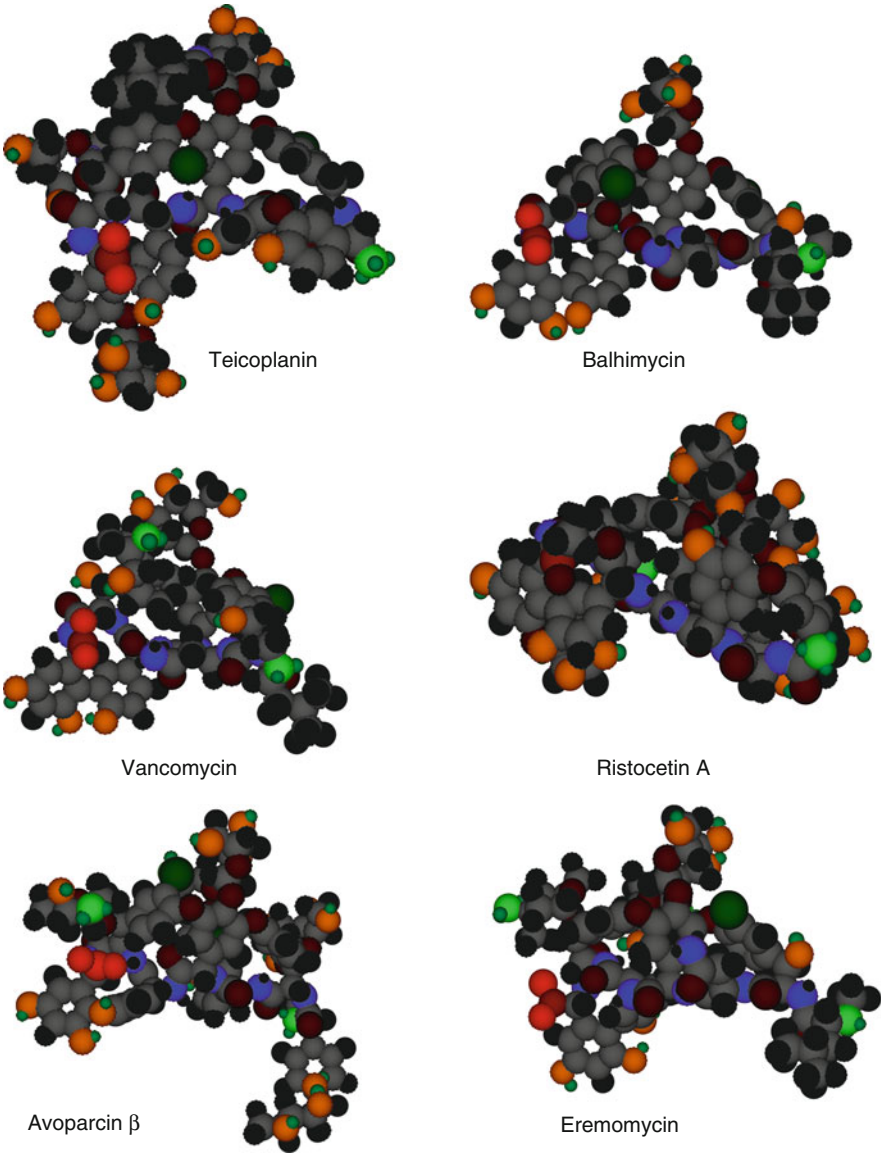


Fig. 3 Three-dimensional representation of the natural macrocyclic glycopeptides chiral selectors whose physicochemical properties are listed in Table 1. The color code is given in Fig. 2 caption

similar aglycon core; they have a very different three-dimensional appearance due to the different attached sugar units. It must be noted that ristocetin is the only selector without a free anionic carboxylate group. This group has the form of an uncharged methyl ester (Fig. 1). Figure 3 shows a calculated possible three-dimensional selector structures in vacuum at the isoelectric point (one positive and one negative charge except for ristocetin shown with one positive charge). This three-dimensional spatial arrangement is certainly different when the molecules are solvated by different mobile phases at various pHs. Mobile phase adjustments on enantioseparations will be described. Alterations in mobile phase composition and/or pH induce conformational changes in the selector structure. However, these changes are mostly reversible [3, 4, 9, 10].

Figures 2 and 3 show that macrocyclic glycopeptides are simple enough so that their molecules can be easily modeled by small computers. They also show the variety of possible selector–selectand interactions including all possible chemical interactions from the strongest charge–charge (coulombic) interaction to the delicate shape recognition interaction within the cavities of the polypeptide aglycon core of the selectors.

3 The Selector Charge–Charge Docking Interaction

Macrocyclic glycopeptides are antibiotics produced by different bacterial strains (Table 1) to inhibit the growth of bacterial competitors. These molecules can target specifically the terminal D-Ala-D-Ala sequence of a protein essential to Gram-positive cell wall building [1]. Not surprisingly, these molecules were found to be excellent chiral selectors in discriminating between amino acid enantiomers [1, 2, 8, 11–14].

3.1 Amino Acid Enantiomer Separation

Table 2 lists a selected set of the considerable data published on the separation of native or derivatized amino acids on macrocyclic glycopeptide CSPs [1, 2, 11–14]. The vancomycin chiral selector was found to have a lower efficacy in the separation of amino acids compared to that of the three other commercialized CSP (Chirobiotic® T, TAG, and R) [14].

The teicoplanin CSPs are recommended for the separation native amino acid enantiomers in reversed-phase mode [11]. It was established that the carbohydrate units on the teicoplanin selector (Fig. 3) were hindering the enantiomer approach making the transient selector–selectand complex more difficult to form [15]. This mechanistic point will be studied in detail later.

The enantioselectivity and enantioresolution factors obtained on the ristocetin A selector are lower than the corresponding ones obtained on teicoplanin and TAG selectors. Eremomycin, a recently introduced macrocyclic glycopeptide very close

Table 2 Enantioseparation of amino acid on macrocyclic glycopeptide chiral selector

		Eremomycin			Ristocetin A			Teicoplanin			Teicoplanin aglycon		
Compound	Mob. phase	k_1	α	Rs	k_1	α	Rs	k_1	α	Rs	k_1	α	Rs
Alanine	RP	0.26	2.4	2.9	0.30	1.3	1.7	0.56	1.8	2.9	0.16	2.7	4.0
Alanine (*N*-benzoyl)	RP				0.21	25.6	8.9	0.51	13	19	1.09	3.6	8.0
Phenylalanine	RP	0.55	4.3	6.6	0.64	1.4	2.5	0.47	1.5	3.1	1.00	3.7	13.7
Phenylalanine methyl ester	RP							0.72	1.0	0	2.19	1.0	0
Phenylalanine amide	RP				0.81	1.0	0	0.69	1.0	0	1.62	1.0	0
Phenylalanine *N*-benzoyl	RP				0.67	13.0	5.8	0.87	4.4	11.4	2.93	1.5	2.6
Tryptophan	RP	1.12	1.81	4.3	1.01	1.2	2.0	0.69	1.4	2.6	1.45	1.8	3.6
Tryptophan	PIM							17.1	1.0	0	6.58	2.2	3.2
Tryptophan methyl ester	RP							0.91	1.0	0	3.0	1.0	0

Column 25 cm × 4.6 mm i.d.; k_1: retention factor of the first enantiomer, α: enantioselectivity factor, Rs: resolution factor between enantiomers. Mobile phases: RP = reversed phase, methanol/buffer pH 4.1 40/60 v/v; PIM = polar ionic mode, methanol/acetonitrile 45/55 v/v with 0.1% acetic acid and 0.1% triethylamine. Data from [1, 2, 12–16].

structurally to vancomycin (Fig. 1), shows promising efficacy toward native amino acid enantiomer separation (Table 2) [8]. The eremomycin enantioselectivity factors of amino acids are comparable if not higher than those of teicoplanin (Table 2).

3.2 The Critical Amino Acid Carboxylic Acid Group

Table 2 shows that the derivation of the amine group of amino acids can improve chiral recognition, e.g., the enantioselectivity factors of alanine are 1.8 and 2.7 on teicoplanin and TAG CSPs; they become 13 and 3.6, respectively, upon derivatization of the amine group forming *N*-benzoyl alanine (Table 2). Enantioselectivity enhancement is very often obtained by N-derivatization of amino acids [14, 16]. Such enantiorecognition enhancement is not an absolute rule as shown by *N*-benzoyl phenylalanine in Table 2. The phenylalanine enantioselectivity factors jumps from 1.5 (native form, $Rs = 3.1$) to 4.4 (N-benzoylated form, $Rs = 11.4$) on teicoplanin CSP. It decreases from 3.7 (native form, $Rs = 13.7$) down to 1.5 ($Rs = 2.6$) after N-benzoylation on the TAG CSP. There is a clear steric effect due to the attached benzoyl group very beneficial for enantiorecognition on teicoplanin CSP and detrimental when the TAG CSP is used.

The picture is completely different when the carboxylic acid group is considered. As an absolute rule, any derivation of this group cancels the enantiomer separation of the amino acid derivatives on macrocyclic glycopeptide CSPs. This point is illustrated by the methyl esterification of phenylalanine and tryptophan canceling enantioselectivity on teicoplanin and TAG CSP (data not found on eremomycin). Similarly, the two phenylalanine amide enantiomers cannot be separated on any CSP (Table 2).

The carboxylic acid groups of amino acids have pK_a values ranging from 1.8 to 2.6 (alanine: 2.3; phenylalanine: 1.8; tryptophan: 2.4) [17]. This ionizable group is in its anionic form, $–COO^-$, between pH 3 and 8, the practical pH range of LC mobile phases. Since all macrocyclic glycopeptide selectors bear charged groups, a positively charged amine group was suspected to be a critical docking point to form the transient enantioselective complex responsible for chiral recognition of the two amino acid enantiomers. Amino acids were used as model examples but the critical charge–charge anchoring is responsible for the chiral separation of many enantiomers containing a carboxylic acid [5–7].

3.3 The Essential Macrocycle Ammonium Docking Site

Vancomycin is able to form a stable complex with copper [18]. This complex involves the secondary amine attached to the peptidic chain of the selector rigid basket and not the very mobile or accessible primary amine of a sugar unit (Fig. 1). Nair et al. established that all enantiorecognition capability of vancomycin disappeared upon copper complexation [18]. The authors concluded that the secondary

amine linked to the vancomycin basket (Fig. 3) was essential in its cationic form for the chiral discrimination of amino acid enantiomers [18].

Berthod et al. also tried copper complexation with teicoplanin and TAG CSPs [19]. Similar results were obtained. Amino acid enantiomers perfectly separated by both teicoplanin and TAG CSP could no longer be separated as soon as copper was present in the mobile phase. The copper–teicoplanin complex is also formed with the primary amine group on the peptidic teicoplanin basket (Figs. 1 and 3). However, unlike the vancomycin–copper complex which was very stable [18], the teicoplanin–copper complex was found to be reversible. Indeed, amino acid enantioselectivity was mostly restored after washing the chiral column with several column volumes of copper-free clean mobile phase [19].

The specificity of the amino acid charge–charge docking interaction must be pointed out. Non-amino acid enantiomers could still be separated by vancomycin and teicoplanin column in presence of copper ions clearly showing the multiple possible mechanisms of the macrocyclic glycopeptide selectors. The two enantiomers of tryptophan ethyl ester, a carboxylic acid blocked amino acid, could be separated on a ristocetin A chiral column in polar organic mode [16]. The separation did not involve the secondary amine on the macrocycle but other possible multiple interactions with the ristocetin A selector.

4 The Role of the Carbohydrate Moieties

Figure 1 selected the teicoplanin aglycon selector as the central molecular structure to clearly show the "basket" structure of the macrocyclic glycopeptide family. All naturally produced macrocyclic antibiotics bear several carbohydrate units whose primary role seems to be the enhancement of water solubility of the central macrocyclic structure (Fig. 3).

4.1 Aglycon Selectors

It was already mentioned that the teicoplanin aglycon selector was superior to teicoplanin itself in the chiral separation of amino acid enantiomers [15]. Table 2 shows this finding for three amino acids. With the same mobile phase, the resolution factor of alanine enantiomers is 2.9 on teicoplanin and 40% higher with 4.0 on TAG. The resolution factors of phenylalanine and tryptophan on teicoplanin CSPs are 3.1 and 2.6, respectively. They become 13.7 (+340%) and 3.6 (+40%) on the TAG CSP. The enantioselectivity factors observed with amino acid enantiomers on the TAG stationary phase were significantly higher than the corresponding factors obtained on native teicoplanin [15]. The carbohydrate units clearly hurt the enantiorecognition of amino acid enantiomers by macrocyclic glycopeptide selectors. They likely interfere either with the charge–charge docking interaction, screening

the positively charged amine group and/or hindering its access, or with secondary interactions that are necessary for the enantiomer recognition.

It was found, however, that those carbohydrate units were helpful in the enantiorecognition of numerous other families of enantiomers. Dihydrofurocoumarin enantiomers were better separated by teicoplanin than by its aglycon [20]. Figure 4 shows the difference ($\alpha_T - \alpha_{TAG}$) between the enantioselectivity factor of the T and TAG selectors. As seen with the Table 2 data, the carbohydrate units hurt the enantioseparations of amino acids that are much better separated by aglycon selectors. These differences in selector–selectand interactions produce negative values for the ($\alpha_T - \alpha_{TAG}$) parameter for amino acid enantiomers and positive values for, e.g., *p*-hydroxy mandelic acid or *N*-benzoyl phenylalanine enantiomers. For compounds such as dihydro angelicin or coumachlor (Fig. 4), the enantioselectivity factors have the same magnitude on the two CSPs. Most often the chiral recognition mechanisms are very different involving different parts or functionalities of the T and TAG selectors. These differences are evidenced by the changes in retention factors and peak efficiencies and, when the information can be obtained, by the changes in enantiomer elution order. Similar results were obtained with vancomycin and its aglycon [21].

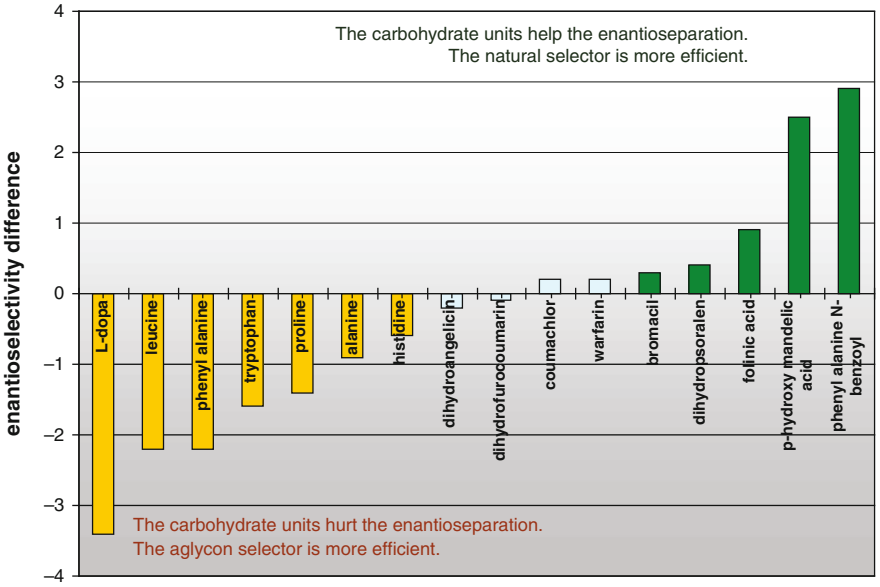


Fig. 4 Enantioselectivity factor difference between the teicoplanin aglycon (TAG) and teicoplanin (T) chiral stationary phase for selected compounds. All experimental conditions were identical: 25 cm × 4.6 mm columns, same mobile phases. Data from [15] and [20]

4.2 Eremomycin Versus Vancomycin

As shown by Figs. 1 and 3, eremomycin and vancomycin are very similar molecules. The eremomycin and vancomycin aglycon parts differ only by a chlorine atom on Ring #1 (Fig. 1). Vancomycin has a chlorine atom on Rings #1 and #3; eremomycin has a chlorine atom on Ring #3 only. As far as carbohydrates are concerned, vancomycin has a hydroxyl group in alpha-position of Ring #1 [22]. This hydroxyl group bears an eremosamine carbohydrate or 2,3,6-trideoxy-3-amino-3-methyl arabino hexopyranose in the eremomycin structure (Fig. 1).

Eremomycin eremosaminyl aglycon was prepared by cleaving the two eremomycin sugars on Ring #2 but keeping its eremosamine sugar on Ring #1 (Fig. 1). The properties of this eremosaminyl aglycon were compared to that of vancomycin in similar conditions [23]. Amino acids were best separated by the eremosaminyl aglycon CSP. However, the native eremomycin CSP had an enantioselectivity toward most amino acids somewhat better than that obtained with the commercialized vancomycin CSP (Chirobiotic® V). This last result was surprising since eremomycin has the same structure as vancomycin but with one more sugar unit on its aglycon basket (Figs. 1 and 3). The access of the amino acid enantiomers to this central part of the molecule should be more difficult with eremomycin than with vancomycin. Since it is not the case, it must be concluded that the eremosamine sugar close to Ring #1 helps the amino acid chiral recognition. The positively charged primary amine group on this sugar may play a role in the chiral recognition mechanisms of amino acids.

5 Polar Ionic Mode

5.1 A Waterless Mobile Phase

The polar organic mode was first termed non-aqueous reversed phase (NARP) when it was introduced in 1978 by Parris [23, 24]. NARP initially used aprotic solvents of intermediate polarity such as dichloromethane or tetrahydrofuran and the polar aprotic solvent acetonitrile. Studies of molecular interaction mechanisms showed the interest of alcohol additions to adjust the elution strength and selectivity [25]. The polar and protic methanol solvent was found to affect H-bond interactions when the polar aprotic acetonitrile solvent was more interacting through $\pi-\pi$ interactions [26]. The polar organic mode uses non-aqueous mobile phases made of 100% organic polar solvents essentially methanol and/or acetonitrile. The polar ionic mode is a variation of the polar organic mode in which the mobile phase acidity is adjusted. It is a very important mode used to separate enantiomers of ionizable molecules such as amino acids, organic acids, and amines.

5.2 Macrocyclic Glycopeptides Bear Ionisable Groups

Since macrocyclic glycopeptides have amine, phenol, and carboxylic acid functionalities, they bear a global charge that depends on the mobile phase pH. Figure 5 shows the total charge borne by the vancomycin selector as a function of pH. The absolute values are indicative; they would be valid in pure water. However, Fig. 5 clearly indicates that the vancomycin chiral selector bears a positive charge in acidic media and passing through the isoelectric point around neutral pH, it can bear up to four negative charges in basic media. In acidic media, the positive charge is due to the sum of two positively charged amine groups and a negatively charged carboxylic group that will maintain its negative charge over the whole 4–13 pH range. At neutral pH, vancomycin has its isoelectric point (pI = 7.2) when the two positive charges of the amine groups are equilibrated by the negatively charged carboxylate and a negatively ionized phenol group. As the pH value increases, the two other phenol groups progressively ionize making the vancomycin global charge negative. Between pH 10 and 12, the two positively charged amine groups lose a proton becoming a neutral primary $-NH_2$ group. Vancomycin bears a -4 negative charge at pH 13 made up by a carboxylate group and three phenate groups (Fig. 5).

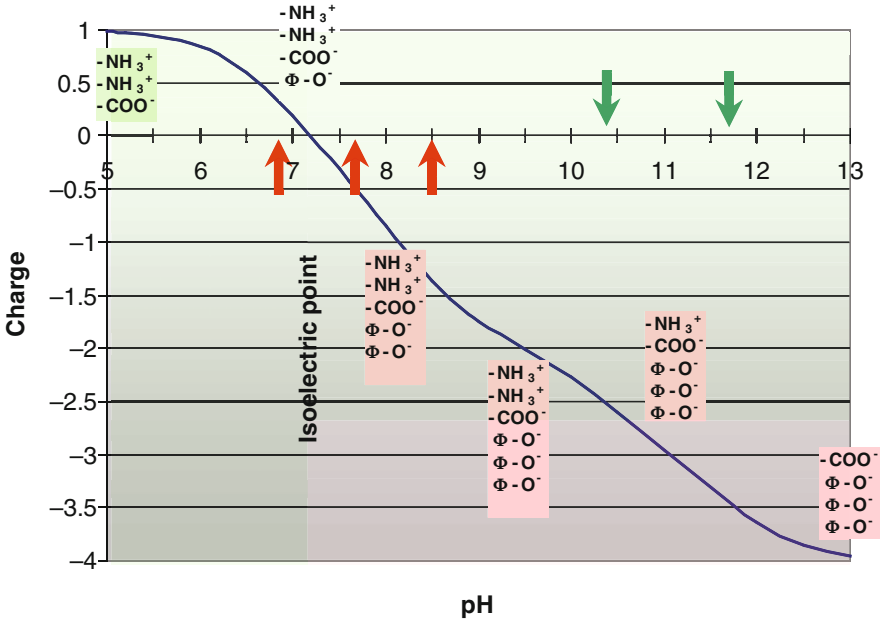


Fig. 5 Total charge of vancomycin as a function of the mobile phase pH. The ionized groups responsible for the vancomycin charge are indicated at the corresponding pH value. The *red arrows* indicate the phenol pK_as, the *green arrows* indicate the primary amines pK_as. The carboxylic acid group has a pK_a value of 2.9 outside the showed pH range

5.3 Utilizing Charge–Charge Interactions

The polar ionic mode is composed of an acid, most often acetic acid, and a base, often triethylamine, added to an alcoholic mobile phase. Table 3 lists the resulting acidity obtained mixing different proportions of acid and base. Two important points must be taken into account: (1) the molar volume of acetic acid and triethylamine being very different, a 1:1 v/v mixture is not neutral but acidic; (2) the solute dissociation constants in methanol can be very different from those in water [27]. The pH scale in pure methanol is 16.7 units wide, meaning that the neutral pH is 8.35. Acetic acid is a very weak acid in pure methanol with a pK_a as high as 8.6, 3.8 pH unit higher than the pK_a in water (Table 3) [28]. Formic acid and ammonium formate are two additives that can be used to adjust pH when working with a mass spectrometer. These additives also have different pK_a values in water and in 100% methanol.

Changing the acid/base ratio in methanol allows for an adjustment of the ionic state of the macrocyclic chiral selector (Fig. 5) and the analyte so that the best enantioselective charge–charge interactions occur between the enantiomers and the chiral selector. Figure 6 shows the significant enantioselectivity and resolution changes observed in the racemic mianserin separation on Chirobiotic® V with the vancomycin macrocyclic selector. The 100/0.05/0.15 polar ionic mode basic composition does not allow for a useful enantioseparations of the mianserin enantiomers on the negatively charged vancomycin selector (Fig. 6, top chromatogram). Increasing the mobile phase acid content produces lower pHs that allow for a baseline separation of the two enantiomers (Fig. 6, bottom chromatograms). The 100/0.1/0.1 composition is the one to choose since a baseline separation is obtained in a minimum of time. If a preparative mianserin separation is needed, it will be effective to work with a lower pH producing a higher enantioselectivity factor and consequently a higher possible column loading while maintaining baseline separation of the two enantiomers.

6 Conclusion: Similarities and Differences

The macrocyclic glycopeptide chiral selectors are now a very important class of CSPs that must be part of the column set of any laboratory involved in enantiomeric separations. The variety of functionalities found in these relatively small molecules allow for many different interactions leading to successful enantioseparations [29]. The similarities between members of this class of chiral selectors produced the "complementary separation" property [14, 30, 31]. If a partial separation of an enantiomeric pair is observed on a macrocyclic selector, say vancomycin, a baseline separation may very likely be observed on a different selector, say teicoplanin. This interesting property in method development illustrates the large number of selector–selectand possible interactions. Such complementarities are due to the

Table 3 Physicochemical parameters useful for pH estimation in polar ionic mode

Data for acetic acid and triethylamine

Compound	MW	*d* (g/mL)	Molar volume (mL/mole)	mM in a mL	pK_a in water pK_i = 14.0	pK_a in methanol pK_i = 16.7
Acetic acid AA	60	1.045	57.4	17.4	4.77	8.60
Triethylamine TEA	101.2	0.727	139.2	7.2	10.75	11.10

Polar ionic mode mobile phase compositions and pH

Mobile phase composition v/v/v	Ratio acid:base v:v	Ratio acid:base (mM:mM)	Excess acid (mM)	Salt TEAA (mM)	Excess base (mM)	pH (H_2O)	pH (methanol)
100/0.05/0.2	1:4	0.87:1.44	0	0.87	0.57	10.6	10.9
100/0.05/0.15	1:3	0.87:1.08	0	0.87	0.21	10.1	10.4
100/0.1/0.2	1:2	1.74/1.44	0.30	1.44	0	5.45	9.2
100/0.1/0.1	1:1	1.74:0.72	1.02	0.72	0	4.6	8.4
100/0.2/0.1	2:1	3.48:0.72	2.76	0.72	0	4.2	8.0
100/0.15/0.05	3:1	2.61:0.36	2.25	0.36	0	3.9	7.7
100/0.2/0.05	4:1	3.48:0.36	3.12	0.36	0	3.8	7.6

pK_i is the solvent autoprotolysis constant (pH scale magnitude). pK_a in solvent taken from [27]. pH is calculated as pK_a + log{[base]/[acid]} taking the solvent pK_a values [28]. TEAA stands for triethylammonium acetate.

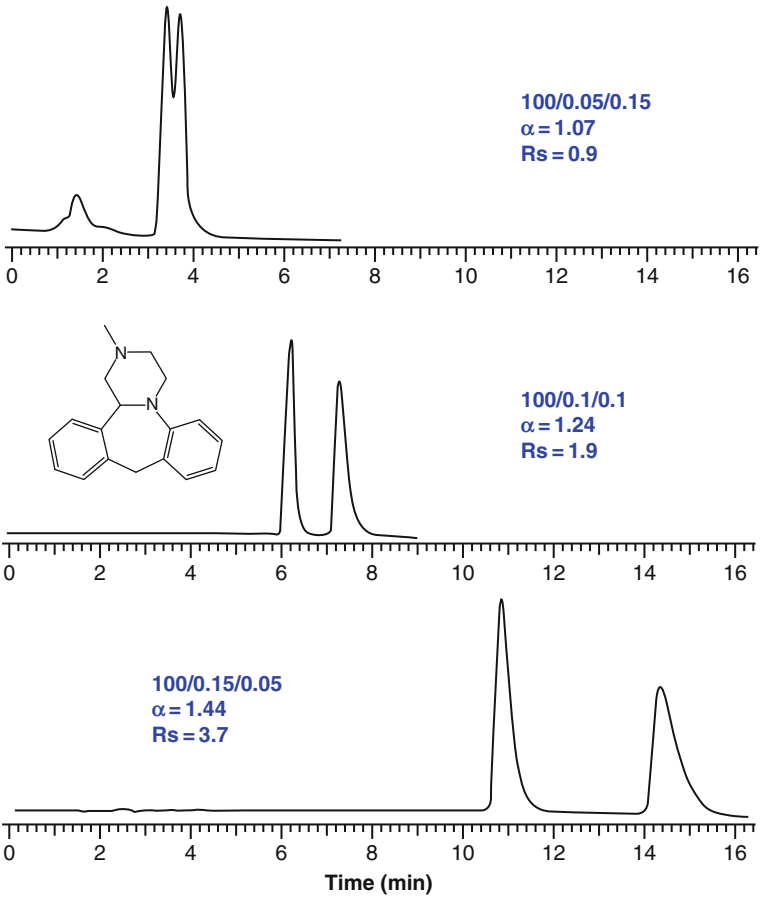


Fig. 6 Separation of mianserin on chirobiotic V in polar ionic mode. Column Chirobiotic® V, 25 cm × 4.6 mm i.d. Mobile phase: polar ionic mode with methanol, acetic acid and triethylamine in indicated v/v proportions, 1 mL/min, room temperature, UV detection 254 nm

small differences in binding sites that lead to higher enantioselectivity between macrocyclic selectors as seen in the molecular structures shown in Fig. 1.

Figure 7 shows the separation of terbutaline on the V and T CSPs with the same mobile phase. The retention, selectivity, and resolution factors are very different for the same solute eluted on the two CSPs. The key point of the example is the reversal of elution order that is observed on the polarimeter trace (Fig. 7). Such reversal signs a different selector–selectand separation mechanism with the same mobile phase at least with one of the interactions needed for enantiorecognition [29].

The most important interaction involved with macrocyclic glycopeptide selectors is the very strong charge–charge interaction that can be modulated playing with the mobile phase pH in the specially developed polar ionic mode. Such charge–charge modulation is possible in reversed-phase mode as well. A recent work showed

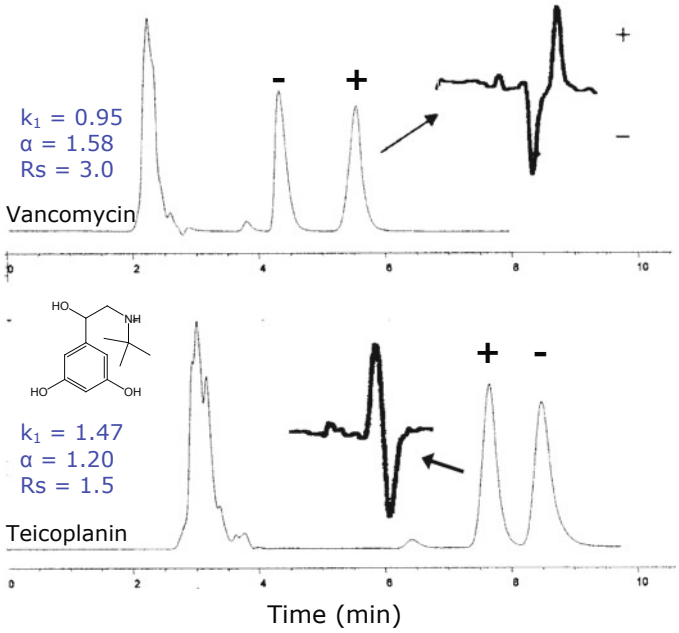


Fig. 7 Separation of terbutaline on a Chirobiotic® V (top chromatogram) and a Chirobiotic® T (bottom chromatogram) 25 cm × 4.6 mm i.d. columns. Mobile phase: polar ionic mode with methanol/ammonium formate 15 mM 1 mL/min. Continuous trace: UV detection 254 nm. Thick trace: polarimetry detection of the two terbutaline peaks. Data from [29].

that charge–charge interactions in RP mode could be modulated adding chaotropic anions such as the perchlorate ClO_4^- or hexafluorophosphate PF_6^- anions to the mobile phase [32]. The chiral discrimination of acid enantiomers on a teicoplanin CSP was significantly improved by chaotropic anions.

The chemistry of the macrocyclic linkage to the silica surface was recently improved and the Chirobiotic® V2 and T2 were proposed along with the classical V and T products. The active macrocyclic selector being the same, the newly proposed CSPs essentially separate the same families of enantiomers, the enantioselectivity factors can be higher. The loading capability of the second-generation CSPs is also higher [29, 31].

References

1. Armstrong DW (1995, 1999, 2003) Macrocyclic antibiotics as separation agents. European Patent EP0748247, 24 Aug 1995, US Patents 5964996 and 6669842, 12 Oct 1999 and 30 Dec 2003
2. Armstrong DW, Tang Y, Chen S, Zhou Y, Bagwill C, Chen JR (1994) Macrocyclic antibiotic as a new class of chiral selectors for liquid chromatography. Anal Chem 66:1473–1484

3. http://www.sigmaaldrich.com/analytical-chromatography/hplc/columns/chiral/chirobiotic.html consulted on 13 Oct 2009
4. Del Rio A, Piras P, Roussel C (2005) Data mining and enantiophore studies on chiral stationary phases used in HPLC separations. Chirality 17:S74–S83
5. Felix G, Berthod A (2007) Commercial chiral stationary phases for the separations of clinical racemic drugs. Part I: From alimentary tract and metabolism to cardiovascular system. Sep Purif Rev 36:285–481
6. Felix G, Berthod A (2008) Commercial chiral stationary phases for the separations of clinical racemic drugs. Part II: From dermatologicals to Sensory organs and various drugs. Sep Purif Rev 37:1–227
7. Felix G, Berthod A, Piras P, Roussel C (2008) Commercial chiral stationary phases for the separations of clinical racemic drugs. Part III supercritical fluid chromatographic separations. Sep Purif Rev 37:229–301
8. Staroverov SM, Kuznetsov MA, Nesterenko PN, Vasiarov GG, Katrukha GS, Fedorova GB (2006) New chiral stationary phase with macrocyclic glycopeptide antibiotic eremomycin chemically bonded to silica. J Chromatogr A 1108:263–267
9. Ilisz I, Berkecz R, Peter A (2009) Retention mechanism of HPLC enantioseparations on macrocyclic glycopeptide-based chiral stationary phases. J Chromatogr A 1216:1845–1860
10. D'Acquarica I, Gasparrini F, Misiti D, Pierini M, Villani C (2008) HPLC chiral stationary phases containing macrocyclic antibiotics: practical aspects and recognition mechanism. Adv Chromatogr 46:109–174
11. Berthod A, Liu Y, Bagwill C, Armstrong DW (1996) Facile liquid chromatography enantioseparations of native amino acids and peptides using a teicoplanin chiral stationary phase. J Chromatogr A 731:123–137
12. Sztojkov-Ivanov A, Lazar L, Fulop F, Armstrong DW, Peter A (2006) Comparison of separation efficiency of macrocyclic glycopeptide-based chiral stationary phases for the LC enantioseparation of β-amino acids. Chromatographia 64:89–94
13. Ilisz I, Berkecz R, Peter A (2006) HPLC separation of amino acid enantiomers and small peptides on macrocyclic antibiotic-based chiral stationary phases: a review. J Sep Sci 29:1305–1321
14. Astec Supelco (2004) Chirobiotic handbook, 5th edn. Supelco, Bellefonte, PA
15. Berthod A, Chen X, Kullman JP, Armstrong DW, Gasparrini F, D'Acquarica I, Villani C, Carotti A (2000) Role of the carbohydrate moieties in chiral recognition on teicoplanin-based stationary phases. Anal Chem 72:1767–1780
16. Ekborg-Ott KH, Liu Y, Armstrong DW (1998) Highly enantioselective HPLC separations using the covalently bonded macrocyclic antibiotic Ristocetin A chiral stationary phase. Chirality 10:434–483
17. Mathews CK, Van Holde KE (1996) Biochemistry, 2nd edn. Benjamin Cummings Publ, Menlo Park, CA
18. Nair UB, Chang SC, Armstrong DW, Rawjee YY, Eggleston DS, McArdle JV (1996) Elucidation of vancomycin's enantioselective binding site using its copper complex. Chirality 8:590–595
19. Berthod A, Valleix A, Tizon V, Leonce E, Caussignac C, Armstrong DW (2001) Retention and selectivity of teicoplanin chiral stationary phases after copper complexation and isotopic exchange. Anal Chem 73:5499–5508
20. Xiao TL, Rozhkov RV, Larock RC, Armstrong DW (2003) Separation of the enantiomers of substituted dihydrofurocoumarins by HPLC using macrocyclic glycopeptide chiral stationary phases. Anal Bioanal Chem 377:639–654
21. Kaplan J, Korty BD, Axelsen PH, Loll PJ (2001) The role of sugar residues in molecular recognition by vancomycin. J Med Chem 44:1837–1840
22. Kustenov MA, Nesterenko PN, Vasiyarov GG, Staroverov SM (2006) Sorbents with immobilized glycopeptide antibiotics for separating optical isomers by HPLC. Appl Biochem Microbiol 42:536–543

23. Parris NA (1978) Non-aqueous reversed-phase chromatography of glycerides using infrared detection. J Chromatogr 149:615–624
24. Parris NA (1983) Isocratic non-aqueous reversed phase liquid chromatography of carotenoids. Anal Chem 55:270–275
25. Tchapla A, Heron S, Lesellier E (1993) General view of molecular interaction mechanisms in RPLC. J Chromatogr A 656:81–112
26. Thevenon-Emeric G, Tchapla A, Martin M (1991) Role of π–π interactions in RPLC. J Chromatogr A 550:267–283
27. Bosch E, Bou P, Allermann H, Roses M (1996) Retention of ionisable compounds on HPLC. pH Scale in methanol-water and the pK and pH values of buffers. Anal Chem 68:3651–5657
28. Subirats X, Roses M, Bosch E (2007) On the effect of organic solvent composition on the pH of buffered HPLC mobile phases and the pKa of analytes. Sep Pur Rev 36:231–255
29. Beesley TE, Lee JT (2009) Method development strategy and applications update for Chirobiotic® chiral stationay phases. J Liq Chromatogr Relat Technol 32:1733–1767
30. Chen S, Liu Y, Armstrong DW, Borell JI, Martinez-Terpel B, Matallama JL (1995) Enantioresolution of Substituted 2-Methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles on macrocyclic antibiotic and cyclodextrin stationary phases. J Liq Chromatogr Relat Technol 18:1495–1507
31. Supelco Analytical (2008) Chirobiotic®, chiral by nature. Sigma-Aldrich Technical bulletin KWY T408131
32. Flieger J (2009) Improvement of chiral discrimination of acidic enantiomers on teicoplanin stationary phase by the use of chaotropic effect. J Liq Chromatogr Relat Technol 32:948–963

Vancomycin Molecular Interactions: Antibiotic and Enantioselective Mechanisms

Timothy J. Ward, Aprile Gilmore, Karen Ward, and Courtney Vowell

Contents

Abstract Medical studies established that vancomycin and other related macrocyclic antibiotics have an enhanced antimicrobial activity when they are associated as dimers. The carbohydrate units attached to the vancomycin basket have an essential role in the dimerization reaction. Covalently synthesized dimers were found active against vancomycin-resistant bacterial strains. A great similarity between antibiotic potential and enantioselectivity was established. A covalent vancomycin dimer was studied in capillary electrophoresis producing excellent chiral separation of dansyl amino acids. Balhimycin is a macrocyclic glycopeptide structurally similar to vancomycin. The small differences are, however, responsible for drastic differences in enantioselectivity in the same experimental conditions. Contributions from studies examining vancomycin's mechanism for antimicrobial activity have substantially aided our understanding of its mechanism in chiral recognition.

T.J. Ward (✉)
Department of Chemistry, Millsaps College, 1701 N. State Street, Jackson, MS 39210, USA
e-mail: wardtj@millsaps.edu

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_8,

1 Introduction

Due to the increased resistance of bacteria to most of the widely used antibiotics and the associated septicemia risk for human health [1], there is an increased urgency to fully understand the mechanism by which antibiotics inhibit bacterial proliferation and growth [2]. With regard to antimicrobial activity, the macrocyclic antibiotics are interacting with the unnatural D-alanine-D-alanine dipeptide in precursors of peptidoglycan (cell wall) of gram-positive bacteria [1]. In addition to serving as antibiotics, the macrocyclic antibiotics have been used for resolving chiral solutes since their introduction by Armstrong in 1994 [3]. The macrocyclic antibiotics interact with chiral solutes to facilitate the separation of enantiomers; a property often referred to as enantioselectivity [3]. As a better understanding of macrocyclic antibiotic activity has been achieved, the properties responsible for their antimicrobial activity also provides additional insight into the chiral recognition process between the macrocyclic antibiotic and chiral molecules.

Several factors have been suggested and studied to explain the mechanisms of chiral separations. Enantioseparations are thought to be possible through mechanisms that include hydrogen bonding, dipole stacking, $\pi-\pi$ complexation, inclusion in a hydrophobic pocket, or through an amalgamation of mechanisms [3]. It has been shown that vancomycin, an important member of the glycopeptide macrocyclic family, forms back-to-back dimers between antiparallel polypeptide backbones via hydrogen bonding as shown in Fig. 1. It thus provides in solution a more confined conformation and forms an aglycon pocket [4]. Also, dimerization of the glycopeptide vancomycin can enhance enantioselectivity, increasing the capillary electrophoresis (CE) resolution factor for dansyl valine from a resolution factor of 1.4 to a resolution factor of 3.7 [5].

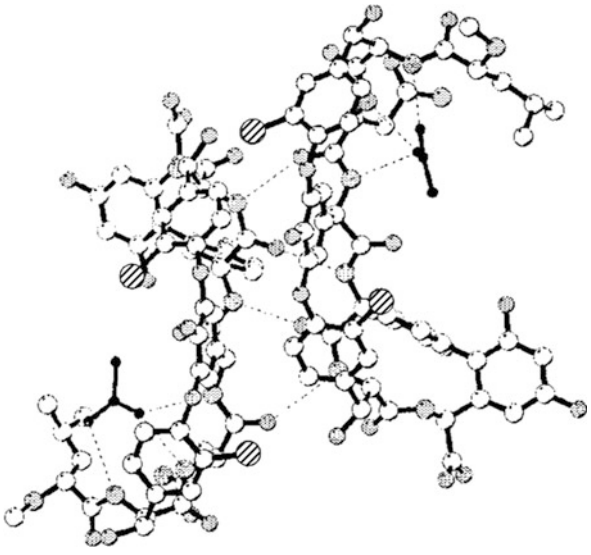


Fig. 1 The back-to-back dimer formed by aglycon monomers 1 and 2. In the aglycon molecules, carbon atoms are colored *white* and heteroatoms are colored *gray*. Two acetate ligands are shown as well and are colored *black*. *Large hatched circles* denote chlorine atoms. Hydrogen bonds are shown as *dashed lines*. Reprinted by permission of the American Chemical Society [4]

Typically the glycopeptide antibiotics have one or more sugar substituents attached to the macrocyclic rings. These sugar units were added by Mother Nature to enhance the aqueous solubility of the active aglycon core of the vancomycin molecule. It has been shown through several studies that the sugar substituent is not necessarily required to be present for antimicrobial activity or for enantioselectivity [4, 6]. This then poses the question about precisely what role the sugar substituent affords in molecular recognition. With regard to antimicrobial activity and enantioselectivity, the importance of the attached sugar moiety has been studied to determine its role in the mechanisms of both processes. The focus of this chapter will be on the vancomycin sugar substituents and the mechanistic role they play in the glycopeptides' antimicrobial activity and ability to affect chiral recognition in CE.

2 Vancomycin

2.1 Physicochemical Properties

The glycopeptide antibiotics are one of the most successful classes of chiral resolving agents used in enantioseparations. The glycopeptides discussed in this chapter include mainly vancomycin, but also ristocetin A, teicoplanin, and balhimycin. The glycopeptides are composed of three to four fused macrocyclic rings composing an aglycon basket-like structure with attached pendant sugar moieties. The sugar moieties attached to the aglycon basket are able to assume a variety of orientations by rotation [7]. Contributing to their use as chiral selectors, glycopeptide antibiotics are amphoteric (contain both acidic and basic ionizable groups), have low background absorbance in the long UV region permitting the use of 280 nm or longer ultraviolet detection, contain hydrophobic and hydrophilic groups resulting in solubility in water and aqueous buffers and partial solubility in some hydroorganic solvents, and show sufficient stability, especially as a solid [8]. The macrocyclic antibiotics are typically soluble in water and some polar aprotic solvents but are insoluble in nonpolar solvents [3, 9]. One of the principle interactions between glycopeptides and solutes in chiral separations is electrostatic, but many other intermolecular interactions, including dipole–dipole, hydrogen bonding, steric repulsion, and hydrophobic interaction often occur between the glycopeptides and chiral solutes [9].

Three of the most common glycopeptide antibiotics, vancomycin, ristocetin A, and teicoplanin, were compared and modeled by Gasper et al. Fig. 2 [9]. The three related glycopeptides show similarities in their enantioselectivity as well as differences in the structure of their aglycon "baskets" and their various substituents. The three glycopeptides used in this study have comparable absorbance spectra between pH of 4 and 8 with the strongest absorption at a wavelength less than 250 nm. All of these glycopeptide antibiotics are the product of fermentation: vancomycin by *Streptomyces orientalis*, ristocetin A by *Nocardia lurida*, teicoplanin by *Actinoplanes teichomyceticus*, and balhimycin was isolated from *Amycolatopsis* sp. fermentation broth [3, 10]. These antibiotics inhibit the cell wall synthesis, primarily affecting the gram-positive bacteria [9].

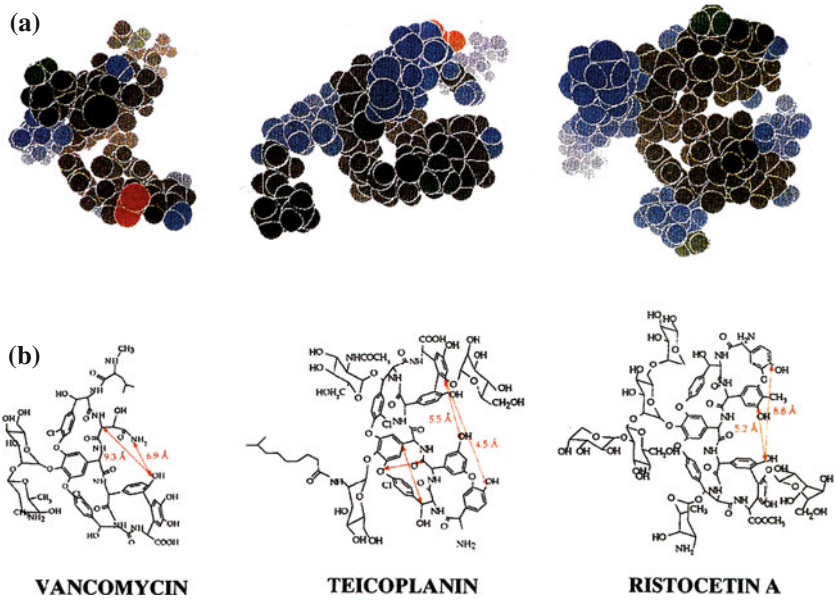


Fig. 2 Structures of the macrocyclic antibiotics vancomycin, teicoplanin, and ristocetin A showing a profile view of the aglycon "basket" using (**a**) space-filling molecular models produced through energy minimization and (**b**) stick figures. The *light gray* atoms in part **a** denote the hydrophilic moieties, while the *black* portion designates the more hydrophobic regions including the aromatic rings, connecting carbons, and amido linkages. Reprinted by permission of the American Chemical Society [9]

Vancomycin is the smallest molecule among the three glycopeptides with a molecular weight of only 1449 and 18 stereogenic centers. Vancomycin is composed of a disaccharide, with monosaccharides D-glucose and vancosamine, and three macrocyclic rings. The ionizable groups on vancomycin include a secondary amino and carboxylic acid on the aglycon portion and amino saccharide moieties [9]. Ristocetin A has a molecular weight of 2066 with 38 stereogenic centers. It has two monosaccharide groups and a pendant oligopolysaccharide as shown in Fig. 2. Included in these carbohydrates are D-arabinose, D-glucose, D-rhamnose, and D-mannose. The ionizable groups of ristocetin A include a primary amine group on the aglycon portion, amino saccharide moieties, and an esterified carboxylic acid group. Teicoplanin is a mixture of five related molecules, each differing by a molecular mass of 20 due to differences in the length and substituents of the amino saccharide's acyl side chain. Overall, teicoplanin is considered to have a molecular weight of approximately 1887 with four fused macrocyclic rings and three carbohydrate groups, two of which are D-glucosamine and D-mannose and contain 23 stereogenic centers. A unique feature of teicoplanin is a "hydrophobic tail" (acyl side chain) which makes the antibiotic surface active inducing the formation of micelles upon aggregation. Teicoplanin's ionizable groups include primary

amine groups on the aglycon portion, two amino saccharides that are N-acetylated, carboxylic acid on the aglycon, and various phenolic moieties [9].

Balhimycin is a newer glycopeptide antibiotic and is considered to be in the same class as vancomycin. Balhimycin has an identical aglycon basket as vancomycin and differs from vancomycin only in the sugar substituents and their location on the fused macrocyclic rings. Balhimycin contains two carbohydrate moieties, D-glucose and dehydrovancosamine and has a molecular weight of 1446 [10].

2.2 Mechanism of Antibiotic Activity

The glycopeptides teicoplanin and vancomycin both have been used clinically as antibiotics and act against gram-positive bacteria by binding to the D-alanine D-alanine terminal dipeptide of cell wall (peptidoglycan) precursors [1]. This prevents the peptidoglycan from maturing in gram-positive bacteria, while gram-negative bacteria remain unharmed by the activity of the antibiotic due to the outer lipopolysaccharide membrane protecting the cell wall. The increased resistance of gram-positive bacteria to the glycopeptide antibiotics is due to the bacteria's production of peptidoglycan precursors terminating in D-alanine D-lactate. This dipeptide interacts weakly with the peptide binding areas of the antibiotics [1]. It is known that bacterial resistance to the glycopeptide antibiotics is a result of the loss of hydrogen bonding between the antibiotic and the peptidoglycan target during cell wall biosynthesis. Subsequently, Loll et al. investigated the importance of hydrogen bonding within vancomycin complexes by comparing crystal structures of vancomycin complexes formed with ligands that mimic peptides of vancomycin-sensitive bacteria to vancomycin-resistant bacteria [11]. The crystal structures indicate that higher affinity ligands undergo a structural change within the vancomycin complex that is not observed with vancomycin binding to low-affinity ligands. A comparison of these interactions allowed for the determination of a minimum set of antibiotic–ligand interactions necessary to give rise to high-affinity binding. It was shown that understanding the differences in binding between vancomycin-sensitive and vancomycin-resistant bacteria with the antibiotic provides vital information to aid in the design of glycopeptide analogues capable of overcoming resistant bacteria [11].

Kerns et al. have examined a mechanism to overcome such resistance by adding a hydrophobic moiety to vancomycin, specifically the vancosamine nitrogen [1]. As it turns out, the glycopeptides with attached hydrophobic substituents are more active against vancomycin-sensitive bacteria and have activity against vancomycin-resistant strains. These modified glycopeptides work by binding to a lipid molecule of the bacterial cell membranes in a transglycosylation process. The glycopeptides without the hydrophobic substituent, like vancomycin, stop cell wall synthesis by binding primarily to an immature peptidoglycan, one that has not yet been cross-linked in a transpeptidation process. The hydrophobic constituent, which was added on the glucose C6 position of vancomycin, works by anchoring the glycopeptide to membranes to increase the closeness and binding to a membrane lipid, the ligand for transglycosylation [1].

2.3 Dimerization's Role in the Mechanism of Antimicrobial Activity

Due to the extensive use of the glycopeptides as antibiotics, the mechanism for the antimicrobial activity of this group of antibiotics has been widely studied. Though hydrogen bonding was known as a predominant interaction in antibiotic activity, the exact role of various groups attached to the antibiotic play was not clear. It has been demonstrated that dimerization plays a significant role in the mechanism of vancomycin when acting as an antibiotic agents [12, 13]. Ristocetin and balhimycin may also form dimers. Teicoplanin is an exception since it does not form homodimers in aqueous solution due to its bulky interfering side chain [14].

In 1971, Nieto and Perkins first noted that vancomycin aggregated in aqueous solution, although the characteristics of the aggregated structure was unknown for nearly two decades [15]. NMR was employed to determine that ristocetin A, ristocetin pseudoaglycon, and eremomycin glycopeptides form back-to-back homodimers held together by hydrogen bonds [12, 13], a concept later applied to members of the vancomycin group [16]. The "back" faces are those referring to the side of the molecule not directly involved in binding of the ligand. Dimerization results in a hydrophobic surface being buried between the two glycopeptides [17]. Mackay et al. determined the various dimerization constants ($K_{\text{dimerization}}$) for vancomycin and derivatives, determining vancomycin's to be 700 M^{-1} [14].

Early on it was recognized that the sugar substituent played a significant role in the glycopeptides solubility. In addition, it was known that the sugar had a role in selectively binding the D-ala D-ala precursor to bacterial cell wall. Further investigating the selectivity role, the sugar moiety began to be linked to various mechanistic aspects of glycopeptides as antibiotics. In 1994, Gerhard et al. noticed a contribution to dimerization from the sugar group, noting that removing the sugar from the molecule resulted in a decrease in the dimerization constant [18].

Dimerization was linked to antimicrobial activity in 1994 by Mackay et al., who noted that dimerization was increased when glycopeptides were in the presence of bacterial cell walls [14]. Although dimerization was not required for antibacterial activity, strong dimerization resulted in higher affinity of the antibiotic for bacterial peptidoglycan precursors [19]. Sundram and Griffin used covalently bound vancomycin dimers linked as depicted in Fig. 3 to further explore the relationship between dimerization and antimicrobial activity [20]. They found that the covalently bound dimers resulted in activity against vancomycin-resistant enterococci strains. This activity against resistant strains further attested to dimerization increasing binding to peptidoglycan precursors, since the antibiotic used in this particular case was completely and irreversibly dimerized due to the covalent link between the two glycopeptides.

Up until 1997, most of the structural studies of glycopeptide dimer formation were accomplished using NMR. That year Loll et al. obtained the first crystal structure of vancomycin. The crystal structure was analyzed with regard to recognition of a carboxylate by vancomycin, a study which ultimately suggested a cooperative mechanism connecting ligand binding and dimerization [21]. Jusuf et al. attributed

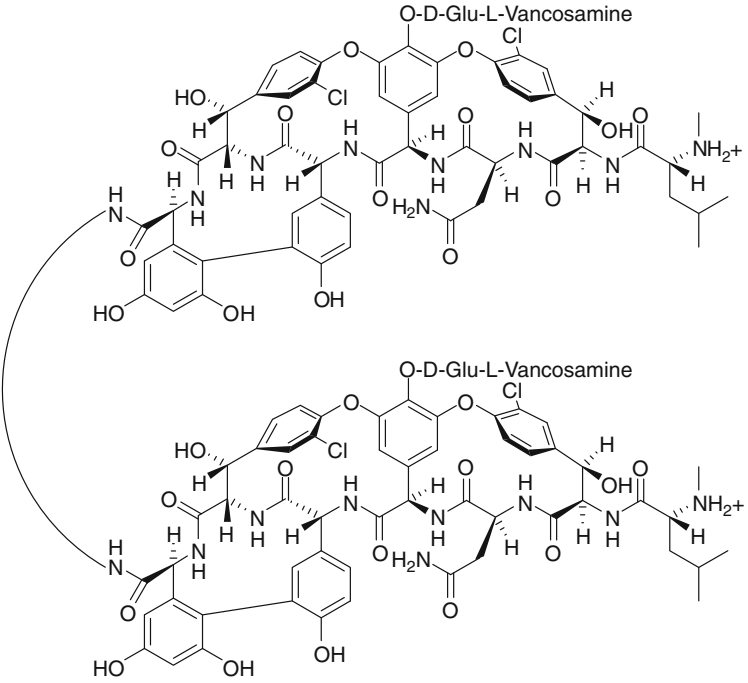


Fig. 3 Bis(vancomycin)carboxamides. The *curved line* locates the linker that could be a simple hexyl chain or a dipropyl- or diethyl-disulfide chain or a dipropylamine chain. Reprinted by permission of the American Chemical Society [20]

the cooperativity that exists between ligand binding and dimerization to an entropy-driven process [22]. After one process (ligand binding or dimerization) sacrifices the entropy of shifting vibrational activity in the antibiotic to lower amplitudes and higher frequencies, that cost does not have to be paid again by the other process because the processes of dimerization and ligand binding both cause the molecule to have the same average structure when fluctuation amplitudes are reduced [22].

Sugar substituent enhancing dimerization paired with the cooperativity between ligand binding and dimerization indicated a significant role of the sugar substituent in antimicrobial activity [23]. In 1998, Golic-Gradadolnik et al. studied differences between vancomycin aglycon and vancomycin to determine the conformational consequences of the sugar moiety and, in turn, the effect of ligand binding. The vancomycin aglycon has a hydroxyl group in place of the sugar group on the fourth residue side chain as shown in Fig. 4. The study found that the vancomycin had two families of conformations, differing in the alignment of the amide protons which take part in the hydrogen bonding with the peptidoglycan precursors and orientation of the aromatic rings in relationship to the backbone (both important in dimerization and cell wall binding). The vancomycin aglycon assumed only one conformation in solution. The two conformations for vancomycin versus one for its aglycon suggest that the sugar substituent introduces mobility into the backbone of the molecule by

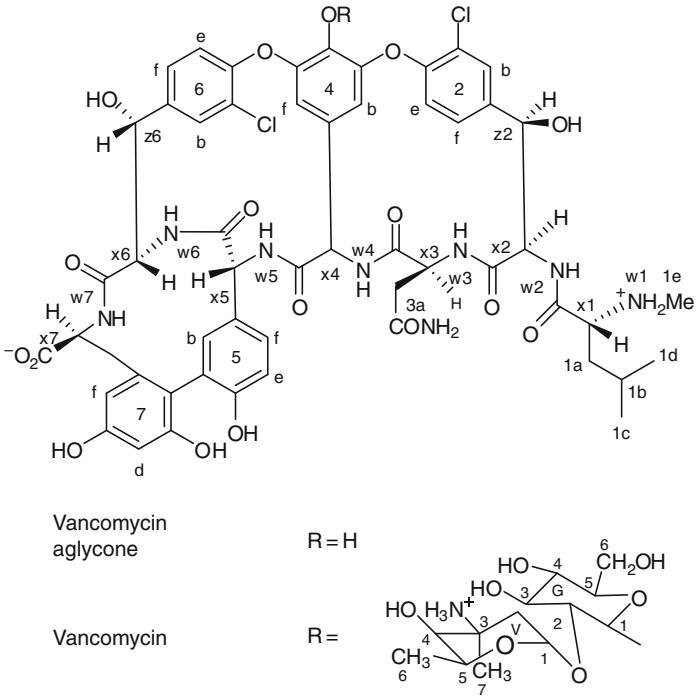


Fig. 4 Molecular structure of vancomycin with the atom nomenclature

switching between the front and back sides of the molecule. This could help assist in ligand binding by allowing needed conformational changes [23]. However, another study found opposing evidence with regard to the sugar substituent's effect on the backbone of the glycopeptide [4]. Kaplan et al. used X-ray crystal structures of vancomycin aglycon and compared it to several vancomycin dimer–ligand complexes. They found that it was the aglycon that exhibited most conformational freedom. This suggested that the sugar moiety increased ligand binding by "locking" the glycopeptides into conformations favorable for molecular recognition. In other words, the sugar group limited movement to keep the molecule in a productive structural conformation [4].

2.4 Mechanism of Chiral Separations

Since the glycopeptides were first introduced as chiral resolving agents in 1994 they have had a significant impact on the field of chiral separations. The properties of the glycopeptides that gave rise to their success as chiral selectors have already been discussed. Vancomycin has been the most widely used glycopeptide for chiral resolution and has been proven especially useful as an enantioselector in capillary

electrophoresis especially for resolving negatively charged chiral compounds [24]. No doubt its low cost has contributed to its widespread use, as it is the least expensive antibiotic at a cost around $165 per gram [25]. Ristocetin A seems to be the most effective chiral resolving agent in CE [9], although it is relatively expensive in comparison with vancomycin at a cost around $3300 per gram (or $830 for 250 mg), which has somewhat limited its popularity [25].

As previously mentioned, teicoplanin and ristocetin A's aglycon "basket" consists of four macrocyclic rings, while vancomycin and balhimycin have only three. The shape and size of this basket structure contribute to the enantioselectivity of the molecule, with the openness of the basket and degree of helical twist being the most important features. "Openness" refers to the distance between the two ends of the aglycon portion, with vancomycin being the most open and teicoplanin being the most closed. The distance between the ends of the aglycon are a result of the helical twist of the molecule, which results in a more closed structure [9].

As chiral selectors in CE, teicoplanin, ristocetin A, and vancomycin all seemed to preferentially separate anionic, acidic, or neutral compounds, with vancomycin consistently producing separations with the longest time for migration. The longer migration times in CE arise primarily from vancomycin's adsorption to the capillary wall resulting in a lower EOF (electroosmotic flow) comparable to ristocetin A and teicoplanin. This additional interaction of vancomycin with the capillary wall often produced separations that resulted in greater band broadening. Vancomycin adheres to the capillary wall to a greater extent than the other glycopeptides primarily due to its smaller size and two amine groups which gave vancomycin a net positive charge under the buffer conditions used in this study [9]. Thus, enantiomeric resolution and migration times increase, and the effective mobility becomes less negative as vancomycin concentration increases due vancomycin's adsorption onto the capillary wall and subsequent reduction in electroosmotic flow velocity. Addition of organic modifiers also decreases EOF velocity and increases analyte migration times. Optimal separations can be obtained by adjusting vancomycin concentration, run buffer pH, or the type and concentration of organic modifier [24].

When using ionizable chiral resolving agents, such as the glycopeptide antibiotics, as noted above pH can have a considerable effect on chiral separations. While it is well understood that the pH of the running buffer affects the charge and migration of the chiral solutes and chiral selectors, vancomycin, ristocetin A, and teicoplanin are limited in the choice of pH since they are stable only in solutions between pH 4 and 9. It is widely known that for optimum chiral separations, the electromigration of the chiral selector and chiral analytes should be opposite to one another [26]. The best separations with vancomycin and ristocetin A were found at a pH just below their p*I* values of 7.2 and 7.5, respectively. In contrast teicoplanin's p*I* is quite dissimilar at 3.8 (some error in measuring due to a zero electrophoretic mobility between pH 3 and 6.5) with the best separations with teicoplanin just above its p*I* value [9]. Figure 5 shows the effect of pH on the electrophoretic mobility of these glycopeptides.

At a pH where vancomycin capillary electrophoresis separations are most effective, at or below its p*I* of 7.2, vancomycin is positively charged. This indicates

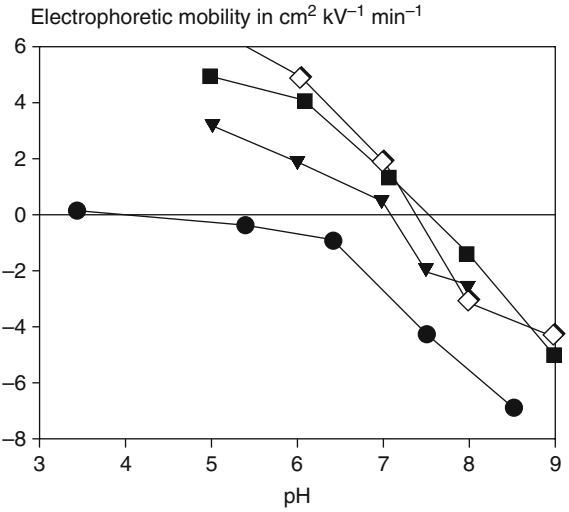


Fig. 5 Plot of the chiral selector electrophoretic mobility versus electrolyte pH. Ristocetin (■), vancomycin (▼), teicoplanin (●), and avoparcin (◇). Reprinted by permission of the American Chemical Society [9]

that ionic interactions appear to play a significant role in the mechanism of enantioselectivity when vancomycin is used as the chiral selector [6]. By complexing vancomycin with Cu^{+2}, Nair et al. showed that vancomycin's selectivity for anionic compounds is related to the presence of the amine group on the disaccharide side chain and the secondary amine on the *N*-methyl leucine side chain located on the aglycon basket [6]. As shown in Fig. 6, complexation of the secondary amine

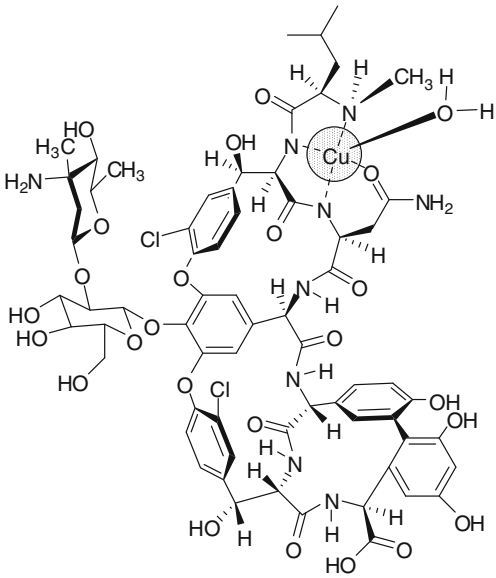


Fig. 6 Structure of the Cu–vancomycin complex. The *dotted circle* shows the copper II ion. Reprinted by permission of Wiley [6]

with copper considerably decreased the chiral recognition ability of vancomycin. Dissociation of the copper complex resulted in improved enantioselectivity, thus indicating that the secondary amine portion of the vancomycin that was complexed to the copper is necessary for the selective interactions with anionic molecules. The primary amine located on the disaccharide side chain did not associate with the copper or any other moiety under the tested conditions, indicating that the primary amine does not appear to play a major role in chiral recognition, though it may be a site for functional secondary interactions [6].

Much of what has been learned about the mechanism and binding interactions of the glycopeptides as antibiotics can also be applied to understanding the mechanism of the glycopeptides as chiral selectors. Extensive NMR studies have shown that vancomycin forms back-to-back dimers in solution [13, 18, 19]. As previously discussed, the glycopeptide antimicrobial mechanism has been examined with respect to the role the sugar moiety plays in this process as well as the glycopeptide's ability to dimerize [14, 19]. Subsequently, dimerization of vancomycin in chiral separations was examined to determine its impact on enantioselectivity.

In 2000, Sun and Olesik demonstrated that addition of vancomycin as a chiral selector in the CE mobile phase resulted in separation of Fmoc amino acids and flurbiprofen and suggested that vancomycin dimerization was related to the separation of the enantiomers [27]. In 2002, Slama et al. used HPLC to study the chiral recognition properties of vancomycin [28]. By using vancomycin as a mobile phase additive, Slama examined its ability to separate D,L-dansyl valine on silica gel stationary phases [28]. Separations of D,L-dansylvaline with the vancomycin were used to develop a retention model to describe the mechanism of the interaction between the vancomycin and chiral solutes. The model considered vancomycin existing as "free" dimers in the mobile phase and as adsorbed dimers on the stationary phase. A model equation, fit to the experimental data, demonstrated that dimerization of glycopeptides increased the enantioselectivity of the chiral solutes by a factor of approximately 3.7. In addition, increasing the concentration of vancomycin in the mobile phase further increased enantioselectivity due to the development of dimers adsorbed onto the stationary phase. Overall, it appeared that dimerization of vancomycin glycopeptides significantly increased the enantioselective characteristics of the chiral selector [28].

Later in 2003, Jourdan et al. used a different chromatographic system, with an amino stationary phase and vancomycin as the chiral resolving agent, to further study the effects of dimerization of glycopeptides on the enantioselectivity of D,L-dansylvaline and D,L-dansylserine [5]. The data were used to derive a basic interaction model that considered only vancomycin dimers forming in the mobile phase. The model effectively described the retention behavior of the chiral analytes, showing that at an eluent pH of 5.5, dimerization of glycopeptides increased enantioselectivity of the amino acid racemate by a factor of 1.5 [5].

Dimerization enhancing enantioselectivity [5, 28] paired with the sugar substituent increasing dimerization [18] suggests that the sugar substituent plays a significant role in enantioselectivity. To further elucidate the role of the sugar substituent in enantioseparations, Kang et al. studied vancomycin and balhimycin [29].

This choice was based on the fact that vancomycin and balhimycin glycopeptides have identical aglycon portions and nearly identical sugar substituents, differing only in the position of the sugar moiety as shown in Fig. 7. Although vancomycin and balhimycin have a high structural similarity, balhimycin has been observed to have an enantioselectivity 2.6 times greater than vancomycin, see Fig. 8 [29].

Blocking the sugar amino group of balhimycin resulted in a drastic decrease in enantioselectivity when compared to vancomycin which remained nearly the same, therefore, providing evidence that the amino sugar and its site of attachment play a major role in enantioseparations (Fig. 9). A dimerization mechanism was suggested to account for this phenomenon since the dimerization properties of balhimycin and

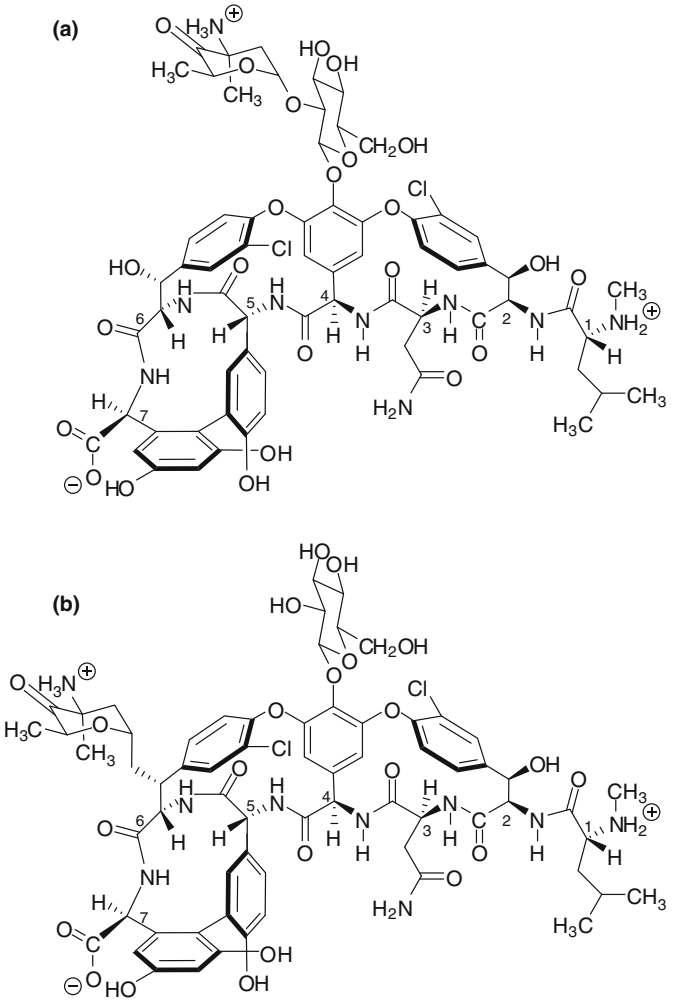


Fig. 7 Structural similarities and differences between **a**, vancomycin and **b**, balhimycin

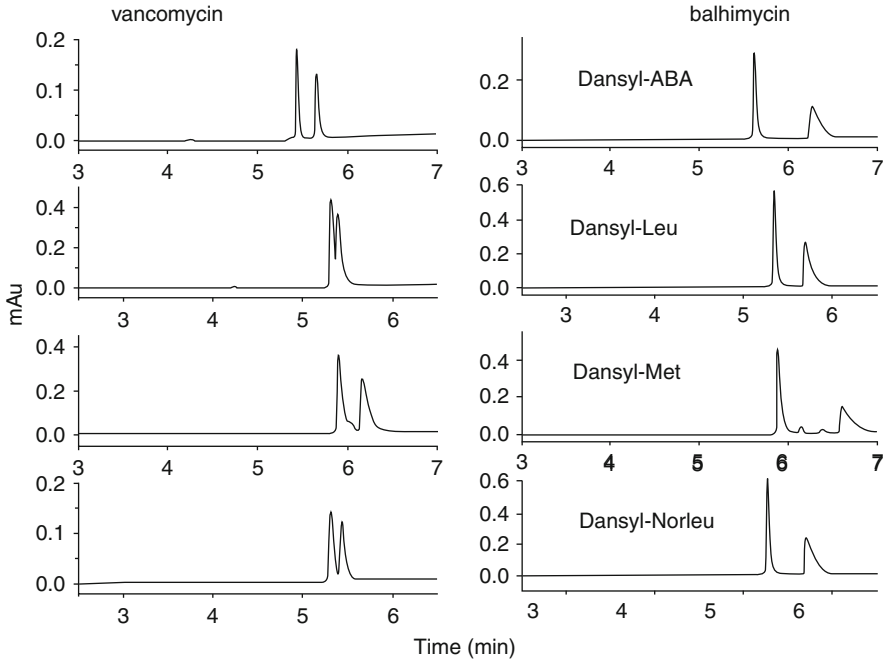


Fig. 8 Electropherograms for comparison of the enantioselectivity between balhimycin and vancomycin. 50 mM phosphate buffer pH 6.0 with 2 mM chiral selector. *Left* electropherograms: vancomycin selector; *right* electropherograms: balhimycin selector. Dansyl-ABA = dansyl-α-aminobutyric acid. Reprinted by permission of the American Chemical Society [29]

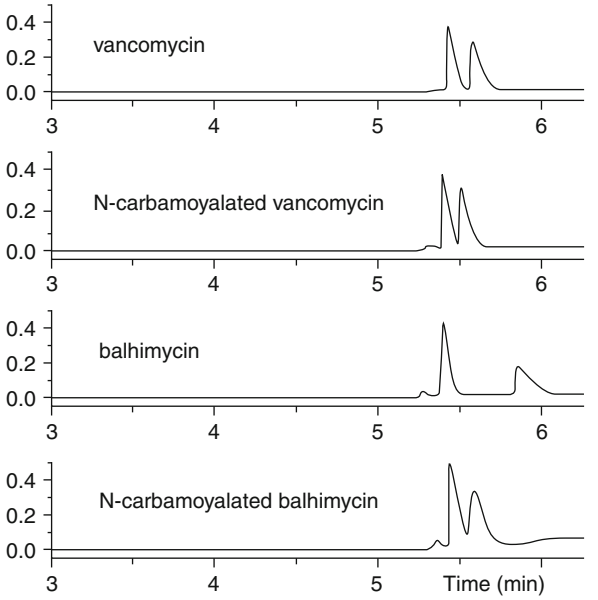


Fig. 9 Electropherograms showing the change in enantioselectivity obtained before and after blocking of the sugar amino groups. Conditions: buffer, 40 mM sodium phosphate buffer pH 6.0 with 2 mM chiral selector. Analyte: dansyl-norleucine. The selector is indicated on each electropherograms. Reprinted by permission of the American Chemical Society [29]

vancomycin are related to their enantioselectivities (the dimerization constant of vancomycin is 78 times less than that of balhimycin). The higher dimer stability leads to a more favorable conformation for chiral recognition [29].

Berthod et al. examined the role of the sugar group and chiral recognition using teicoplanin and teicoplanin aglycon stationary phases with HPLC [30]. Enantiomeric separations showed that the aglycon stationary phase resolved alpha-amino acids, thus demonstrating that the sugar moiety of teicoplanin is not required for chiral separations of most alpha-amino acid racemates. However, several non-amino acid chiral solutes were resolved better or exclusively with the teicoplanin stationary phase [30].

3 Covalently Bound Vancomycin Dimer

Previous work from Sundram and Griffin [20] used covalently bound vancomycin dimers to further explore the relationship between dimerization and antimicrobial activity and found that covalently bound dimers resulted in increased activity against vancomycin-resistant enterococci strains. Ward and workers examined the use of covalently bound dimers to further examine the effects of dimerization on enantiomeric separation. The covalent vancomycin dimer in this study was produced by bonding two vancomycin monomers together via their primary amine functional group on the sugar moiety. Blocking the primary amine should not have a negative effect on chiral separations because previous studies have shown that it is only the secondary amine on the vancomycin molecule necessary for chiral separations [6].

Covalent vancomycin dimers were obtained from Astec (Sigma-Aldrich, Bellefonte, PA), where the dimers were synthesized using a coupling reagent as follows: first 1.5 g of vancomycin was dissolved in 75 mL of anhydrous DMF at 50°C in a 250 mL round-bottomed flask. The solution was cooled to room temperature and 1 mL of triethylamine was added. In a separate beaker, 0.173 g of bis-*N*-hydroxysuccinimidyl glutarate (coupling reagent) was dissolved in 28 mL of DMF. The coupling reagent/DMF was added to the vancomycin/DMF solution dropwise while stirring. The solution was then heated to 50°C and stirred for 3 h. Afterward, the solution was again cooled to room temperature and the solvent was evaporated to dryness with a rotovap. The residual was extracted with diethyl ether (two times, using 20 mL each time), decanting the solvent. A volume of 50 mL of ACN was then added to the residual and dried in a vacuum over P_2O_5 at 50°C overnight. This compound was used without further purification.

By plotting electrophoretic mobility versus pH as shown in Fig. 10, the p*I* of the synthesized dimer was found to be approximately 4.9. The dimer was incorporated into the running buffer of 0.10 M sodium phosphate buffer (pH adjusted between 4.5 and 7.0) at a concentration of 1 mM and amino acids prepared at 1 mg/mL in methanol. Bonding through the primary amine group, loss of polarity, and an additional ionizable group resulted in poor solubility in aqueous solutions. This required the addition of organic modifiers (4% methanol and 8% DMSO) to the aqueous

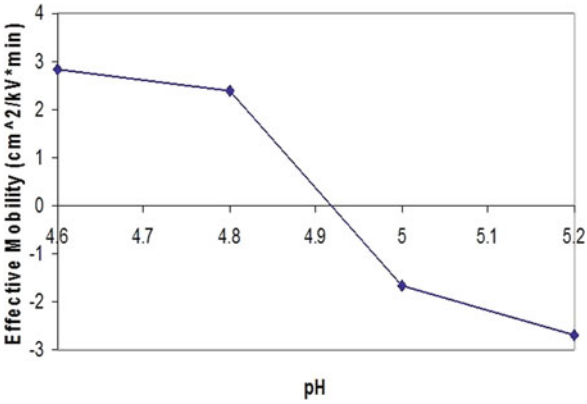


Fig. 10 EOF versus pH for vancomycin-covalent dimer

buffer to adequately solubilize the vancomycin dimer into the run buffer. Voltage was set at 10.0 kV, temperature at 22°C, detection at 230 and 250 nm, and a fused silica capillary 50 μm i.d. × 30 cm with polarity set positive to negative was used.

Table 1 shows the results of pH on the separation of the test solute dansyl-D, L-valine. The study examined the resolution of the test solute in the pH range of 4.5–7.0, and as shown in Table 1, the optimum resolution value was achieved at an approximate pH of 6, with Rs decreasing to a value of 0.2 at pH 7 and decreasing again at pH values below 5. It is not particularly surprising that the optimum pH was slightly above the p*I* of the chiral selector. Previous studies [31] showed that the charge on the chiral selector as well as the analyte plays a crucial role in enantioselectivity with ionizable compounds. At pH below the p*I* of 4.9, the carboxylic acid moiety on the test solute begins to protonate, partially removing the negative charge from the analyte. As pH is increased the solute begins to acquire a complete negative charge, but as the pH is raised the amine portion of the chiral selector begins to deprotonate and lose its positive charge, resulting in a near-complete loss of enantioselectivity at pH of 7. From previous work it has been established that electrostatic interactions play a significant role in chiral recognition with vancomycin,

Table 1 Resolution of dansyl-D,L -valine versus pH obtained by CE separation with vancomycin dimer chiral selector

pH	Resolution	EOF (min)[a]	Migration time (min)[a]
4.5	1.1	5.93	10.58
5	1.3	5.24	8.77
6	1.5	5.6	10.08
6.5	1.2	4.63	7.03
7	0.2	3.58	5.47

[a]First eluting isomer
30 cm × 50 μm i.d. fused silica capillary; voltage 10.0 kV; temperature 22°C; UV detection 250 nm.

Table 2 Enantiomeric resolutions, migration times, and apparent mobilities of racemates using a covalently bound vancomycin dimer as chiral resolving agent

Amino acid	Rs	Migration time (min)[a]	EOF (min)[a]	μ apparent[a]	μ_{EOF}	μ effective[a]
Dansyl valine	1.6	10.10	5.60	7.72	13.93	–6.21
Dansyl serine	1.3	11.17	5.83	6.98	13.38	–6.4
Dansyl methionine	1.3	12.82	5.29	6.08	14.74	–8.66
Dansyl phenylalanine	0.25	5.82	4.05	13.4	19.26	–5.86
Dansyl leucine	1.4	12.34	4.92	6.32	15.85	–9.53
Dansyl aspartic acid	0	12.50	4.52	6.24	17.26	–11.02
Suprofen	1.2	8.29	4.40	9.41	17.73	–8.32

[a]First eluting isomer
μ is the electrophoretic mobility in $mm^2\ min^{-1}\ V^{-1}$; EOF = electroosmotic flow; 30 cm × 50 μm i.d. fused silica capillary; voltage 10.0 kV; temperature 22°C; UV detection 250 nm.

and a pH value of 6 appears to provide the optimum balance between the charge on the solute and chiral selector, thus further studies were conducted at a pH value of 6.

Six dansylated amino acids with differing *R* groups were chosen as test solutes to examine resolving ability of the covalently bound vancomycin dimer versus previous separations performed with vancomycin. As shown in Table 2, the covalently bound vancomycin dimer separated to some degree all the solutes tested except for dansyl aspartic acid. These amino acid solutes have differences with respect to their polarity/hydrophobicity and size of their *R* group. There is not a discernable trend among the small set of test solutes. Dansyl phenylalanine is the bulkiest of the compounds and had the poorest resolution; this could be attributable to a size effect with respect to interacting with the dimer, but more compounds of systematically varying sizes would need to be examined to evaluate such a trend.

While the covalently bound dimer is presumed to exist solely as a dimer due to the covalent bond between the primary amines, derivatizing through the primary amine on the sugar moiety does not appear to preclude chiral recognition. However, the covalently bound vancomycin dimer does not appear to offer different or significantly better enantioselectivity as compared to free vancomycin-based separations. While it is quite clear that dimerization plays a major role in chiral separation, attempting to increase dimerization through covalent linking of vancomycin molecules does not necessarily appear to result in increased enantioselectivity. There are other considerations such as proximity of functional groups that can hydrogen bond as well as interact electrostatically with vancomycin that are essential for chiral recognition.

4 Conclusion

Contributions from studies examining vancomycin's mechanism for antimicrobial activity have substantially aided our understanding of its mechanism in chiral recognition. While studies seem to indicate that the pendant sugar moieties do not directly

participate in chiral recognition, their role in forming dimers in solution and conformational considerations are essential for chiral recognition in CE separation. This is not particularly surprising given that the primary mechanism is by binding to the D-alanine D-alanine terminal dipeptide of cell wall (peptidoglycan) precursors. Still, it is interesting that studies in the medical literature with respect to its antimicrobial mechanism have substantially aided separation scientists in our quest to explain its chiral recognition mechanism in separations. No doubt as future studies examine other antibiotics, interactions, and mechanisms as clinical agents, other fields such as separations will continue to draw from this knowledge as we explore the mechanism of separation for other antibiotics and macromolecules currently used as chiral resolving agents in CE or other techniques.

Acknowledgement We thank Erin Redman for her invaluable assistance in the preparation of this chapter.

References

1. Kerns R et al (2000) The role of hydrophobic substituents in the biological activity of glycopeptide antibiotics. J Am Chem Soc 122:12608–12609
2. O'Brien DP et al (1999) High affinity surface binding of a strongly dimerizing vancomycin-group antibiotic to a model of resistant bacteria. J Am Chem Soc 121:5259–5265
3. Armstrong DW et al (1994) Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography. Anal Chem 66:1473–1484
4. Kaplan J et al (2001) The role of sugar residues in molecular recognition by vancomycin. J Med Chem 44:1837–1840
5. Jourdan E et al (2003) Use of an amino stationary phase to study the vancomycin dimerization dependence on solute enantioselectivity. J Liq Chromatogr Relat Tech 26:1027–1039
6. Nair U et al (1996) Elucidation of vancomycin′s enantioselective finding site using its copper complex. Chirality 8:590–595
7. Ward T, Oswald T (1997) Enantioselectivity in capillary electrophoresis using the macrocyclic antibiotics. J Chromatogr A 792:309–325
8. Armstrong DW, Nair U (1997) Capillary electrophoretic enantioseparations using macrocyclic antibiotics as chiral selectors. Electrophoresis 18:2331–2342
9. Gasper M et al (1996) Comparison and modeling study of vancomycin, ristocetin A, and teicoplanin for CE enantioseparations. Anal Chem 68:2501–2514
10. Chatterjee S et al (1994) Balhimycin, a new glycopeptide antibiotic with an unusual hydrated 3-amino-4-oxoaldopyranose sugar moiety. J Org Chem 59:3480–3484
11. Loll P et al (1999) Vancomycin binding to low-affinity ligands: delineating a minimum set of interactions necessary for high-affinity binding. J Med Chem 42:4714–4719
12. Batta G et al (1991) NMR Study of eremomycin and its derivatives. Full 1H and 13C assignment, motial behaviour, dimerization and complexation with Ac-D-Ala-D-Ala. J Antibiotics 44:1208–1221
13. Waltho J, Williams DH (1989) Aspects of molecular recognition: solvent exclusion and dimerization of the antibiotic ristocetin when bound to a model bacterial cell-wall precursor. J Am Chem Soc 111:2475–2480
14. Mackay J et al (1994) Dissection of the contributions toward dimerization of glycopeptide antibiotics. J Am Chem Soc 116:4572–4580
15. Nieto A, Perkins H (1971) Physicochemical properties of vancomycin and iodovancomycin and their complexes with diacetyl-l-lysyl-d-alanyl-d-alanine. Biochem J 123: 773–787

16. Marion D, Wuthrich K (1983) Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurement of 1H-1H spin-spin coupling constants in proteins. Biochem Biophys Res Commun 113:967–974
17. Williams DH et al (1993) Toward an estimation of binding constants in aqueous solution: studies of associations of vancomycin group antibiotics. Proc Natl Acad Sci U S A 90:1172–1178
18. Gerhard U et al (1993) The role of the sugar and chlorine substituents in the dimerization of vancomycin antibiotics. J Am Chem Soc 115:232–237
19. Mackay J et al (1994) Glycopeptide antibiotic activity and the possible role of dimerization: a model for biological signaling. J Am Chem Soc 116:4581–4590
20. Sundram U, Griffin J, Nicas T (1996) Novel vancomycin dimers with activity against vancomycin-resistant enterococci. J Am Chem Soc 118:13107–13108
21. Loll P et al (1997) Simultaneous recognition of a carboxylate-containing ligand and an intramolecular surrogate ligand in the crystal structure of an asymmetric vancomycin dimer. J Am Chem Soc 119:1516–1522
22. Jusuf S, Loll P, Axelson BA (2003) Configurational entropy and cooperativity between ligand binding and dimerization in glycopeptide antibiotics. J Am Chem Soc 125:3988–3994
23. Golic-Gradadolnik S, Pristovsek K, Meirke D (1998) Vancomycin: conformational consequences of the sugar substituent. J Med Chem 41:2090–2099
24. Armstrong DW, Rundlett K, Chen J (1994) Evaluation of the macrocyclic antibiotic vancomycin as a chiral selector for capillary electrophoresis. Chirality 6:496–509
25. Aldrich Catalog (2009) Handbook of fine chemicals, Sigma-Aldrich Chemical, Milwaukee, WI
26. Vespalec R, Bocek T (2000) Chiral separations in capillary electrophoresis. Chem Rev 100:3715–3753
27. Sun Q, Olesik S (2000) Chiral separation by simultaneous use of vancomycin as stationary phase chiral selector and chiral mobile phase additive. J Chromatogr B 745:159–166
28. Slama I et al (2002) Vancomycin dimerization and chiral recognition studied by high-performance liquid chromatography. Anal Chem 74:5205–5211
29. Kang J et al (2004) A mechanistic study of enantiomeric separation with vancomycin and balhimycin as chiral selectors by capillary electrophoresis. Anal Chem 76:2387–2392
30. Berthod A et al (2000) Role of the carbohydrate moieties in chiral recognition on teicoplanin-based LC stationary phases. Anal Chem 72:1767–1780
31. Ward T, Dann C, Blaylock A (1995) Enantiomeric resolution using the macrocyclic antibiotics rifamycin B and rifamycin SV as chiral selectors for capillary electrophoresis. J Chromatogr A 715:337–344

Enantioselective Recognition in Solution: The Case of Countercurrent Chromatography

Núria Rubio and Cristina Minguillón

Contents

Abstract Countercurrent chromatography (CCC) is a preparative separation technique that works with a liquid stationary phase. Biphasic liquid systems are needed to perform a separation. Since a chiral selector is required to perform enantiomer separations, special requirements are imposed in CCC. The chiral selector (CS) must be located in the stationary phase since partitioning with the mobile phase would cause losses of the valuable chiral selector in the mobile phase. Sulfated cyclodextrins and proteins were used as polar CS located in the polar stationary phase (reversed phase mode). Apolar CSs such as *N*-dodecyl-L-proline

C. Minguillón (✉)
Faculty of Pharmacy, Laboratory of Medicinal Chemistry, University of Barcelona, Parc Científic de Barcelona, Baldiri Reixac, 10, E 08028, Barcelona, Spain
e-mail: cminguillon@pcb.ub.es

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_9, © Springer-Verlag Berlin Heidelberg 2010

3,5-dimethylanilide or Whelk-O selectors, quinine and quinidine derivatives, cellulose or amylose apolar derivatives were used located in the apolar stationary phase (normal phase mode). The special CCC displacement method called pH-zone refining was found useful in the increase of the loading capacity for cellulose, quinine, quinidine, and proline-derived selectors. Dual and multidual mode uses of CCC could produce an increase in peak separation thereby broadening the applicability of moderately enantioselective CSs.

1 Introduction

Countercurrent chromatography (CCC) is an effective separation technique based on the differences in partitioning of solutes in a sample between two non-miscible solvents or solutions which constitute the solvent system [1–4]. Therefore, the mobile and the stationary phases in the chromatographic process are liquids. The main advantages of CCC over conventional solid–liquid chromatography, but also the encountered difficulties in being more broadly known and applied, are the consequence of the lack of solid support. The usual way to maintain one of the liquids stationary, while the other is passing through, requires a more sophisticated engineering work than simply making a liquid phase percolate a solid. In CCC the "column" is substituted by a device that produces a centrifugal field used to maintain the stationary liquid phase stable. Therefore, moving parts, rotors, gears, rotating seals, are required to handle the liquid system. This has been one of the causes of the slow development of CCC. However, due to the advantageous characteristics of CCC as a purification/isolation process, the number of reports on this technique, mainly applied to natural compounds [5], has recently experienced a great increase. The more than one hundred CCC publications per year released in the last 4 years constitute an evidence of the interest that the technique arouses.

The liquid–liquid constitution of the chromatographic system makes CCC highly versatile. Uncountable combinations of readily accessible solvents can be used as solvent systems. Their composition can be finely tuned and adapted to the compounds to be separated. Only some conditions have to be fulfilled. The stationary phase has to remain stable inside the CCC instrument and emulsification has to be avoided in the working conditions. The solvent system also has to be adapted to the analytes. It is considered that distribution coefficients ranging between 0.5 and 2 are the most appropriate to obtain good selectivity/resolution values. In a solvent system where the stationary phase is the more lipophilic phase, a higher distribution coefficient will lead to long retention times and, consequently, broad peaks, while lower distribution coefficient values will result in poor access of the analytes to the stationary phase and poor selectivity. Although, systematic solvent system selection protocols have been studied [6, 7], the broad choice can make this stage a major time consuming step when developing a separation.

As in HPLC, the chromatographic CCC system can be eluted isocratically, or using a gradient that can be continuous [8] or stepwise [9]. Normal phase mode (the more lipophilic phase being the mobile phase) or reversed-phase mode can be also used [4]. The good point in CCC is that to switch from one mode to the other does not imply a change in hardware but simply to switch the position of a valve, switching the phase role. A displacer agent can be added to the mobile phase to produce the selective elution of the analytes retained in the stationary phase. In this case CCC turns into a kind of displacement chromatography. This is the principle of the pH-zone-refining mode [10] applicable to ionisable compounds. Moreover, multidimensional CCC, which involves the use of two CCC devices, has also been described [11].

Additionally, the fact that there is no solid support makes several other eluting modes possible. Among them [12], the mobile and the stationary phases can be switched during the run to avoid a too long retention time for solutes highly retained in the stationary phase. This is the so-called dual-mode [13]. The process can be repeated several times (multidual mode) [14], promoting the elution of compounds with either one or the other of the two liquid phases. The stationary phase can be extruded after a certain time of classical elution in the elution-extrusion mode [15]. Alternatively, the two phases can be flowed through the column in a real counter current mode (dual CCC, [16]) or in a cocurrent mode at different flow rates [17].

Even though certain CCC features, such as the relatively low efficiency when compared to HPLC, are considered drawbacks for this technique, they can be compensated by the characteristic selectivity and high loading capacity. Thus, the volume ratio of active stationary phase/mobile phase in the CCC column, usually around 80% in contrast to the less than 10% of HPLC columns, and the accessibility of this liquid stationary phase, lead to a much higher loading capacity with lower solvent consumption for a given amount of product processed in CCC. Moreover, problems related to adsorption of analytes on the support are avoided and the whole amount of sample injected can be recovered. These characteristics make CCC specially suited for preparative purposes whose scalability as process technique has already been demonstrated [18, 19].

Regarding to the separation of enantiomers, the preparative application of CCC can be of great interest since this technique offers the possibility to produce enantiomerically pure compounds at a lower cost compared to conventional liquid chromatography. As for other enantioselective separation techniques, in CCC, a chiral selector (CS) is needed. To produce the enantioselective environment able to separate enantiomers, it is preferably added to the liquid stationary phase. The chiral selector is designed to be confined in the stationary phase thanks to its solubility properties, while the racemate is partitioned between the two phases of the biphasic solvent system. Moreover, the CS must preserve its enantioselectivity in the biphasic liquid chosen. Encountering a combination of solvent system/CS, adapted to the analyte under study, which fulfill the specified requirements is not an easy task. This is the main reason for the few publications released in this field [20, 21].

2 Nomenclature and Instrumentation

Controversies about nomenclature have accompanied CCC since the early development of the first apparatus. The very name of the technique has been a matter of controversy as in most cases there is absolutely no countercurrent circulation: only one of the liquids flows, while the other is stationary. Moreover, a significant number of reports uses the countercurrent chromatography terms when referring to simulated moving bed (SMB), a liquid–solid continuous modality of liquid chromatography [22, 23].

Modern CCC apparatus were developed by Ito et al. in the late 1960s [24], who named the technique after the *countercurrent partition method* of Craig, based also on partitioning between two liquid phases [25]. The CCC apparatus, which constitutes just the "column" of the chromatographic system, generates a centrifugal force capable of maintaining the liquid stationary phase inside the device. The CCC "columns" have been classified into two categories considering the nature, constant or not, of the gravitational field generated by the instrument [1–4].

2.1 Hydrostatic CCC Columns

These machines have only one rotating axis that generates a constant gravitational field. The column is the adapted rotor of a centrifuge apparatus, being the rotating axis central to this rotor. The liquid phases are contained in a series of channels connected by ducts which are engraved in separated disks. A number of disks are connected to each other to constitute a cartridge (the rotor of the centrifuge) (Fig. 1). The mixture of phases takes place in the channels, while the ducts conduct the mobile phase from one channel to the next. This type of instrument is often referred to as centrifugal partition chromatograph (CPC) and needs rotary seals to connect rotating with static conductions [3].

2.2 Hydrodynamic CCC Columns

The column consists in Teflon tubing wound around a cylindrical holder which is submitted to a planetary movement obtained with two rotating axes. One or several spools or bobbins thus constructed rotate around their own axis (axis of rotation) and around a second axis (axis of revolution), located in the center of the centrifuge (Fig. 2). The two rotating movements have the same angular velocity and direction, which permits to avoid rotary seals. This motion originates inside the column a variable gravitational field with zones where the field reverses and the two liquid phases mix and zones of high field, where the two phases separate. The instruments made under these principles are commonly called countercurrent chromatographs and have been extensively described in reviews and monographs [2, 26].

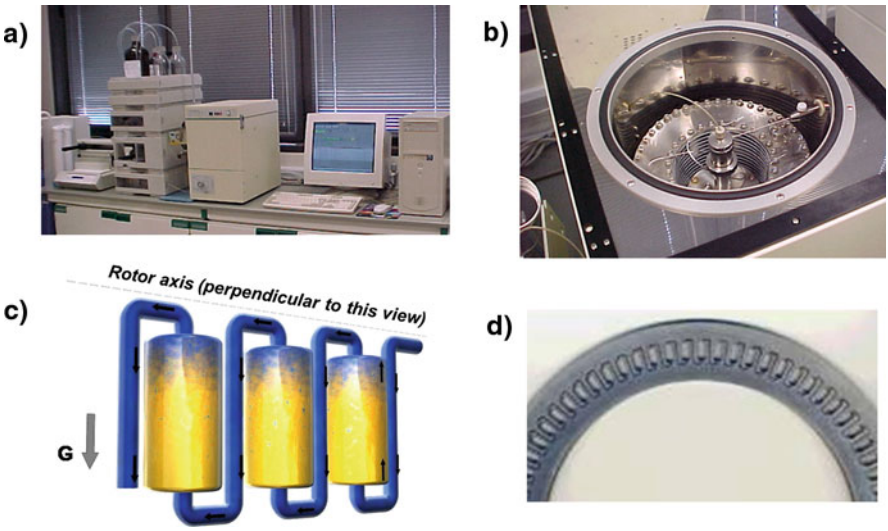


Fig. 1 Hydrostatic CCC column. (**a**) Complete chromatographic system including a CPC column. (**b**) Detail of the rotor of a CPC column. The rotor has a single vertical rotating axis. (**c**) Schematic representation of the ducts and channels engraved in the disks that constitute the rotor. (**d**) Image of one of these disks

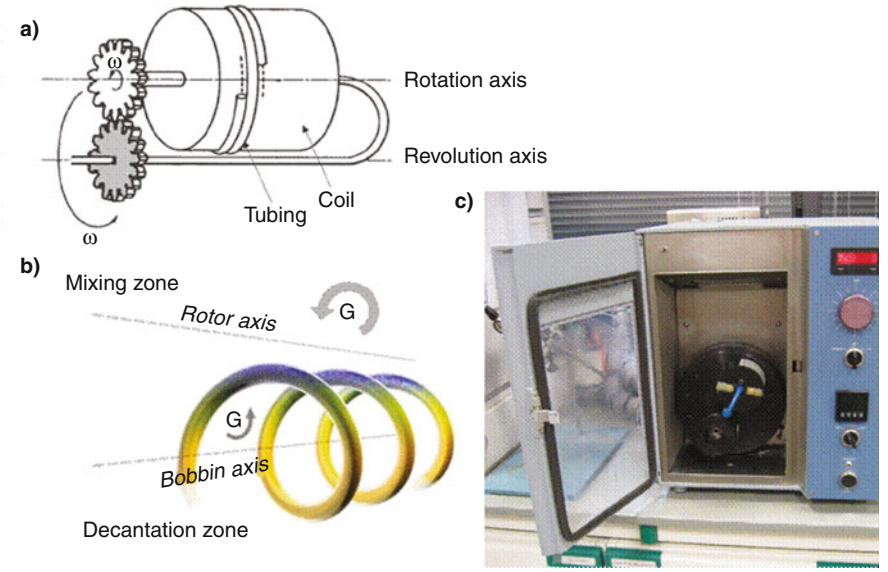


Fig. 2 Hydrodynamic CCC column. (**a**) Scheme of a J-type planetary motion multilayer coil. The two rotating axis produce a synchronous planetary motion. (**b**) Scheme showing the mixing and decantation zones within the coil. (**c**) CCC device. A single coil and a counterweight are visible on the rotor

Despite the technical differences and the underlying different fluid dynamics for the two types of systems and their consequences in the process of mixing and separation of the two liquid phases, the principle of chiral separations is the same for the two kinds of columns: the preferential association of one of the enantiomers of the chiral compound considered with the chiral selector (CS) involved in the process.

3 CCC Enantioseparation

As in HPLC, the application of CCC to enantioseparation involves the use of a chiral stationary phase. In the former, enantioseparation arises as a difference in the affinity of the *R* and *S* enantiomers, in the liquid mobile phase, for the chiral selector (CS) included in the solid support that is called a chiral stationary phase (CSP). The association constants Ka_R and Ka_S govern the position of these equilibria.

$$\begin{aligned} R + \mathrm{CSP} &\overset{Ka_R}{\rightleftharpoons} R\text{–CSP} \\ S + \mathrm{CSP} &\overset{Ka_S}{\rightleftharpoons} S\text{–CSP} \\ & Ka_R \neq Ka_S \end{aligned} \tag{1}$$

The retention factor, k', can be expressed as a function of the association constants for the two equilibria, ϕ being the phase ratio.

$$k'_R = Ka_R \cdot \phi \; ; \quad k'_S = Ka_S \cdot \phi \tag{2}$$

In HPLC the selectivity factor is defined as the ratio of retention factors for two peaks. When considering enantioseparation this is referred to as *enantioselectivity factor*, α. Assuming *S* to be the most retained enantiomer and therefore $Ka_S > Ka_R$, this factor can be expressed as

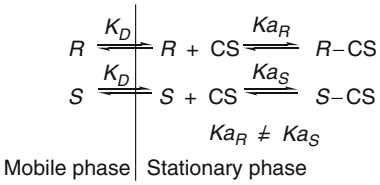
$$\alpha_{\mathrm{HPLC}} = \frac{k'_S}{k'_R} = \frac{\phi Ka_S}{\phi Ka_R} = \frac{Ka_S}{Ka_R} \tag{3}$$

According to this expression, α_{HPLC} is only dependent on the ratio of association constants of the two isomers with the CS in given conditions. Moreover, the affinity between the CS and *R* and *S* enantiomers is determined by the free energy changes between the free and the complexed states, which are also related to the association constants.

$$\begin{aligned} \Delta G^\circ_S &= \Delta H^\circ_S - T\Delta S_S = -RT \ln Ka_S \\ \Delta G^\circ_R &= \Delta H^\circ_R - T\Delta S_R = -RT \ln Ka_R \\ \Delta\Delta G^\circ &= -RT \ln \frac{Ka_S}{Ka_R} = -RT \ln \alpha_{\mathrm{HPLC}} \end{aligned} \tag{4}$$

The value of these constants depends on the nature of the CS and the enantiomers under study, which will determine the concrete interactions established between the two species – such as hydrogen bonding, π-stacking, or van der Waals interactions – and also on the environment in which the association occurs, which includes the effect of the solvation of the two species by the solvent used as a mobile phase [27].

In CCC the separation of analytes is determined by differences in distribution between the two liquid phases. While in HPLC the phase ratio, ϕ, is constant, this is not the case in CCC. Therefore k' is not a typical reference retention parameter in CCC as it is in HPLC. The distribution ratio, K_D, is preferred instead. Therefore, the selectivity factor, α_{CCC}, is defined as the ratio of distribution constants for each analyte. However, for a chiral compound in the presence of a CS, two co-existing processes affect distribution of the enantiomers: the partition equilibrium between phases and the association with the CS in the stationary phase. Assuming that the CS is confined in this later phase [20, 28]:



(5)

If the CS and its complexes do not partition to the mobile phase, the distribution ratios for each enantiomer in the presence of the CS, K_{DR}, and K_{DS}, can be expressed as:

$$K_{DS} = \frac{[S]_{SP} + [S-CS]_{SP}}{[S]_{MP}} = K_D(1 + [CS]\,Ka_S) \qquad \text{and}$$
$$K_{DR} = \frac{[R]_{SP} + [R-CS]_{SP}}{[R]_{MP}} = K_D(1 + [CS]\,Ka_R) \tag{6}$$

and considering S the most retained enantiomer, α_{CCC} can be expressed as follows:

$$\alpha_{CCC} = \frac{K_{DS}}{K_{DR}} = \frac{1 + [CS]\,Ka_S}{1 + [CS]\,Ka_R} \tag{7}$$

where $[CS]$ is the concentration of chiral selector that remains free in the stationary phase and in the presence of the enantiomers. That is, α_{CCC} is not only dependent on the ratio between association constants but also dependent on the magnitude of these constants. The latter will determine the concentration of the CS that remains free in the stationary phase. α_{CCC} increases with this concentration up to a limit (Fig. 3). The highest α_{CCC} value attainable in given chromatographic conditions is the ratio of CS/enantiomer association constants [20].

Although high stability of the complexes does not necessarily imply high enantioselectivity, the Ka_S/Ka_R ratio is likely to be greater when binding is stronger [29]. This principle, known in pharmacology as Pfeiffer's rule [30], has also been

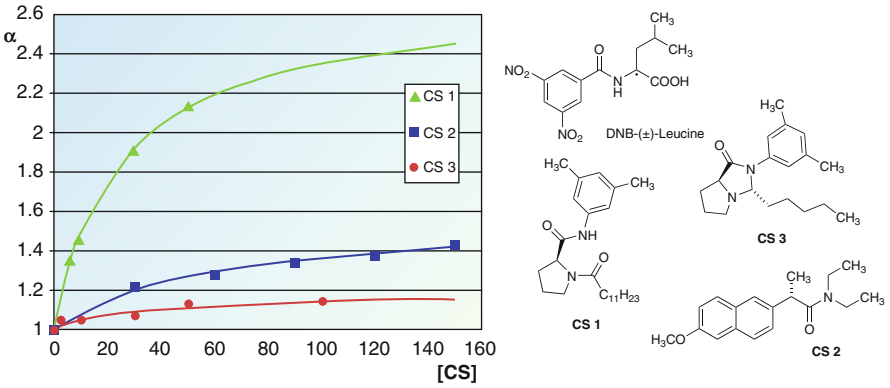


Fig. 3 Dependence of the α_{CCC} value on the free CS concentration. The experimental values were obtained in the separation of DNB-(±)-Leu enantiomers (75 mg) using the same solvent system and the three CS shown at different mM concentrations. Solvent system: MTBE-sodium phosphate buffer 50 mM pH 6.0

observed in chromatography [31]. Nevertheless, from expression (7) it can be inferred that the effect of the free CS concentration on selectivity will be more significant for high Ka_S/Ka_R ratios and low association constants.

The resolution factor (R_S) is often used in HPLC to quantify the quality of an analytical separation. A *Rs* value of 1.5 indicates that two consecutive peaks are completely resolved, while lower values imply the existence of an overlapping region between the two eluting compounds. When considering preparative separations it is not necessary to obtain absolutely resolved peaks because peak-shaving or recycling techniques are common practice to improve the purity of the recovered compounds and can also be applied to CCC. However, it is clear that identifying the main factors affecting resolution is also important. One of the broadly accepted expressions for resolution in CCC is Eq. (8) [12], where K_{D1} is the distribution ratio for the first eluting compound, N is the average peak efficiency and S_f is the ratio V_S/V_C, the liquid stationary phase volume over the column volume.

$$Rs = \frac{1}{4} S_f \sqrt{N} \frac{K_{D2} - K_{D1}}{1 - S_f \left(1 - \frac{K_{D2}+K_{D1}}{2}\right)} \tag{8}$$

From Eq. (8) some of the factors affecting resolution can be identified. First, technical factors, such as hydrodynamic parameters and centrifuge design, affect the efficiency of the column (N) [32]. Unfortunately, users have a limited access to modify these factors. Only column length, which also affects resolution [33], can be easily modified in certain devices.

Peak resolution can be improved by increasing the volume of liquid stationary phase retained in the column, S_f. This volume can be increased up to a maximum value [34] by reducing the flow rate or increasing the rotation speed of the centrifuge [35]. Moreover, even at constant S_f, sample resolution improves at higher

rotation speed and lower flow rate since mixing and consequently efficiency is enhanced [36].

Distribution of the analyte also affects efficiency. Although CCC is a technique in which the absorption of components is considered to be reduced to a minimum, and the solvent system should promote a fast exchange between phases, the introduction of a CS in the system modifies the distribution of the analyte. As a consequence the exchange of analyte between phases becomes slower inducing a lower efficiency and peak resolution decreases [37, 38]. Therefore, to counteract low *Rs* the use of highly enantioselective CSs is of interest.

4 Chiral Selectors Used in CCC Enantioseparation

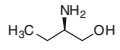
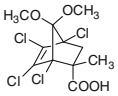
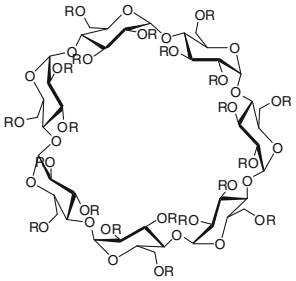
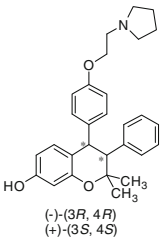
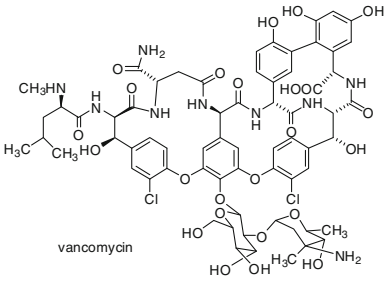
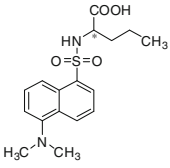
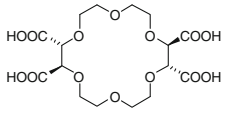
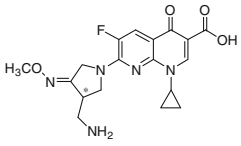
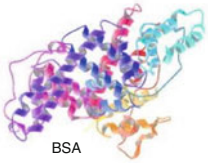
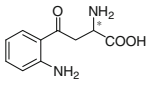
Generally, the two-phase solvent systems used in CCC enantioseparations are constituted by an organic solvent or mixture, the more lipophilic phase, and a strongly polar solution, often aqueous. Enantiorecognition occurs more often in the stationary phase, either organic or aqueous, where the CS is retained. In this chapter we will focus on the environment where the recognition occurs, since the enantiorecognition mechanism is highly dependent on this environment. Therefore, CSs have been categorized according to the nature of the stationary phase in which they are retained. Most of the CSs applied to CCC came from other separation techniques such as HPLC or capillary electrophoresis (CE). Accordingly, there is considerable knowledge available about recognition mechanisms acting in particular cases and about the experimental conditions required to make these CSs applicable to enantioseparations.

4.1 Chiral Recognition in the Polar Phase

Most of the CSs used in the aqueous phase of the CCC solvent system had been previously used in capillary electrophoresis (CE). Like CCC, CE does not involve a solid support and the CS-enantiomer association takes place in an aqueous or strongly polar environment. The information obtained in CE separations has been useful to further develop CCC methods for certain compounds and to elucidate the recognition mechanism of the selector.

The CSs used in the polar phase have some similar structural features (Table 1). Their structure includes polar functional groups, necessary for their solubilization in the aqueous solution. Nevertheless, the chiral recognition occurs in a lipophilic cavity or microenvironment which is present in a single CS molecule or originated by the complexation of two or more CS units. In these lipophilic sites, weak hydrophobic interactions are reinforced. This contributes to the stabilization of the CS-enantiomer complexes and allows for chiral discrimination.

Table 1 Enantioseparation by CCC. Chiral recognition in the polar phase

Chiral selector	Racemate	Solvent system	Ref.
 (R)-2-aminobutanol		Chloroform – methanol – phosphate buffer	[39]
 S-β–CD R: SO_3H (7-11) H (10-14)	 (-)-(3*R*, 4*R*) (+)-(3*S*, 4*S*) 7-DMO	Ethyl acetate – methanol – triethylammon–ium acetate buffer (TEAA) (10:1:9)	[41]
 vancomycin	 DNS-(±)-Nle	Toluene – aqueous solution pH 4.7	[45]
 $18C6H_4$	 (±)-gemifloxacin	1-Butanol – ethyl acetate – (Bis-Tris) acetate buffer pH 6.0	[37]
 BSA	 (±)-kynurenine	(ATPS) 10% PEG and 5% dibasic sodium phosphate in water	[50]

The first separation of enantiomers by CCC in which the enantiomer recognition takes place in the polar phase was reported by Oya and Snyder in 1986 [39]. The separation of several racemic bicyclo[2.2.1]hept-5-ene-2-carboxylic acids was obtained using (*R*)-2-aminobutanol as CS in the aqueous phase of a chloroform–methanol–phosphate buffer system. The authors employed one of the first CCC devices with a low-phase mixing efficacy and, as a consequence, the separation took more than 2 days.

The outstanding results obtained for the separation of 7-desmethylmeloxifene (7-DMO) using sulfated β–cyclodextrin (*S*-β-CD) with a resolution factor, *Rs*, greater than 100 in CE [40] encouraged Breinholt et al. to apply CCC in an attempt to overcome the inherent loading capacity limitation of CE. The complete separation of 7-DMO enantiomers using S-β-CD, as CS in the lower aqueous phase of an ethyl acetate-methanol-triethylammonium acetate buffer (TEAA) solvent system (10:1:9), was attained. The study constitutes the first successful application of modern CCC including the CS in the aqueous phase [41]. The separation was optimized in an analytical CCC device (6.5 mL of internal volume) and was further scaled up to a preparative CCC instrument with an internal volume of 320 mL. Baseline separation was achieved in both cases.

The recognition mechanism is based on the formation of a cyclodextrin-enantiomer inclusion complex, which is stabilized partially by hydrophobic and ion pairing interactions. The increasing polarity of the solvent system was shown to stabilize this complex. The influence of CS concentration in the stationary phase on enantioselectivity has also been studied. The increase of the CS/racemate molar ratio from 10- to 160-fold resulted in an improvement of enantioselectivity and resolution.

Vancomycin is one of the macrocyclic antibiotics introduced by Armstrong and co-workers as broad-application CSs for analytical separation techniques. Before being tested in CCC, vancomycin had previously shown to be applicable in aqueous solution in other techniques such as CE [42], thin-layer chromatography (TLC) [43], and in HPLC [44]. In CCC, the separation of dansyl-norleucine enantiomers was achieved using vancomycin (140 mg/mL) as CS in a biphasic solvent system constituted by a mixture of toluene-aqueous buffer solution at pH 4.7 [45]. The separation was first performed in a CCC device (internal volume 13 mL) and then in a CPC device (90 mL) where the resolution of 50 mg of the racemate was achieved in a single run. The dual eluting mode was applied to shorten run time due to the strong retention of the second eluting enantiomer (DNS-D-Nle). In this mode mobile and stationary phases are switched after the complete elution of the first enantiomer. As a consequence, the second enantiomer elutes together with the CS (Fig. 4).

Some insights of the enantiomer recognition mechanism of vancomycin were previously studied in test tubes where the partition of the product is reproduced. These studies concluded that the recognition promoted by vancomycin is highly dependent on the solvent mixture where the association CS-enantiomers takes place, since slight variations in the composition of this mixture leads to a decrease or even the suppression of the enantiorecognition of the DNS-(±)-Nle enantiomers. The use of an aromatic solvent, such as toluene, as a component of the solvent system

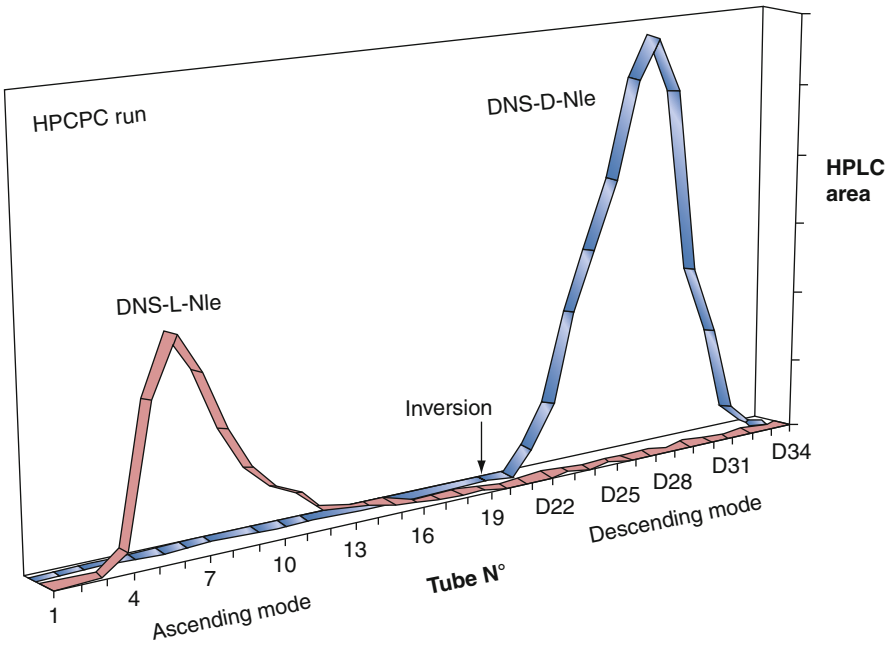


Fig. 4 Separation of DNS-(±)-Nleu using vancomycin as CS. Dual-mode elution was applied to shorten run time due to the strong retention of the second eluting enantiomer. DNS L-Nleu was eluted in ascending mode and DNS-D-Nleu was eluted in descending mode. Solvent system: toluene–vancomycin in aqueous buffer adjusted at pH 4.7. Adapted from [45]

was found to be essential. A non-aromatic solvent used in place of toluene reduced enantiorecognition significantly. Even more, the addition of some co-solvents such as acetonitrile to the solvent system also produced a drop of enantioselectivity. This observation indicates that enantiorecognition for vancomycin in solution follow different rules than when it is bonded to silica gel in a CSP for HPLC, since acetonitrile is one of the best solvents when using this kind of CSPs.

An inverse relationship between enantioselectivity and temperature was observed while enantioseparation was only detected within the pH 4.0−6.0 range for DNS-(±)-Nle. However, in spite of the narrow range of conditions in which vancomycin can be used as a CS in CCC, the major drawbacks for its extensive use are the high molecular weight combined with the involvement of more than one molecule of vancomycin in the solute–CS complex which compromises loading capacity.

One of the most recently reported CCC chiral separations where chiral recognition occurs in the aqueous phase involves the separation of gemifloxacin enantiomers using (+)-(18-crown-6)-tetracarboxylic acid ($18C_6H_4$) as CS [37]. This CS has been previously used in CE to resolve the enantiomers of chiral primary amines. The macrocyclic polyether ring in $18C_6H_4$ structure forms stable inclusion complexes with protonated primary amines. This interaction is the basis of the chiral recognition mechanism.

Following the information obtained from CE separations, Chung et al applied $18C_6H_4$ to CCC, using a CCC analytical device with a toroidal coil column (internal volume 7.4 mL). The baseline resolution of small amounts of gemifloxacin was achieved in a biphasic solvent system consisting of 1-butanol – ethyl acetate – bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris) acetate buffer. The presence of multiple ionisable groups in the derivatized crown ether CS and in the racemate makes pH an important factor in this separation. A pH value around 6.0 was determined to be optimal. The influence of CS concentration on enantioselectivity was also studied.

Bovine serum albumin (BSA) as high molecular weight CS was first used in HPLC enantioseparations [46, 47]. Although its application domain is considerably wide, the low loading capacity of the resulting chromatographic systems restricts its applicability to analytical applications. Nevertheless, CCC was considered to extend the application of BSA to preparative purposes by using aqueous biphasic solvent systems (aqueous two-phase systems, ATPS). ATPSs consist in mixtures of two different polymers, or a polymer and a salt, in water. Unfortunately, attempts to separate DL-tryptophan [48] and ofloxacin [49] by counter-current distribution resulted in poor resolutions. The high viscosity of the liquid phases, which hinders mass transfer, and the use of a CCC device of the first generation were considered the causes of the low efficiency observed.

In spite of these results Shinomiya et al. undertook the application of BSA to CCC using a cross-axis hydrodynamic column equipped with a coil of 28 mL internal volume for the separation of kynurenine enantiomers [50]. The solvent system used consisted of 10% polyethylene glycol 2000 (PEG), 5% dibasic sodium phosphate in water, where 6% of BSA was solubilized. A partial separation of 2.5 mg of racemic kynurenine was reported. Besides the viscosity of the solvent mixture, the main drawback in the use of BSA as CS is the high molecular weight of the protein; even more when considering that it is likely that a single molecule only contains a single recognition site for the analyte. A particularity of this separation was the fact that BSA partitioned the mobile phase. Beside the continuous loss of CS, this produced the elution of the enantiomer that had a higher affinity for the CS, L-kynurenine, at a shorter time.

4.2 Chiral Recognition in the Organic Phase

Most of the CSs included in this section have been developed as CSPs used in HPLC. In this case, the chiral recognition takes place in the apolar environment provided by the organic phase. In contrast to what is observed in aqueous phase, in an apolar environment polar interactions are reinforced. The relevance of these interactions is evidenced by the reduction in enantioselectivity observed when CSPs containing this kind of CSs are used in HPLC under reversed-phase conditions [51, 52]. To this respect, it has to be considered that the organic lipophilic phase of an organic-aqueous biphasic solvent system contains variable amounts of solubilized

water. This water contained in the organic phase of the solvent system may act by screening the hydrogen bonding ability of the CS, thereby preventing H-bond association with the analyte.

As mentioned, enantioselectivity as well as loading capacity are dependent on the concentration of the CS in the stationary phase. In order to dissolve the CS appropriately, the lipophilic stationary phase must have a certain degree of polarity. However, solubility is attained by solvating the polar groups of the CS in the lipophilic environment, a process that may affect interaction with analytes and therefore enantioselectivity [53]. In order to keep polarity of the organic phase low while allowing the solubilization of the CS, a long hydrophobic chain is often introduced in the structure of the selector. However, the simultaneous partition of the analytes, organic compounds of medium/low polarity, has to be ensured. In the case of ionisable analytes, partition can be controlled by adjusting the pH of the mobile phase or by introducing a suitable counter ion. In spite of these opposing arguments, a number of successful CCC enantioseparations have been attained using diverse CSs in the organic phase of a biphasic organic-aqueous solvent system (Table 2).

In 1982 the enantioseparation of racemic norephedrine in a 1,2-dichloroethane-water solvent system using the highly lipophilic (*R,R*)-di-5-nonyl tartrate as CS was reported [54]. This CS had been previously used in liquid–liquid partition experiments [55]. Hexafluorophosphate was used as a chaotropic (apolar) anion to facilitate the partition of the positively ionisable racemate.

L-Proline derivatives have profusely been used with enantioselective purposes including synthetic or separation processes. The first application of an L-proline derivative as CS in CCC was reported in 1984 by Takeuchi et al. [56]. *N*-Dodecyl-L-proline (C_{12}-Pro) was used as a ligand-exchange CS in the separation of several racemic amino acids. The recognition mechanism studied in depth in HPLC involves the use of a mobile aqueous phase containing Cu(II) ions. C_{12}-Pro provided successful CCC separations when used in the organic phase of a butanol-aqueous Cu(II) containing solution solvent system. Among, the baseline separation of (±)-isoleucine was attained.

N-Acyl-L-proline-3,5-dimethylanilide was first introduced by Pirkle et al. bonded to silica gel as CSP for HPLC [57]. This CSP exhibits a remarkable enantioselectivity for analytes containing a π-acceptor group. The π−π interaction was considered one of the major interactions between CS and analytes. In 1994, Oliveros et al. used a soluble form of this CS, *N*-dodecyl-L-proline-3,5-dimethylanilide, as a CS for CPC. The complete resolution of *N*-(3,5-dinitrobenzoyl)-*tert*-butyl-(±)-valinamide and *N*-(3,5-dinitrobenzoyl)-*tert*-butyl-(±)-leucinamide was achieved. The solvent system was constituted by a quaternary mixture of heptane-ethyl acetate–methanol–water in a ratio (3:1:3:1, v/v). This was the first complete resolution reported of non-ionisable racemates by CPC. However, due to the similar polarity of the CS and the analytes, a slight leak of the CS to the mobile phase could not be prevented [58].

The use of acidic racemates and the modification of the solvent system allowed Ito et al. [59] to avoid the problem. Using a mixture of hexane–ethyl acetate–methanol–10 mM hydrochloric acid (8:2:5:5, v/v) as solvent system, the

Table 2 Enantioseparation by CCC. Chiral recognition in the organic phase

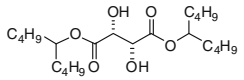
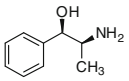
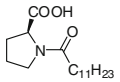
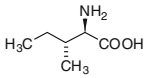
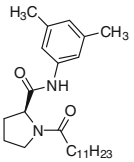
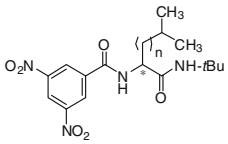
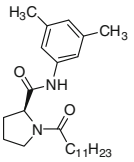
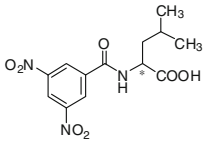
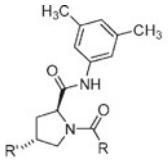
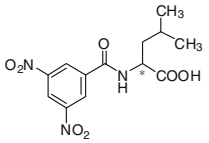
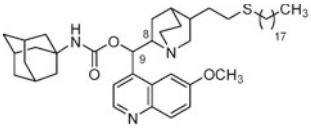
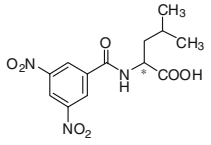
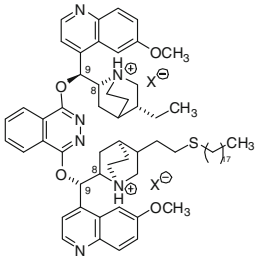
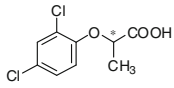
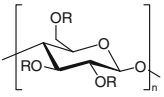
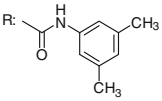
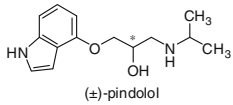
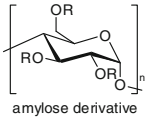
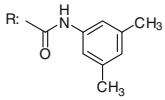
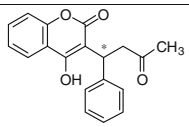
Chiral selector	Racemate	Solvent system	Ref.
 (R,R)-di-5-nonyl tartrate	 (±)-norephedrine	1,2-Dichloroethane – water	[54]
 C_{12}-Pro	 (±)-isoleucine	Butanol – Cu(II) aqueous solution	[56]
 N-dodecyl-L-proline-3,5-dimethylanilide	 n:0, valine derivative n:1, leucine derivative	Heptane – ethyl acetate – methanol – water (3:1:3:1, v/v)	[58]
 N-dodecyl-L-proline-3,5-dimethylanilide	 DNB-(±)-Leu	Hexane – ethyl acetate – methanol – 10mM hydrochloric acid (8:2:5:5, v/v)	[59]
 R: $C_{11}H_{23}$ or 3,5-$Me_2C_6H_3$ R': H or OH	 DNB-(±)-Leu	MTBE – sodium phosphate buffer 0.1 M (pH 6.0, 6.7) MTBE – ammonium acetate buffer 0.1 M (pH 6.0, 6.7)	[53]
 (8S,9R), Adamantylcarbamoyl QN-CS1 (8R,9S), Adamantylcarbamoyl QD-CS2	 DNB-(±)-Leu	MIBK – acetone – ammonium acetate buffer 0.1 M pH 8.0 (2:1:2)	[64]

Table 2 (continued)

Chiral selector	Racemate	Solvent system	Ref.
 (DHQD)$_2$PHAL	 (±)-dichlorprop	MTBE – sodium phosphate buffer 100 mM pH 8.0	[65]
 cellulose derivative		 (±)-pindolol	MIBK – sodium phosphate buffer 50 mM pH 7.0	[38]
 amylose derivative		 (±)-warfarin	MTBE – sodium phosphate buffer 50 mM pH 9.0 (v/v)	[38]

enantioselectivity of the above mentioned CS was tested in the separation of several *N*-(3,5-dinitrobenzoyl)-(±)-amino acids. The influence of the CS concentration on the loading capacity was considered. The separation of 1 g of racemic DNB-(±)-Leu was successfully achieved when using a 60 mM CS concentration in a CCC device of 330 mL. Taking this separation as an example, some parameters related to the mechanism of chiral separation were established [28]. These studies reveal the influence of the amount of CS on enantioselectivity and resolution. Also the inverse relationship between enantioselectivity and hydrophobicity of the solvent system was observed.

A chromatographic method for the calculation of the association constants CS-enantiomers, $Ka_{R/S}$, assuming 1:1 complexes, was described by the same authors [60]. Some modifications on the anilide ring of the CS had been introduced to further elucidate the mechanism of enantiorecognition. The studies corroborate the key role of the π–π-interaction between the π-donor group of the CS and the π-acceptor group of the analyte in enantiorecognition.

In an attempt to improve enantioselectivity several L-proline and (4*R*)-hydroxy-L-proline derivatives, structurally related to *N*-dodecyl-L-proline-3,5-dimethylanilide, were synthesized. The resulting CSs were tested using a simple binary biphasic solvent system [53]. The introduction of a second π-donor group

on the structure of CS was found to have a beneficial effect on enantioseparation. Unfortunately the solubility in certain solvents of this CS was limited, which restricted its application for preparative purposes. Separations in a less polar environment (MTBE) afforded better resolution than when more polar solvents (MIBK) were used. The pH of the aqueous buffer was adjusted to promote partition of the racemates. However, strong effects on the separation were observed not only by changing pH but also when modifying buffer nature (Fig. 5).

Chinchona alkaloid derivatives are another class of natural selectors used as basic chiral skeletons for enantioselective purposes. When covalently bonded to silica gel, as CSP for HPLC, they showed good enantioselectivity for racemic acidic compounds acting through an anion-exchange mechanism in aqueous-alcoholic mobile phases [61]. They were used in solution as CS in liquid–liquid extraction experiments [62] or in CE [63]. All this applications provide valuable information when considering the use of chinchona derivatives as CS in CCC. The mechanism of enantiorecognition involves the ion pair formation between the ionized basic nitrogen atom in the CS and carboxylic group of the analyte. Other interactions, such as hydrogen bonding or π-staking, condition the discrimination of enantiomers.

In 2002 the application of quinine and quinidine derivatives as CS in CCC was first reported [64]. Considering the significance of the positively charged nitrogen atom on recognition, the CS was provided with a long hydrocarbon chain to

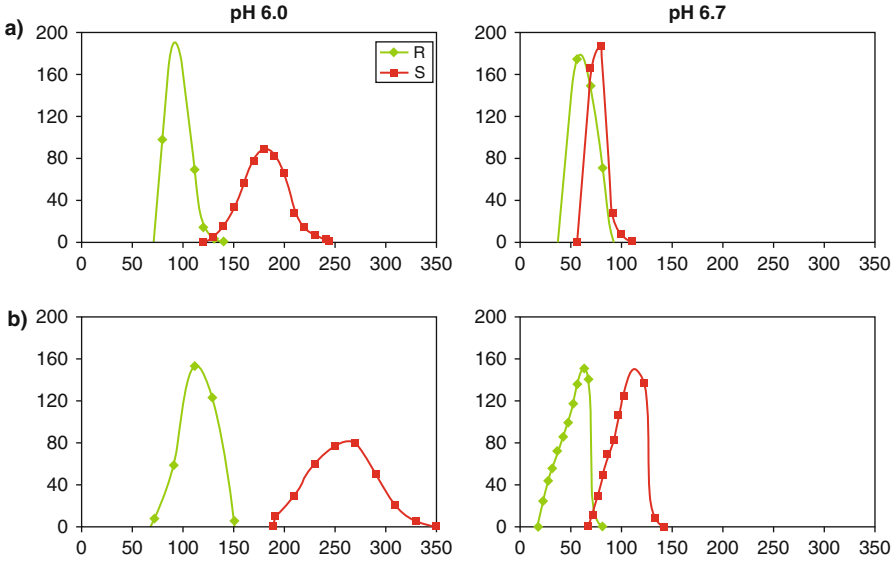


Fig. 5 Elution profiles corresponding to the separation of 75 mg (0.23 mmol) of DNB-(±)-Leu using *N*-dodecyl-L-proline-3,5-dimethylanilide as CS (30 mM) [53]. Influence of the pH and the nature of the buffer used as a mobile phase on enantioselectivity. (**a**) MTBE-sodium phosphate buffer 0.1 M. (**b**) MTBE-ammonium acetate buffer 0.1 M. Vertical axis, arbitrary absorbance units; horizontal axis, time (min)

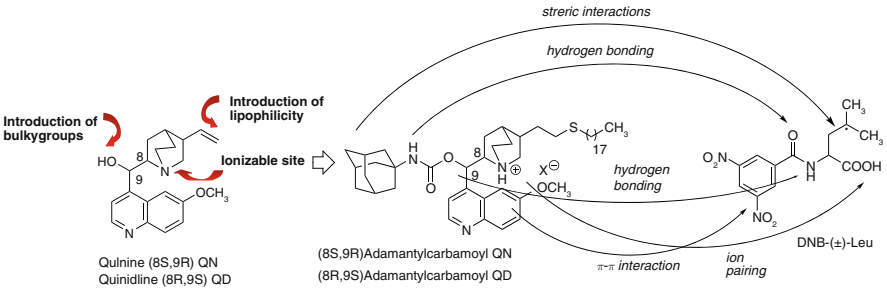


Fig. 6 Chemical structure of quinine (QN)- and quinidine (QD)-CSs used in ref. [64]. The structural modifications introduced on the alkaloids structure and the recognition model described for DNB-(±)-Leu are indicated

avoid partitioning to the aqueous mobile phase even in conditions where the CS was ionized. Also, an adamantylcarbamoyl group was introduced to enhance enantioselectivity, since it was reported that bulky groups in the 9 position of the alkaloid skeleton improved chiral recognition (Fig. 6). Liquid–liquid extraction tests were performed in order to screen analytical conditions such as the influence of the solvent system composition. Several acidic racemates, among them some amino acid derivatives and some pesticides such as dichlorprop, were tested.

All experiments were performed using a CPC hydrostatic column with 10 mM CS concentration in the upper organic layer of a ternary solvent system composed of a mixture of MIBK – acetone − ammonium acetate buffer 0.1 M pH 8.0. Baseline separations of DNB-(±)-Leu and DNZ-(±)-NPG, among others, were achieved. After the optimization of conditions (Fig. 7), the best separations obtained were scaled up in order to determine loading capacity for the analyte. This resulted to be in the order of the equimolar amount than the CS involved in the separation.

Two years later a report from the same group described a comparative study CPC/HPLC for the separation of the herbicidal agent dichlorprop (2-(2,4-dichlorphenoxy)propionic acid). A dimer compound derived from bis-1,4-(dihydroquinidinyl)phtalazine ($(DHQD)_2PHAL$) was used as CS [65]. This compound showed an extraordinary degree of enantioselectivity toward dichlorprop ($\alpha = 15.3$) in HPLC when bonded to silica gel. This result made it a promising CS candidate for CCC. A 10 mM concentration of CS in a MTBE-sodium phosphate pH 8.0 solvent system were determined to be adequate conditions to perform the separation in previous test-tube partition experiments and the baseline separation of dichlorprop enantiomers was attained. The loading capacity of the system was determined. Even when the molar ratio CS/racemate was reduced from the initial value of 2 to equimolar 1, the baseline separation was maintained. The preparative separation was compared to HPLC concluding that higher loading capacity with lower solvent consumption was attained for CPC. Nevertheless, CPC gave a lower specific productivity than HPLC in the experimental conditions used.

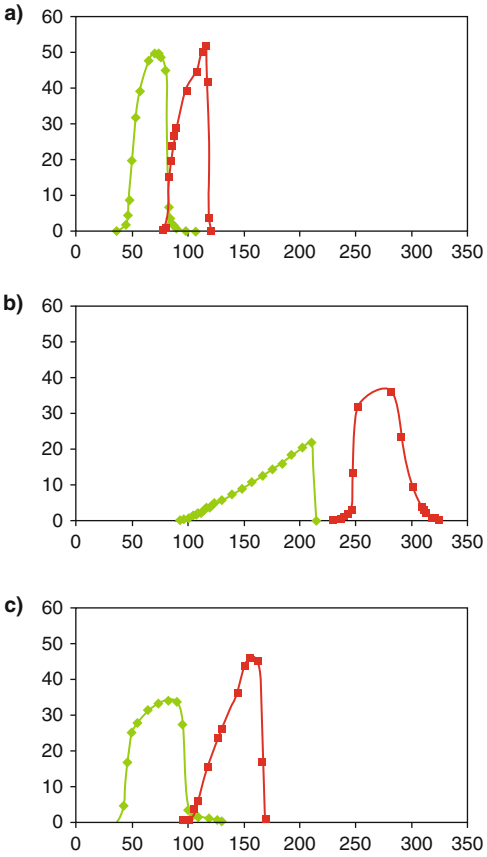


Fig. 7 Elution profiles corresponding to the separation of 150 mg (0.46 mmol) of DNB (±) Leu using 10 mM QN-derived CS [64]. The optimization of elution conditions is indicated. Solvent system: (**a**) MIBK ammonium acetate buffer 0.1 M pH 8.0; (**b**) MIBK ammonium acetate buffer 0.1 M pH 6.9; (**c**) MIBK-acetone-ammonium acetate buffer 0.1 M pH 8.0 (2:1:2, v/v). In all cases the first eluting isomer corresponds to the *R* enantiomer. Vertical axis, arbitrary absorbance units; horizontal axis, time (min)

Polysaccharide derived CSPs are among the most used for HPLC enantioseparation due to their broad application domain, either in normal [66] or reversed-phase [52, 67] conditions, and their high loadability [66]. These features make them CSPs of first choice for LC and CCC preparative purposes. The first application of polysaccharide derivatives to CCC was reported in 2006 [38]. Cellulose and amylose 3,5-dimethylphenylcarbamates, the CS contained in the well-know Chiralcel® OD and Chiralpak® AD CSPs, were used. Although solubility of these CSs limits the use of coated polysaccharide-derivatives-containing CSPs in HPLC, their macromolecular structure prevents the formation of real solutions with solvents. Instead, these compounds swell and originate colloid solutions or suspensions of enough stability to be used in CCC.

A partial separation of racemic pindolol was achieved with cellulose 3,5-dimethylphenylcarbamate (7.5 mg/mL) in a MIBK-phosphate buffer pH 7.0 solvent system. A slightly better separation was obtained for warfarin enantiomers using amylose 3,5-dimethylphenylcarbamate (7.6 mg/mL) in a MTBE-phosphate buffer pH 9.0. However, racemates such as propranolol, structurally close to pindolol, naproxen, and DNB-(±)-Leu, which were easily resolved in HPLC using

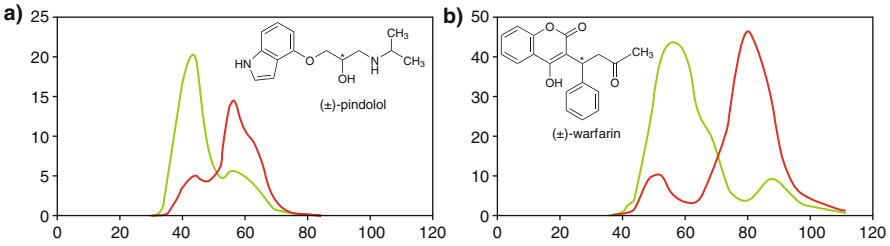


Fig. 8 Elution profiles corresponding to the separation of (**a**) 40 mg (0.16 mmol) of pindolol using cellulose 3,5-dimethylphenylcarbamate (7.5 mg/mL) as CS; solvent system MIBK-sodium phosphate buffer 50 mM pH 7.0 and (**b**) 50 mg (0.16 mmol) of warfarin using amylose 3,5-dimethylphenylcarbamate (7.5 mg/mL) as CS; solvent system MTBE-sodium phosphate buffer 50 mM pH 8.0. Vertical axis, arbitrary absorbance units. Horizontal axis, time (min). Adapted from [38]

the same CSs, could not be separated (Fig. 8). Moreover, only slight enrichments were observed for warfarin and pindolol enantiomers when using 3,5-dichlorophenylcarbamate of cellulose. As the involvement of the secondary and supramolecular structures of these CSs in enantiorecognition is generally accepted [68, 69], the reduction in enantioselectivity observed for these materials in the CCC conditions could be related to a decrease in the molecular order. It is worth noting that the maximum stable concentration for the suspensions/colloidal solutions prepared is below the range at which polysaccharide derivatives attain a semi-ordered liquid-crystallinity in liquid phase [70].

In an attempt to increase the amount of the polysaccharide derived CSs loaded in the stationary liquid phase, double derivatized materials containing dodecanoyl chains in addition to 3,5-dimethylphenylcarbamate groups were tested [71]. Although solubility increased notably, it was observed that a high degree of dodecanoyl chains induced a decrease in the enantioselectivity. It has been suggested that dodecanoyl chains might interact in a non-enantioselective way with racemates thereby producing broader peaks and the co-elution of the enantiomers. Nevertheless, this double modification in polysaccharides allowed a substantial increase in loadability, notably when using pH-zone-refining conditions.

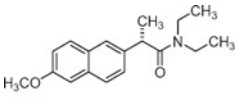
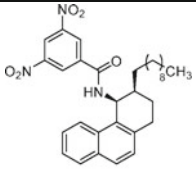
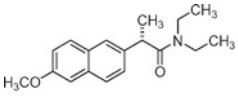
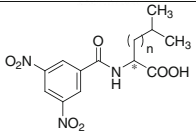
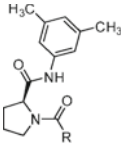
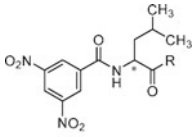
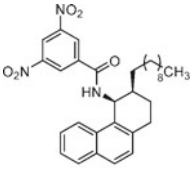
4.3 Recently Applied CSs and Solvent Systems

Among brush-type CSPs for HPLC, the Whelk-O® CSP, first designed for naproxen enantioseparation, has shown an outstanding applicability for a broad diversity of racemic compounds [72]. Nowadays it has become one of the CSPs of reference included in most screening processes addressed to the search of adequate conditions for enantioselective separation in drug discovery [73, 74]. A CS containing the Whelk-O® skeleton, and a lipophilic chain aimed to enhance solubility

in organic phases of organic-aqueous biphasic solvent systems, was planned. The conventional synthetic pathway leading to this compound produced the racemic mixture of the Whelk-O® selector analogue, (±)-(WSA), whose enantioseparation was undertaken by CPC [75]. One of the difficulties encountered when searching the appropriate solvent system was the high lipophilicity of the racemic mixture that has to partition between the two phases. Additionally, an adequate CS was chosen among several (*S*)-naproxen derivatives.

Unfortunately, the highly polar (*S*)-naproxen did not result effective as CS in the organic-aqueous solvent systems tested. However, the diethylamide derivative of (*S*)-naproxen was found useful although an acceptable compromise between promoting the partition of the analyte while retaining the CS in the polar phase had

Table 3 Recently applied CSs and solvent systems in CCC separations

Chiral selector	Racemate	Solvent system	Ref.
 (S)-naproxen diethylamide	 (±)-WSA	Heptane – ethyl acetate – methanol – water (9:1:9:1, v/v)	[75]
 (S)-naproxen diethylamide	 n:0, valine n:1, leucine	MTBE – sodium phosphate buffer 50 mM pH 6.0	[77]
 R: $C_{10}F_{21}$, $C_{11}H_{23}$,	 R:OH, NH^tBu	Ethoxynonafluorobutane (ENFB) – 2-propanol – water (25:35:40, v/v)	[80]
 (*R*,*R*)-WSA	Naproxen Naproxen diethylamide Warfarin Troger's base	hexane – ethyl acetate – methanol – water (1:1:1:1, v/v)	[76]

to be attained. Finally a solvent system composed by a mixture of hexane–ethyl acetate–methanol–water in ratio 9:1:9:1, in which the CS was located in the more polar aqueous lower phase, was used in the separation. The optimal CS concentration was determined to be 100 mM. At this concentration of the CS the maximum loading capacity of the system was attained when a molar ratio CS/racemate of 6.9 was used (900 mg in a single run using a 190 mL volume CPC system). The inversion of the elution order of enantiomers, compared to that observed in HPLC on a CSP containing the same CS, was observed. This was attributed to the elution of the more stable adsorbate CS-(*R,R*)WSA, instead of the usual elution of the least adsorbed enantiomer at a shorter elution time. When the CPC separation was compared to semipreparative HPLC, even though only a partial separation was attained when using the former, the global process resulted highly advantageous to CPC from the point of view of time and solvent consumption. The preliminary test of (*R,R*)-WSA as CS in CCC afforded the partial separation of racemic naproxen, naproxen diethylamide, warfarin, and Tröger's base when using a mixture of hexane–ethyl acetate–methanol–water (1:1:1:1) in which the CS is located in the organic phase [76]. Moreover, (*S*)-naproxen diethylamide has also been used as a CS for the separation *N*-(3,5-dinitrobenzoyl)-(±)-leucine enantiomers in a binary solvent system composed by a mixture of MTBE-phosphate buffer pH 6.0. In this system, the CS was located in the upper more lipophilic phase [77] (Table 3).

A major problem in the application of CCC to enantioseparation arises when CS and no-ionisable analytes have similar polarities. As in the above described case, the search of an appropriate solvent system becomes a difficult task. In order to

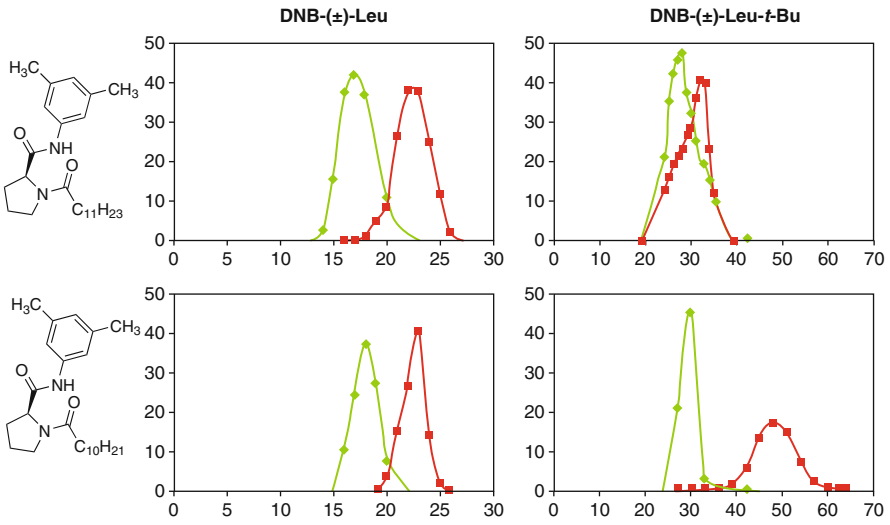


Fig. 9 Elution profiles corresponding to the separation of of DNB-(±)-Leu and DNB (±) Leu-tBu in a fluorinated solvent system consisting in a mixture of ethoxynonafluorobutane-2 propanol-water (25:35:40, v/v). Analogous L-proline-derived CSs have been used. Vertical axis, arbitrary absorbance units; horizontal axis, time (min). Adapted from [80]

overcome this problem the particular affinity properties of fluorinated solvents can be a solution. Thus, the retention of the CS in an organic fluorinated phase can be promoted by the introduction of a fluorinated group on its structure. To this aim, ethoxynonafluorobutane (ENFB), a fluorinated solvent recently introduced in chromatography as a substituent of hexane [78, 79], has been used as a component of a biphasic solvent system. The concept has been proved for a CS analogous to *N*-dodecyl-L-proline-3,5-dimethylanilide, containing a *N*-undecenoyl perfluorinated group. This CS resulted enantioselective for DNB-(±)-Leu-*t*-butylamide in a solvent system constituted by ENFB–2-propanol–water (25:35:40). The fluorinated CS was successfully retained in the organic lower phase that contains the fluorinated solvent (Fig. 9) [80].

5 Alternative Elution Modes Applied to Enantioseparation

As stated in the introduction, one of the advantages of CCC over conventional solid-liquid chromatography is not only the possibility to use of a great variety of solvent systems but also the diversity of elution modes applicable, some of which are not possible when a solid support is present. Among the diverse eluting modes possible in CCC only pH-zone refining, dual and multidual mode have been applied up to now to enantioseparation.

5.1 pH-Zone Refining Mode: Increasing Loading Capacity

pH-zone refining is a kind of displacement chromatography introduced by Ito et al. in the early 1990s [81]. It promotes the separation of ionisable compounds as a function of their pK_a and polarity [10]. The methodology consists in using a retainer agent in the stationary phase to retain the sample constituents in the column at the beginning of the experiment. The nature of the retainer agent has to be acid or basic, depending on the nature – acid or basic, respectively – of the analytes. A displacer agent, opposite in nature to the retainer agent – basic or acid, for acids or basic analytes, respectively – is added to the mobile phase to promote the elution of the sample components. The most outstanding advantage of this approach over the classical elution mode is the increase in loading capacity produced, which is very convenient for preparative purposes. In contrast, it is not possible to perform repetitive injections, a common way to proceed when the preparative purification of a particular analyte is the goal. The constitution of the stationary phase evolves during elution and starting conditions have to be ensured before undertaking the next run. Also advantages of this approach are the concentration at which the analytes elute and the possibility to monitor the elution of compounds by monitoring the pH of the eluate.

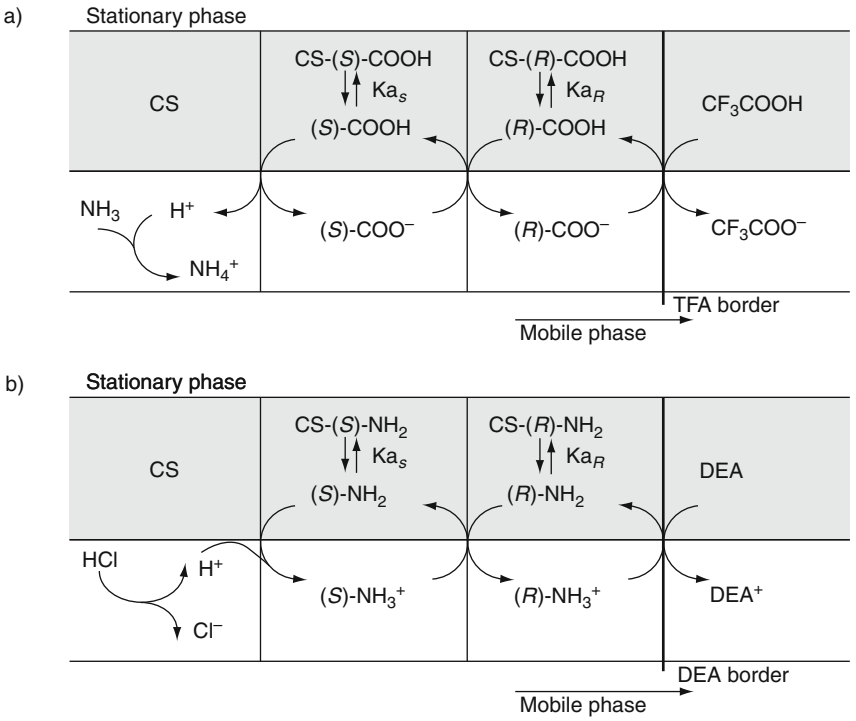


Fig. 10 Scheme of the pH-zone-refining mechanism in enantioselective applications. (**a**) The separation of acidic analytes is considered. Retainer agent: trifluoroacetic acid; displacer agent: ammonia. The enantiomer more strongly associated to the CS is retained longer. (**b**) Separation of basic analytes. Retainer agent: diethylamine; displacer agent: hydrochloric acid

When enantioseparations are considered, the CS added to the stationary phase contributes to the preferential retention of one of the enantiomers, thereby conditioning the elution order. The process occurring in the column during elution of enantiomers in pH-zone refining conditions is schematized on Fig. 10. Generally, improved separations are obtained when applying pH-zone refining with respect to classical mode with ionisable enantiomers.

Ito et al. described the first application of pH-zone refining to chiral separation using *N*-dodecanoyl-L-proline-3,5-dimethylanilide as CS (40 mM) in the separation of DNB-(±)-Leu enantiomers [59]. A binary solvent system composed by a mixture of MTBE-water has been used. Due to the acidic character of the analyte, trifluoroacetic acid was added as a retainer agent to the organic MTBE stationary phase and ammonia was added to the aqueous mobile phase as a displacer agent. In the described conditions the separation of 2 g of leucine racemate (CS/racemate molar ratio: 1.30) in a single run was possible in a CCC device of 330 mL.

Since then pH-zone refining eluting mode has been applied to several chiral separations (Table 4). Adamantylcarbamoyl QN-CS, solubilized at a 10 mM concentration in the organic phase of a MIBK-water solvent system permitted the

Table 4 Chiral separations where pH-zone refining was applied

Chiral selector	Racemate	Solvent system	Retainer (mM)	Displacer (mM)	Ref.
N-dodecanoyl-L-proline-3,5-dimethylanilide	DNB-(±)-Leu	MTBE/water	TFA (40 mM)	NH_4OH (20 mM)	[59]
Adamantyl carbamoyl QN-CS	DNB-(±)-Leu	MIBK/water	TFA (10 mM)	NH_4OH (20 mM)	[64]
Cellulose 3,5-dimethyl phenylcarbamate	(±)-pindolol	MIBK/water	DEA (10 mM)	HCl (5 mM)	[38]
Cellulose dodecanoyl /dimethyl phenylcarbamate	(±)-pindolol	MTBE/water	DEA (10 mM)	HCl (5 mM)	[71]
Amylose 3,5-dimethyl phenylcarbamate	(±)-warfarin	MTBE/water	TFA (10 mM)	NH_4OH (2.5 mM)	[38]

resolution of 900 mg of DNB-(±)-Leu in a 190 mL CPC device [64]. This amount of racemate corresponds to a CS/racemate molar ratio of 0.66, which is twice the amount of the racemate resolved using classical elution conditions. Considering the mechanism governing the elution of enantiomers in pH-zone refining displacement mode and assuming a highly enantioselective CS (high difference between the two association constants Ka_S and Ka_R) the highest sample loading attainable corresponds to a CS/racemate molar ratio of 0.5 in the case of racemates (Fig. 11). However, loadability is dependent on the amount of the most retained enantiomer contained in the sample. Therefore, this figure may be even lower in the case of samples enriched in the less retained enantiomer.

Polysaccharide derivatives have also been used in pH-zone-refining displacement conditions. Amylose and cellulose 3,5-dimethylphenylcarbamates and cellulose dodecanoyl/dimethylphenycarbamate were used to resolve pindolol and warfarin [38, 71]. Given the basic nature of pindolol, diethylamine, an organic base was used as a retainer agent in the organic stationary phase. In these conditions an improvement of enantioseparation over that obtained in the classical eluting mode was obtained. This result was attributed to the retention of the analyte in its neutral form in the stationary phase, which contributes to a proper interaction with the CS. In classical elution mode the pH of the mobile phase is set to obtain feasible retention times for analytes. Therefore, depending on the pK_a and lipophilicity of each particular compound, a partial ionization may be produced.

When compared to HPLC, the CPC separation required a lower amount of CS for a given amount of analyte, what was attributed to a better accessibility of the CS in the liquid stationary phase. Moreover, the loadability of the system was even improved when the doubly derivatized cellulose derivatives were used [71].

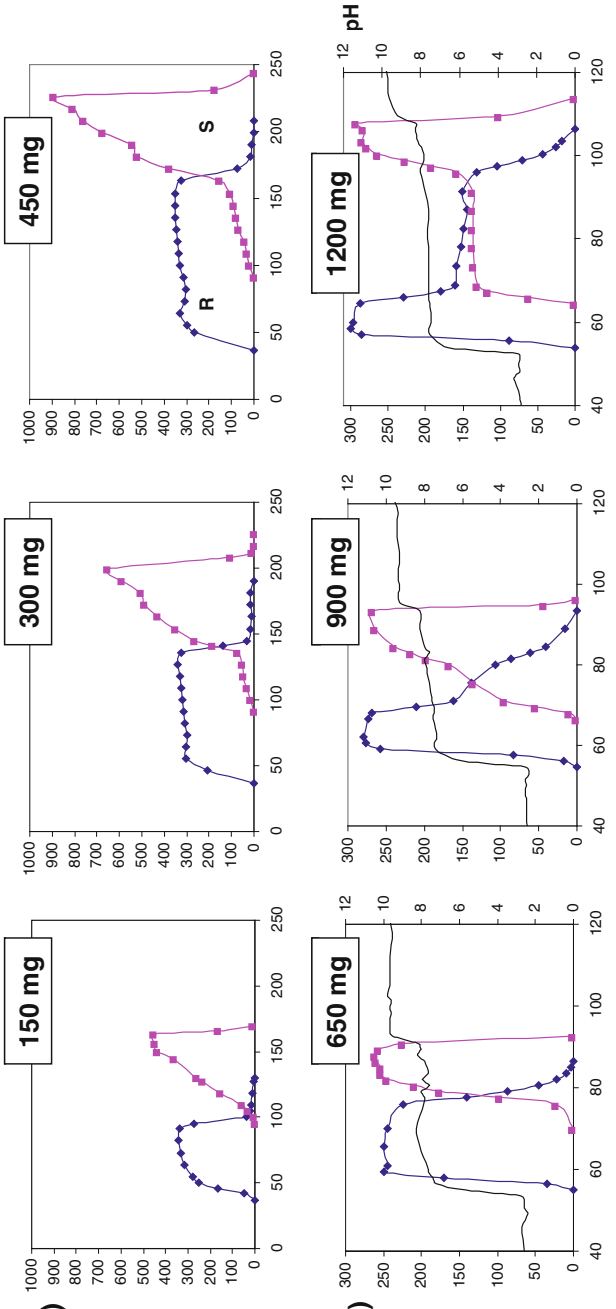


Fig. 11 Elution profiles corresponding to the separation of increasing amounts of DNB (±)-Leu using a quinine derivative as CS. (**a**) Classical elution mode: CS/racemate molar ratios: 2.6, 1.3, and 0.8, respectively. Solvent system: MIBK-acetone-ammonium acetate buffer 0.1 M pH 8.0 (2:1:2), 10 mM CS. (**b**) pH-Zone-refining. Stationary phase, MIBK containing TFA (10 mM) and the CS (10 mM); mobile phase, water containing ammonia (20 mM). CS/racemate molar ratios: 0.85, 0.61, and 0.45, respectively. In all cases the first eluting isomer corresponds to the *R* enantiomer. Vertical left axis, arbitrary absorbance units. Vertical right axis, pH; Horizontal axis, time. Adapted from [64]

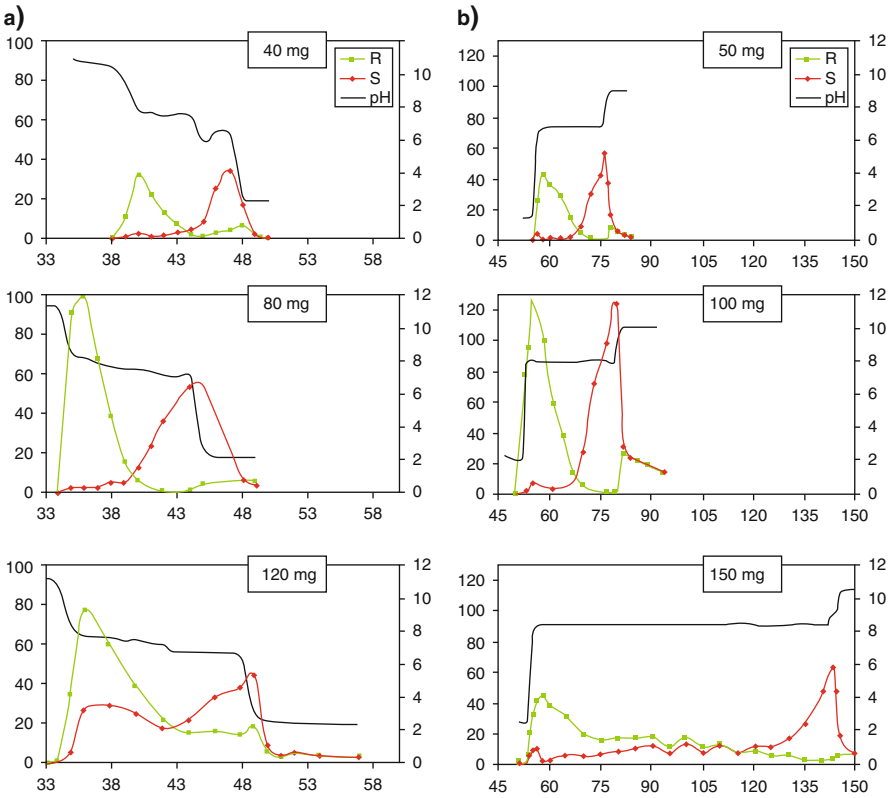


Fig. 12 Elution profiles corresponding to the separation of increasing amounts of (**a**) pindolol (solvent system: MIBK-water; stationary phase: 10 mM DEA and 7.5 mg/mL of cellulose 3,5-dimethylphenylcarbamate; mobile phase: 5 mM HCl) and (**b**) warfarin (Solvent system: MTBE-water, stationary phase: 10 mM TFA and 7.6 mg/mL of amylose 3,5-dimethylphenylcarbamate; mobile phase: 2.5 mM NH_4OH). Left vertical axis, arbitrary absorbance units; right vertical axis, pH. Horizontal axis, time (min). Adapted from [38]

Amounts in the order of 200 mg of racemic pindolol were resolved in a single run (6 mg/mL of CS) while only 80 mg were resolved using an equivalent amount of the homogeneously derived cellulose 3,5-dimethylphenylcarbamate as CS (Fig. 12).

5.2 Dual-Mode and Multidual Mode: Increasing Resolution

Dual-mode is an eluting mode applicable to CCC because only liquid phases are involved. It consists in switching the mobile and stationary liquid phase roles at a certain point within the experiment. This approach permits to ensure the complete elution of all components in complex mixtures comprising a broad range of

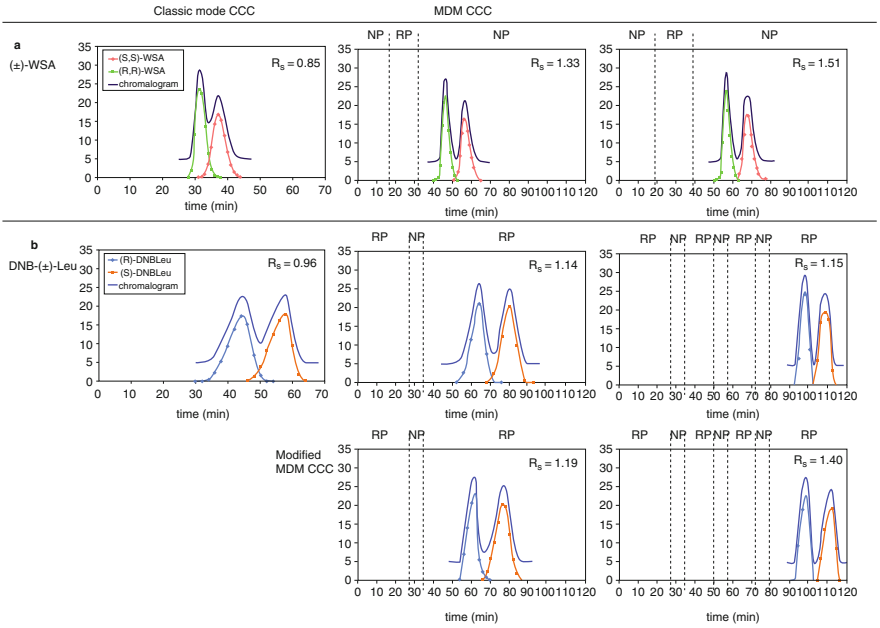


Fig. 13 Elution profiles corresponding to the classical mode and MDM separation of: (**a**) (±)-WSA (solvent system, hexane-ethyl acetate-methanol-water (9:1:9:1, v/v); CS, 100 mM (*S*)-naproxen diethylamide in the upper phase). An increase in *Rs* is observed in MDM over classical mode which is dependent on the RP period. (**b**) DNB-(±)-Leu (solvent system: MTBE-sodium phosphate buffer 50 mM pH 6.0; CS, 90 mM (*S*)-naproxen diethylamide in the lower phase). The separation does not improve even after three complete cycles of MDM. The modification in MDM, which consists of stopping the centrifugal rotation during NP periods, results in an improved separation. Differences observed are the result of the partition of the CS/enantiomer complexes between the two liquid phases in the case of (±)-WSA. Adapted from [77]

polarities. Moreover, eluting times are reduced for those sample components of high affinity for the stationary phase. Various applications show its usefulness in reducing analysis time and increasing the resolution factor in preparative CCC [13]. Dual-mode (DM) has also been applied to enantioseparation to promote the elution of the second eluting enantiomer highly retained by the CS. However, it has to be taken into account that, when applied to enantioseparations, the most retained enantiomer elutes in the phase containing the CS. This was the case of vancomycin in the separation of DNS-(±)-Nle (Fig. 4) [45].

The extension of the DM methodology by performing several phase inversion cycles leads to what is known as multidual mode (MDM) [14]. This eluting mode consists in performing several changes between normal and reversed-phase modes. Therefore, if the process starts in reversed-phase mode, being the lower aqueous phase the mobile phase, it then switches to normal phase, with the upper organic phase acting as a mobile phase. Further switches will alternate normal phase and reversed-phase conditions. The application of MDM CCC to enantioseparation

permits to avoid the elution of enantiomers in the phase containing the CS. By performing an even number of phase inversions the system returns to the starting mobile phase conditions.

MDM CCC has been recently applied for the first time to chiral CCC [77]. *S*-Naproxen diethylamide was used as a CS for the separation of (±)-WSA and DNB-(±)-Leu (Table 3). Despite the improvement in resolution produced for (±)-WSA enantioseparation, no improvement was observed for DNB-(±)-Leu (Fig. 13). The difference in the result obtained was explained on the basis of the difference in the enantiorecognition mechanism. As for most of CCC enantioseparations described, enantiomer recognition in the separation of DNB-(±)-Leu takes place in the stationary phase, where the CS is confined. This is not the case for (±)-WSA [75], whose more stable adsorbate *S*-naproxenamide/(*R,R*)-WSA undergoes partition to the mobile phase during the eluting process. Nevertheless, a modification in MDM, consisting in stopping the device rotation during the reversed-phase elution, permits the desired improvement in resolution for the DNB-(±)-Leu separation.

5.3 Continuous Operation

The separations described up to this point, although providing higher loadability than conventional HPLC, correspond to discontinuous chromatographic processes. Regarding HPLC, it is widely accepted that continuous chromatography technology, the so-called simulated moving bed (SMB), boosts productivity over traditional batch processes. Therefore, the envisagement of a continuous CCC or CPC technology should improve the capacity and the productivity of the technique. In this regard, a new system was recently patented [82], which describes a continuous CPC process with an indubitable applicability to the separation of enantiomers. This system is characterized by the injection of the sample at an intermediate point of the "column," followed by sequential pumping of a dense solvent phase and a light solvent phase from opposite ends of the column. Up to now this system has been applied to a single conventional separation by CCC [83].

6 Conclusions and Future Perspectives

Countercurrent chromatography, the separation technique using a liquid stationary phase, has been reported to be suitable for preparative purposes due to the advantages derived from the fact of being a support-free technique. When applying CCC to chiral separations we must consider three fundamental points: the structure of the CS, which has to be complementary to the racemate to be separated, the biphasic solvent system, since enantioselectivity is highly dependent on the environment where it occurs, and the efficiency of the instrument, because it must ensure an effective mixing and settling of the liquid phases.

Given the reduced efficiency of CCC, highly enantioselective CSs are desirable. This property is related to the complementarity CS-enantiomers. Moreover, although CSs with broad applicability would be desirable, most of the CSs described up to now are suitable only for a few racemic compounds. Nevertheless, a certain compromise between these two properties (high enantioselectivity and broad applicability) should be reached when searching for new CSs.

The solvent system, and in particular the composition of the phase that contains the CS, affects greatly enantiorecognition. In this context, the use of new solvents, such as ionic liquids [84] or ordered liquid phases (lyotropic liquid crystals), extended to chiral separations could provide new properties and selectivities. Fluorinated solvents and their particular miscibility properties can also extent the applicability of diverse chiral selecting moieties.

Finally, all these progresses should be accompanied by technical improvements in instrumentation to optimize efficiency and phase mixing. This will contribute to broaden the feasibility of CCC enantioseparations by using CSs of moderate enantioselectivity.

Acknowledgments Financial support from the Ministerio de Educación y Ciencia of Spain and from the European Regional Development Fund (ERDF) (project number CTQ2006-03378/PPQ) is gratefully acknowledged. N. Rubio thanks the Ministerio de Educación y Ciencia for a doctoral fellowship. The authors thank A. M. Pérez and Dr. M. D. Llongueras for fruitful discussions.

References

1. Mandava NB, Ito Y (eds) (1985) Countercurrent chromatography, theory and practice, chromatographic science series, vol 44. Marcel Dekker, New York
2. Conway WD (1990) Countercurrent chromatography, apparatus, theory and applications. VCH Publishers, New York
3. Foucault AP (ed) (1995) Centrifugal partition chromatography. Chromatographic science series, vol 68. Marcel Dekker, New York
4. Berthod A (ed) (2002) Countercurrent chromatography, the support free liquid stationary phase. Comprehensive analytical chemistry, vol 38. Elsevier Science, Amsterdam
5. Marston A, Hostettmann K (2006) Developments in the application of counter-current chromatography to plant analysis. J Chromatogr A 1112:181–194
6. Friesen JB, Pauli GF (2007) Rational development of solvent system families in counter-current chromatography. J Chromatogr A 1151:51–59
7. Dubant S, Mathews B, Higginson P, Crook R, Snowden M, Mitchell J (2008) Practical solvent system selection for counter-current separation of pharmaceutical compounds. J Chromatogr A 1207:190–192
8. Leitao GG, El-Adji SS, Lopes de Melo WA, Leitao SG, Brown L (2005) Separation of free and glycosylated flavonoids from *Siparuna guianensis* by gradient and isocratic CCC. J Liq Chromatogr Relat Technol 28:2041–2051
9. Jin W, Tu P-F (2005) Preparative isolation and purification of trans-3,5,4'-trihydroxystilbene-4'-O-β-D-glucopyranoside and (+)-catechin from *Rheum tanguticum Maxim*. ex Balf. Using high-speed counter-current chromatography by stepwise elution and stepwise increasing the flow-rate of the mobile phase. J Chromatogr A 1092:241–245
10. Ito Y, Ma Y (1996) pH-Zone-refining countercurrent chromatography. J Chromatogr A 753:1–36

11. Yang F, Quan J, Zhang TY, Ito Y (1998) Multidimensional counter-current chromatographic system and its application. J Chromatogr A 803:298–301
12. Berthod A, Maryutina T, Spivarov B, Shpigun O, Sutherland IA (2009) Countercurrent chromatography in analytical chemistry. Pure Appl Chem 81:355–387
13. Agnely M, Thiébaut D (1997) Dual-mode high-speed counter-current chromatography: retention, resolution and examples. J Chromatogr A 790:17–30
14. Delannay E, Toribio A, Boudesocque L, Nuzillard J-M, Zèches-Hanrot M, Dardennes E, Le Dour G, Sapi J, Renault J-H (2006) Multiple dual-mode centrifugal partition chromatography, a semi-continuous development mode for routine laboratory-scale purifications. J Chromatogr A 1127:45–51
15. Berthod A, Ruiz-Angel MJ, Carda-Broch S (2003) Elution-extrusion countercurrent chromatography. Use of the liquid nature of the stationary phase to extend the hydrophobicity window. Anal Chem 75:5886–5894
16. Lee YW, Cook CE, Ito Y (1988) Dual countercurrent chromatography. J Liq Chromatogr Relat Technol 11:37–53
17. Berthod A, Hassoun M (2006) Using the liquid nature of the stationary phase in countercurrent chromatography IV. The cocurrent CCC method. J Chromatogr A 1116:143–148
18. Sutherland IA, Audo G, Bourton E, Couillard F, Fisher D, Garrard I, Hewitson P, Intes O (2008) Rapid linear scale-up of a protein separation by centrifugal partition chromatography. J Chromatogr A 1190:57–62
19. Sutherland IA, Hewitson P, Ignatova S (2009) Scale-up of counter-current chromatography: demonstration of predictable isocratic and quasi-continuous operating modes from the test tube to pilot/process scale. J Chromatogr A 1216:8787–8792
20. Foucault AP (2001) Enantioseparations in counter-current chromatography and centrifugal partition chromatography. J Chromatogr A 906:365–378
21. Pérez E, Minguillón C (2007) Countercurrent chromatography in the separation of enantiomers. In: Subramanian G (ed) Chiral separation techniques. A practical approach, 3rd edn. Wiley-VCH, Weinheim
22. Kessler LC, Seidel-Morgenstern A (2006) Theoretical study of multicomponent continuous countercurrent chromatography based on connected 4-zone units. J Chromatogr A 1126: 323–337
23. Lim BG, Ching CB (1996) Preliminary design of a simulated counter-current chromatographic system for the separation of praziquantel enantiomers. J Chromatogr A 734:247–258
24. Ito Y, Weinstein MA, Aoki I, Harada R, Kimura E, Nunogaki K (1966) The coil planet centrifuge. Nature 212:985–987
25. Ito Y (2002) Foreword. In: Berthod A (ed) Countercurrent chromatography: the support-free liquid stationary phase. Comprehensive analytical chemistry, vol 38. Elsevier, Amsterdam, pp xix–xx
26. Ito Y (1992) Countercurrent chromatography. In: Heftmann E (ed) Chromatography, 5th edn. J Chromatogr Lib, vol 51A, Elsevier, Amsterdam
27. Maier NM, Lindner W (2006) Stereoselective chromatographic methods for drug analysis. In: Francotte E, Lindner W (eds) Chirality in drug research. Methods and principles in medicinal chemistry seriers, vol 33. Wiley-VCH, Weinheim
28. Ma Y, Ito Y (1995) Chiral separation by high speed countercurrent chromatography. Anal Chem 67:3069–3074
29. Lämmerhofer M, Lindner W (2000) Recent developments in liquid chromatographic enantioseparation. In: Valkó K (ed) Separation methods in drug synthesis and purification, handbook of analytical separations, vol 1. Elsevier, Amsterdam
30. Pfeiffer CC (1956) Optical isomerism and pharmacological action, a generalization. Science 124:29–30
31. Lämmerhofer M, Lindner W (1996) Quinine and quinidine derivatives as chiral selectors. I. Brush type chiral stationary phases for high-performance liquid chromatography based on cinchonan carbamates and their application as chiral anion exchangers. J Chromatogr A 741:33–48

32. Berthod A, Billardello B (2000) Test to evaluate countercurrent chromatographs liquid stationary phase retention and chromatographic resolution. J Chromatogr A 902:323–335
33. Ignatova SN, Sutherland IA (2003) A fast, effective method of characterizing new phase systems in CCC. J Liq Chromatogr Relat Technol 26:1551–1564
34. Foucault AP, Bousquet O, Le Goffic F (1992) Importance of the parameters Vm/Vc in countercurrent chromatography: tentative comparison between instrument designs. J Liq Chromatogr 15:2691–2706
35. Maryutina TA, Fedotov PS, Spivakov BY (1999) Application of countercurrent chromatography in inorganic analysis. In: Menet JM, Thiebaut D (eds) Countercurrent chromatography, chromatographic science series, vol 68. Marcel Dekker, New York
36. Maryutina TA, Ignatova SN, Spivakov BY, Sutherland IA (2003) The efficiency of substance separation in countercurrent liquid chromatography. J Anal Chem 58:762–767
37. Kim E, Koo YM, Chung DS (2004) Chiral counter-current chromatography of gemifloxacin guided by capillary electrophoresis using (+)-(18-crown-6)-tetracarboxylic acid as a chiral selector. J Chromatogr A 1045:119–124
38. Pérez E, Santos MJ, Minguillón C (2006) Application of cellulose and amylose arylcarbamates as chiral selectors in counter-current chromatography. J Chromatogr A 1107:165–174
39. Oya S, Snyder JK (1986) Chiral resolution of a carboxylic acid using droplet counter-current chromatography. J Chromatogr 370:333–338
40. Bergholdt BA, Lehmann SV (1998) High-speed separation of ormeloxifene enantiomers using sulfated β-cyclodextrin in capillary electrophoresis. Chirality 10:699–704
41. Breinholt J, Lehmann SV, Varming AM (1999) Enantiomer separation of 7-des-methyl-ormeloxifene using sulfated β-cyclodextrin in countercurrent chromatography. Chirality 11:768–771
42. Armstrong DW, Rundlett KL, Chen J-R (1994) Evaluation of the macrocyclic antibiotic vancomycin as a chiral selector for capillary electrophoresis. Chirality 6:496–509
43. Armstrong DW, Zhou Y (1994) Use of a macrocyclic antibiotic as the chiral selector for enantiomeric separations by TLC. J Liq Chromatogr 17:1695–1707
44. Armstrong DW, Tang Y, Chen S, Zhou Y, Bagwill C, Chen JR (1994) Macrocyclic antibiotics as a new class of chiral selectors for liquid chromatography. Anal Chem 66:1473–1484
45. Duret P, Foucault A, Margraff R (2000) Vancomycin as a chiral selector in centrifugal partition chromatography. J Liq Chromatogr Relat Technol 23:295–312
46. Stewart KK, Doherty RF (1973) Resolution of DL-triptophan by affinity chromatography on bovine-serum-albumin-agarose columns. Proc Natl Acad Sci U S A 70:2850–2852
47. Allenmark S, Bomgren B, Borén H (1983) Direct liquid chromatographic separation of enantiomers on immobilized protein stationary phases III. Optical resolution of a series of N-aroyl D,L-amino acids by high performance liquid chromatography on bovine serum albumin covalently bonded to silica. J Chromatogr 264:63–68
48. Ekberg B, Sellergren B, Albertsson PA (1985) Direct chiral resolution in an aqueous two-phase system using the counter-current distribution principle. J Chromatogr 333:211–214
49. Arai T, Kuroda H (1991) Distribution behaviour of some drug enantiomers in an aqueous two-phase system using counter current extraction with protein. Chromatographia 32:56–60
50. Shinomiya K, Kabasawa K, Ito Y (1998) Enantiomeric separation of commercial D,L-kynurenine with an aqueous two-phase solvent system by cross-axis coil planet centrifuge. J Liq Chromatogr 21:135–141
51. Pirkle WH, Welch CJ (1992) Effect of superfluous remote polar functionality on chiral recognition. J Chromatogr A 589:45–51
52. Perrin C, Matthijs N, Mangelings D, Granier-Loyaux C, Maftouh M, Massarta DL, Heyden YV (2002) Screening approach for chiral separation of pharmaceuticals Part II. Reversed-phase liquid chromatography. J Chromatogr A 966:119–134
53. Delgado B, Pérez E, Santano MC, Minguillón C (2005) Enantiomer separation by counter-current chromatography. Optimisation and drawbacks in the use of L-proline derivatives as chiral selectors. J Chromatogr A 1092:36–42

54. Domon B, Hostettmann K, Kovacevic K, Prelog V (1982) Separation of the enantiomers of (±)-norephedrine by rotation locular counter-current chromatography. J Chromatogr 250:149–151
55. Prelog V, Stojanac Z, Kovacevic K (1982) Separation of enantiomers by partition between liquid phases. Helv Chim Acta 65:377–384
56. Takeuchi T, Horikawa R, Tanimura T (1984) Complete resolution of DL-isoleucine by droplet counter-current chromatography. J Chromatogr 284:285–288
57. Pirkle WH, Murray PG (1993) Chiral stationary phase design. Use of intercalative effects to enhance enantioselectivity. J Chromatogr 641:11–19
58. Oliveros L, Franco P, Minguillón C, Camacho-Frias E, Foucault AP, Le Goffic F (1994) Donor-acceptor chiral centrifugal partition chromatography: complete resolution of two pairs of amino-acid derivatives with a chiral II donor selector. J Liq Chromatogr 17:2301–2318
59. Ma Y, Ito Y, Foucault A (1995) Resolution of gram quantities of racemates by high-speed counter-current chromatography. J Chromatogr A 704:75–81
60. Ma Y, Ito Y, Berthod A (1999) A chromatographic method for measuring kf of enantiomer-chiral selector complexes. J Liq Chromatogr Relat Technol 22:2945–2955
61. Lämmerhofer M, Lindner W (1996) Quinine and quinidine derivatives as chiral selectors I. Brush type chiral stationary phases for high-performance liquid chromatography based on cinchonan carbamates and their application as chiral anion exchangers. J Chromatogr A 741:33–48
62. Kellner KH, Blasch A, Chmiel H, Lämmerhofer M, Lindner W (1997) Enantioseparation of N-protected α-amino acid derivatives by liquid-liquid extraction technique employing stereoselective ion-pair formation with a carbamoylated quinine derivative. Chirality 9:268–273
63. Zarbl E, Lämmerhofer M, Franco P, Petracs M, Lindner W (2001) Development of stereoselective nonaqueous capillary electrophoresis system for the resolution of cationic and anphoteric analytes. Electrophoresis 22:3297–3307
64. Franco P, Blanc J, Oberleitner WR, Maier NM, Lindner W, Minguillón C (2002) Enantiomer separation by countercurrent chromatography using cinchona alkaloid derivatives as chiral selectors. Anal Chem 74:4175–4183
65. Gavioli E, Maier NM, Minguillón C, Lindner W (2004) Preparative enantiomer separation of dichlorprop with a cinchona-derived chiral selector employing centrifugal partition chromatography and high-performance liquid chromatography: a comparative study. Anal Chem 76:5837–5848
66. Okamoto Y, Kaida Y (1994) Resolution by high-performance liquid chromatography using polysaccharide carbamates and benzoates as chiral stationary phases. J Chromatogr A 666:403–419
67. Tachibana K, Ohnishi A (2001) Reversed-phase liquid chromatographic separation of enantiomers on polysaccharide type chiral stationary phases. J Chromatogr A 906:127–154
68. Yashima E, Yamamoto C, Okamoto Y (1996) NMR studies of chiral discrimination relevant to the liquid chromatographic enantioseparation by a cellulose phenylcarbamate derivative. J Am Chem Soc 118:4036–4048
69. Schulz L, Seger B, Burchard W (2000) Structures of cellulose in solution. Macromol Chem Phys 201:2008–2022
70. Terbojevich M, Cosani A, Focher B, Gastaldi G, Wu W, Marsano E, Conio G (1999) Solution properties and mesophase formation of 4-phenylbenzoylcellulose. Cellulose 6:71–87
71. Pérez E, Minguillón C (2006) Optimisation of the derivatization in cellulose-type chiral selectors for enantioseparation by centrifugal partition chromatography. J Sep Sci 29:1379–1389
72. Pirkle WH, Welch CJ, Lamm B (1992) Design, synthesis, and evaluation of an improved enantioselective naproxen selector. J Org Chem 57:3854–3860
73. Andersson S (2006) Preparative chiral chromatography – a powerful and efficient tool in drug discovery. In: Subramanian G (ed) Chiral separation techniques. A practical approach, 3rd edn. Wiley-VCH, Weinheim

74. Francotte E (2001) Enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers. J Chromatogr A 906:379–397
75. Rubio N, Minguillón C (2010) Preparative enantioseparation of (±)-N-(3,4-cis-3-decyl-1,2,3,4-tetrahydrophenanthren-4-yl)-3,5-dinitrobenzamide by centrifugal partition chromatography. J Chromatogr A 1217:1183–1190
76. Rubio N, Minguillón C, in preparation
77. Rubio N, Ignatova S, Sutherland I, Minguillón C (2009) Multiple dual-mode counter-current chromatography applied to chiral separations. J Chromatogr A 1216:8505–8511
78. Kagan M, Chlenov M, Kraml CM (2004) Normal-phase high-performance liquid chromatographic separations using ethoxynonafluorobutane as hexane alternative II. Liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry applications with methanol gradients. J Chromatogr A 1033:321–331
79. Ding J, Desai M, Armstrong DW (2005) Evaluation of ethoxynonafluorobutane as a safe and environmentally friendly solvent for chiral normal-phase LC-atmospheric pressure chemical ionization/electrospray ionization-mass spectrometry. J Chromatogr A 1076:34–43
80. Pérez AM, Minguillón C (2010) New biphasic solvent systems containing a fluorinated component and its use in countercurrent chromatography for the separation of enantiomers. J Chromatogr A 1217:1094–1100
81. Weisz A, Scher AL, Shinomiya K, Fales HM, Ito Y (1994) A new preparative-scale purification technique: pH-zone-refining countercurrent chromatography. J Am Chem Soc 116:704–708
82. Couillard F, Foucault A, Durand D (2005) Process and device for separation of the components to a liquid load by centrifugal liquid-liquid chromatography. Patent WO2005011835
83. Van den Heuvel R, Mathews B, Dubant S, Sutherland I (2009) Continuous counter-current extraction on an industrial sample using dual-flow counter-current chromatography. J Chromatogr A 1216:4147–4153
84. Berthod A, Carda-Broch S (2004) Use of the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate in countercurrent chromatography. Anal Bioanal Chem 380:168–177

Enantioselective Properties of Nucleic Acid Aptamer Molecular Recognition Elements

Eric Peyrin

Contents

Abstract Target-specific chiral selectors, which are characterized by a predictable elution order depending on the target enantiomer employed for the selection of the chiral selector, have recently received much attention in the enantioselective analysis field. In this context, bioaffinity-based molecular recognition tools such as nucleic acid aptamers have notably demonstrated very attractive features for the chiral discrimination of active molecules. In this chapter, the enantioselective properties of aptamer chiral selectors and the major factors that control and modulate the liquid chromatography and capillary electrophoresis enantiomer separation are addressed.

E. Peyrin (✉)
Département de Pharmacochimie Moléculaire UMR 5063 CNRS, Institut de Chimie Moléculaire de Grenoble FR 2607, Université de Grenoble I, Bât E (C) André Rassat, Domaine Universitaire, 301 avenue de la Chimie, BP 53, 38041 Grenoble Cédex 9, France
e-mail: eric.peyrin@ujf-grenoble.fr

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_10,

1 Introduction

Various chiral selectors (CSs) have been introduced in liquid chromatography (LC), capillary electrophoresis (CE), and capillary electrochromatography (CEC) for the separation and quantification of enantiomers. To date, cyclodextrins, ligand-exchange mechanism-based selectors, crown ethers, cellulose and amylose derivatives, macrocyclic antibiotics, synthetic polymers, alkaloids or proteins constitute the most widely used CSs for the chiral discrimination of active molecules [1]. The main strategy employed to resolve a racemic compound of interest is typically based on the rational screening of such "conventional," commercially available CSs and/or the use of database. This can be time consuming and, in some cases, the enantiomeric separation cannot be achieved. Furthermore, the elution/migration order of enantiomers is generally not predetermined and the optimization of the separation operating conditions may be difficult to attain. In this context, an alternative approach, which is related to the development of CSs specifically designed against the racemate to resolve (target-specific CSs), has gained increasing interest over the last years. Two kinds of target-specific CSs have been reported: the molecular imprinted polymers (MIPs) and the bioaffinity systems such as antibodies and nucleic acid aptamers. The applications and properties of MIPs in LC and CE chiral separations have been extensively studied, and recent detailed reviews have been published [2, 3]. Recently, the use of enantioselective antibodies in the analytical field, including notably the LC applications of the antibody-based CSPs, has been exhaustively reviewed by Hofstetter et al. [4].

Aptamers are single-stranded DNA or RNA oligonucleotides (from 20 to 70 bases in general) which are generally isolated by the systematic evolution of ligands by exponential enrichment (SELEX) procedure. The SELEX process is a combinatorial technique for the screening of very large libraries of oligonucleotides by an iterative process of in vitro selection and amplification. The random sequence of the oligonucleotide library is incubated with a target of interest. Oligonucleotides with an affinity toward the target are isolated from the tremendous number of species in the library, the unbound fraction being separated by separation techniques such as chromatography, magnetic bead technology, and capillary electrophoresis. The bound oligonucleotides are then isolated and amplified to obtain an enriched library which is used for a next selection/amplification cycle. The efficiency of enrichment of binders is governed by the stringency of the selection of each round. After around 10–15 cycles, the enriched library is cloned and sequenced. More details on the principle of the SELEX technology can be found in the literature [5, 6].

Like antibodies, aptamers are characterized by very impressive, unsurpassed affinity, and selectivity properties. Such remarkable feature has determined immense potentialities in the diagnostic field and various analytical systems have been developed, notably in the field of biosensors, ELISA-type assays, flow cytometry, or separation techniques [6, 7]. Specifically, aptamers constitute, with antibodies, the most popular affinity reagent employed for the development of bioaffinity-based LC and CE methodologies. However, the aptamers present many advantages over antibodies. Aptamers can be regenerated within minutes via a denaturation–renaturation

step and are, at least for DNA aptamers, relatively stable over the time. The in vitro selection can be manipulated to obtain binding properties desirable for specific assays. The aptamers can be produced by chemical synthesis resulting in little or no batch-to-batch variation and reporter or functionalizing molecules can be attached to aptamers at precise locations. Furthermore, they are produced through an in vitro process which does not require animals.

In this chapter, we focus particularly on the enantioselective properties of aptamers and their recent use as target-specific CSs in LC and CE. The main features of the aptamer-based chiral separations and the influence of two essential factors, i.e., the separation medium composition and the column/capillary temperature, on the enantiodiscrimination were especially emphasized.

2 Enantioselective Properties of Aptamers

2.1 General Characteristics of the Binding Mechanism for Small Molecule Target

Aptamers are characterized by a specific and complex three-dimensional shape and contain various structural motifs that can be stems, loops, bulges, hairpins, pseudoknots, triplexes, or quadruplexes. Aptamers can bind to a wide variety of targets from single molecules to whole organisms. Numerous aptamers have been selected toward small molecules including nucleotides, nucleosides, cofactors, amino acids, drugs, carbohydrates. The small molecule–aptamer complexes display generally dissociation constants in the micromolar range. In rare cases, lower K_d values have been observed, a dissociation constant as low as ~800 pM has even been reported for the tetracycline binding to its corresponding RNA aptamer [8].

Such impressive affinity is expected to originate from the unique conformational flexibility and adaptability features of nucleic acid aptamers. A conformational transition is classically associated with the aptamer–target complex formation, i.e., an induced-fit binding mode [9]. In the more generally accepted binding model, the target associates to the aptamer that exists as multiple interconverting conformers, one of this conformer being competent for binding. The mechanism between the aptamer and its cognate ligand is then assumed to be governed by at least two critical steps [10]. The first target-independent step is related to the conformational change from inactive species to the active aptamer. The second step is related to the binding of the target to the active aptamer conformer. This is governed by slow association and dissociation rate constants: association rate constant values as small as 10^4–10^6 M^{-1} s^{-1} have been reported for the small target binding to aptamer associated to dissociation rate constant comprised between 10^{-2} and 10^{-4} s^{-1}.

From a structural point of view, the nucleic acid receptor folds upon small molecule binding from a more or less disordered structure into a well-defined binding pocket, encapsulating the target. Numerous studies have shown that such structural reorganization of the functional nucleic acid, mediated by the small

target complexation, can imply base rearrangements, sugar–phosphate backbone distortions, structural motif reorientations, three-dimensional topology changes, and gyration radius variations [11–13]. For example, it has been demonstrated that the radius of gyration of the anti-argininamide DNA aptamer would decrease by nearly 20% when it is in its ligated form (Fig. 1) [12]. Such argininamide encapsulation would be accompanied by a strong reduction of the solvent accessible surface area upon complex formation in the order of 1000 $Å^2$ [12]. Another analysis by circular dichroism has shown that the anti-tyrosinamide DNA aptamer undergoes a DNA B-form to A-form transition upon the target binding [13].

This induced-fit mechanism is of crucial importance as it governs the adjustment of the recognition surface of the aptamer binding pocket with the target. So, an optimal shape complementarity between the two partners is established allowing tightly packed contacts between them. Dependent on the target nature and its functional groups as well as the SELEX procedure used, hydrogen bonds, electrostatic interactions, stacking interactions, and hydrophobic effect are the more common driving forces which are involved in the molecule binding to the aptamer. For example, the interaction between aromatic targets such as theophylline, flavin mononucleotide,

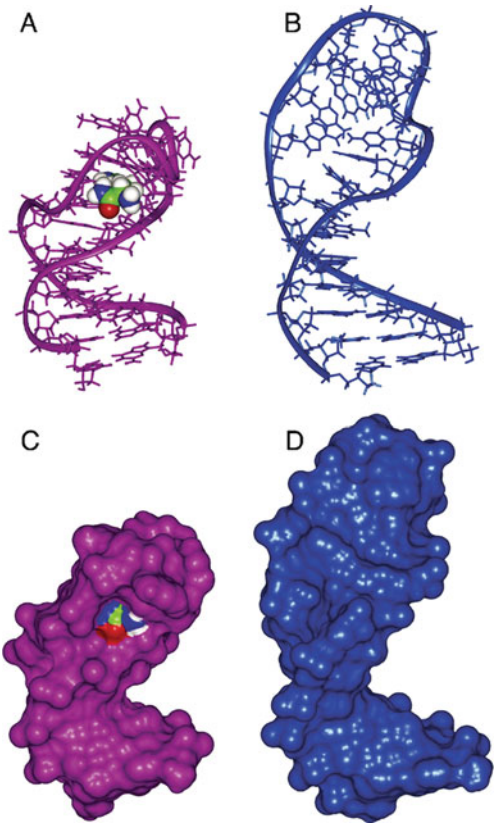


Fig. 1 The L-argininamide bound (**a, c**) and unbound (**b, d**) forms of anti-L-argininamide DNA aptamer. From [12]

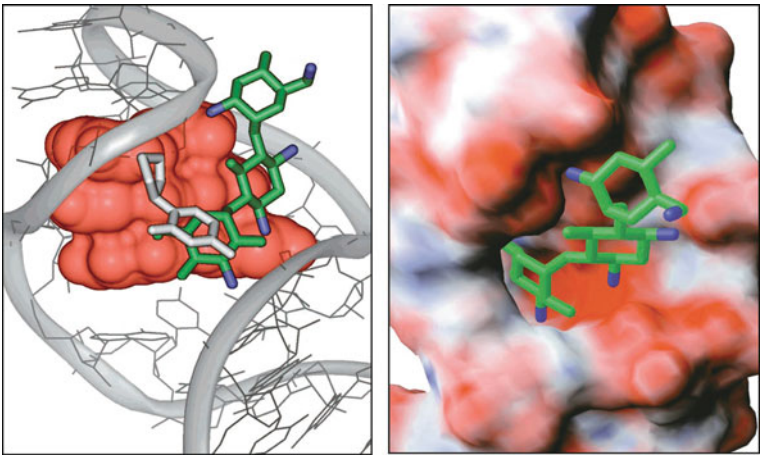


Fig. 2 Molecular recognition of the aminoglycoside antibiotic tobramycin (*green*) by an RNA aptamer. From [9]

or AMP and their corresponding aptamers is based on a similar mechanism which involves stacking interactions with a planar surface into the binding pocket and additional intermolecular hydrogen bonding contacts which contribute to the selectivity [9]. On the other hand, aminoglycosidic targets are typically recognized by their aptamers through a combination of electrostatic and shape complementarity along with distinct hydrogen bonds involving polar groups in the antibiotics [9]. A representative example of resolved structure for the tobramycin target–RNA aptamer complex is presented in Fig. 2.

2.2 Enantioselectivity

Although the aptamers were not selected, in most cases, for a selective binding, the efficient monitoring of the SELEX procedure has often allowed a very high specificity exemplified by the capability of the aptamer to bind specifically the target. For example, the theophylline-binding RNA aptamer displays an affinity for its cognate ligand 10,000 times that of caffeine, which differs from theophylline by only a single methyl group [14]. In this context, aptamers selected against a chiral target appear to be very frequently enantiospecific. A necessary condition is that the target immobilization to the matrix (chromatographic support, magnetic beads, filter, etc.) must allow an adequate exposure of the enantiomer key functional groups to the oligonucleotide pool during the in vitro selection. If necessary, and in order to isolate nucleic acid sequences with higher enantioselectivity, a SELEX methodology involving a counter-selection with the non-target enantiomer can be performed so that non-enantioselective sequences were successfully removed from

the nucleic acid pool [15]. Usually, the in vitro selection of aptamers has been carried out using an enantiopure target immobilized on a matrix. Alternatively, it has been shown recently by Sawai and coworkers that enantiospecific aptamers can also be isolated from a racemic mixture of targets [16]. It has been shown that, although the selection is carried out using a racemic thalidomide derivative as target, the selected aptamer clone shows high binding affinity for the *R*-form of thalidomide but not for the *S*-form. This result is of crucial importance as it becomes therefore possible to generate target-specific enantioselective CSs even using a non-resolved racemic mixture of compounds. The various DNA and RNA aptamers isolated against chiral compounds display commonly very important enantioselectivity. In some cases, extreme enantioselectivity ranging from about 100 to more than 10,000 have been reported for compounds such as amino acids (histidine, tryptophan, arginine), nucleosides (adenosin e), drugs (thalidomide), or oligopeptides (vasopressin) [15–20]. From a mechanistic point of view, it is very likely that such stereoselectivity originates from the unique binding mechanism of aptamers. The non-target enantiomer is assumed to fit less adequately to the aptamer binding pocket, engaging less stabilizing contacts with the aptamer and/or being submitted to significant steric hindrance or electrostatic repulsion. This results in the lower stability of the diastereoisomeric complex formed. In the future, experiments allowing the elucidation of the three-dimensional structures of a complex between an aptamer and a non-target enantiomer could be of great interest to understand in details the fine enantioselective interaction balance mediating the chiral discrimination mechanism.

3 The Aptamer-Based Chiral Discrimination in LC and CE

3.1 Main Features of the Aptamer-Based Chiral Separation

Aptamers have been successfully exploited in target-specific chiral separations both in LC (immobilized CS as chiral stationary phases, CSPs) and CE (CS added to the background electrolyte) [21–28]. Table 2 summarizes the different aptamer-based chiral stationary phases and the CE-based assays reported at the present time and the related chromatographic and electrophoretic results.

Three major aspects can be emphasized. First, and as expected from the aptamer properties evoked above, a predetermined enantioselectivity is already observed. The target enantiomer presents more affinity for the CS than the non-target enantiomer so that it is more retained on the aptameric column or migrated more slowly in the capillary during the electrophoretic run [21–28]. High to very high separation factors are commonly observed in LC. In some cases, the α-value cannot be even determined accurately due to the fact that the non-target enantiomer does not interact significantly with the aptamer (Table 1). For example, D-vasopressin and L-arginine were eluted roughly in the void volume on the anti-L-vasopressin or the anti-D-arginine aptamer chiral stationary phases (CSPs), respectively [21, 24]. The relatively low α-values reported to date have been obtained with the anti-tyrosine

Table 1 Main features of the LC and CE aptamer-based chiral separations

Aptamer chiral stationary phase						
Target [ref]	Aptamer	Immobilization type[a]	Binding capacity[b]	Separation factor (α)[c]	Target efficiency (h)[b,d]	Stability[e]
Vasopressin [21]	DNA (55 mer)	NC	nd	na	35–40 (20°C)	≥ 5 months
Adenosine [22]	DNA (37 mer)	NC	nd	3.4 (24°C)	nd	≥ 2 months
Adenosine [23]	DNA (37 mer)	C	3.4 nmol/100 μL	3.3 (24°C)	30 (24°C)	≥ 6 months
Tyrosinamide [22]	DNA (49 mer)	NC	nd	80 (20°C)	nd	1 month
Arginine [24]	RNA (44 mer)	NC	nd	na	nd	Few days
Arginine [24]	L-RNA (44 mer)	NC	nd	na	nd	≥ 3 months
Histidine [25]	L-RNA (40 mer)	NC	3.3 nmol/100 μL	20 (10°C)	nd	≥ 2 months
Aromatic amino acids and derivatives: tyrosine, tryptophan, DOPA [26]	L-RNA (63 mer)	NC	nd	1.3–30 (10°C)	25–120 (20°C)	≥ 3 months
CE aptamer chiral additive						
Target	Aptamer	CE mode	Aptamer concentration	Selectivity factor[f]	Target efficiency (N)[d]	Stability[g]
Arginine [27]	L-RNA (53 mer)	Partial-filling CZE	80 μM	1.05 (60°C)	2000–10,000 (55–60°C)	~1 week
Nucleotides: AMP, ADP, ATP [28]	DNA (37 mer)	Partial-filling MEKC	200 μM	1.10 (30°C)	1000–20,000 (10–40°C)	~1 week

[a] *NC*: Non-covalent, biotin–streptavidin link between the aptamer CS and the chromatographic support; C: covalent, amide bond for the aptamer CS immobilization to the chromatographic support; AMP, ADP, ATP: adenosine mono, di, tri-phosphate

[b] nd: Not determined

[c] Separation factor: Ratio of the enantiomer retention factors; na: not available, the too weak retention factor of the non-target enantiomer cannot allow calculating with accuracy the enantioselectivity

[d] h: Reduced theoretical plate height; N: theoretical plate number

[e] Stability of the CSP over the time under conventional chromatographic use and storage

[f] Selectivity factor: Ratio of the enantiomer migration times

[g] Time of use of the same aptamer CS solution employed as CE chiral additive

Table 2 Thermodynamic parameters associated with the enantiomer retention on some aptamer CSPs

Target [ref]	Aptamer CSP	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	ΔCp (kJ/mol.K)
L-Vasopressin [21]	Anti-L-vasopressin	−23.4 (25°C)	3.0 (25°C)	–3.78
L-Tyrosinamide [22]	Anti-L-tyrosinamide	−139.4	nd	/
D-Adenosine [22]	Anti-D-adenosine	−71.4	nd	/
L-Adenosine [22]	Anti-D-adenosine	−65.6	nd	/
L-Histidine [25]	Anti-L-histidine	−69.5	nd	/

nd: Not determined

aptamer column for related compounds such as tryptophan and its derivatives and DOPA. Moreover, this is the first example of an aptamer CSP which is able to separate the enantiomers of several compounds, i.e., a class-specific aptameric CSP [26]. The immobilization strategy of the aptamer to the chromatographic support does not affect significantly the chiral recognition properties of the oligonucleotide [23]. Both non-covalent (biotin–streptavidin link) and covalent (amide bond) immobilization have been evaluated for the anti-adenosine aptamer and similar apparent enantioselectivity were reported (Table 1).

Another important aspect is related to the separation efficiency. As reported above, the induced-fit mechanism of aptamer is characterized by slow association and dissociation rate constants. For the separation applications, this determines very poor efficiency performances for the target enantiomer. In some chromatographic experiments, the values of reduced plate height (h) can be as high as 100 and the theoretical plate number (N) can be as low as 1000 in CE separation (Table 1). As previously expected [28], it is possible that the presence of different aptamer conformers with distinct affinity and kinetic properties for the target enantiomer could be responsible for a heterogeneous binding system, participating also to the low efficiency value.

Finally, the DNA aptamer CSs appear to be of acceptable stability over the time under usual chromatographic/electrophoretic utilization and storage (Table 1). In contrast, the RNA aptamer CSs are rapidly degraded by RNases [24]. To overcome this severe limitation for a practical use, the mirror image strategy can be applied, using an L-RNA aptamer as CS. L-RNA aptamer is the mirror image of the "natural" D-RNA aptamer. Due to the stereospecificity of the RNases, the L-RNA aptamer CS is unsusceptible to the naturally occurring enzymes and then can be used both as CSP or chiral additive in CE during an extended period of time (typically several months) without degradation [24].

3.2 Role of the Separation Medium Composition on the Chiral Separation

From the various studies performed during these last years, it appears that the binding and chiral recognition properties of aptameric CSs are highly dependent on the

separation medium composition. First of all, the chromatographic/electrophoretic separation can be achieved only under reversed-phase conditions as the presence of water and mono- and/or divalent cations is a necessary condition to maintain the active, functional conformation of the nucleic acid CS. It is well known that cations promote folding by reducing the repulsion between nucleic acid phosphates. Generally, millimolar Mg^{2+} concentrations are able to stabilize tertiary structures and it is well established that Mg^{2+} promotes the formation of the active aptamer from the inactive conformers [29, 30]. To note, for the particular case of the quadruplex-based aptamers, the 3D structure is commonly stabilized by the presence of a monovalent cation (especially potassium) in the center of the tetrads. Thus, the Mg^{2+} concentration, in the mobile phase or the background electrolyte is of major importance. For example, the chiral chromatographic separation of histidine is very strongly increased when the $MgCl_2$ concentration in the mobile varied from 0 to 10 mM (Fig. 3) [25]. Furthermore, it has been shown that the enantioseparation of adenosine using an anti-adenosine aptamer CSP is completely abolished when the column is flushed with a buffer containing 5 mM of EDTA [23]. In addition, the variation of the ionic strength of the mobile phase or electrolyte can affect the direct association between a cationic target compound and the aptamer. If Coulomb interactions are involved in the binding process, a decrease in the equilibrium association constant is observed with increasing salt concentration. This has been exemplified with the chiral separation of vasopressin on an aptamer CSP. The increase in the NaCl concentration from 25 to 100 mM in the mobile phase is responsible for a reduction of the target enantiomer retention factor [21]. Moreover, from the data, it has been deduced that a charge–charge interaction is involved in the target binding.

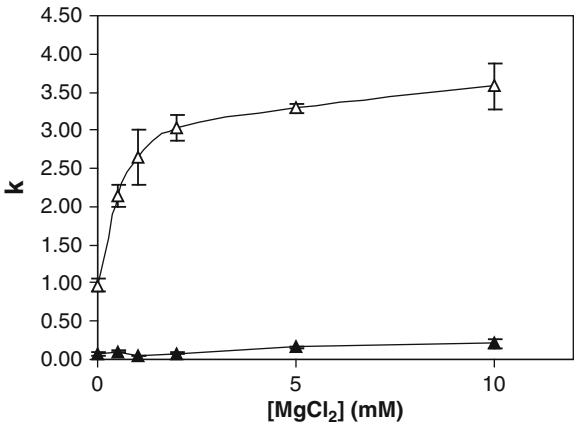


Fig. 3 Plots of retention factor *k* versus mobile phase $MgCl_2$ concentration for D-histidine (*open* symbol) and L-histidine (*filled* symbol) using an anti-D-histidine L-RNA aptamer CSP. Column: 260 × 0.80 (i.d) mm; mobile phase: Hepes buffer 50 mM, NaCl 250 mM, pH: 6.5; column temperature: 20°C; flow rate: 15 μL/min. Adapted from [25]

Besides the nature and the concentration of salt in the separation medium, the mobile phase or background electrolyte pH can also be an important criterion to modulate the interaction of ionizable targets with their corresponding aptamers. As a representative example, the histidine enantiomers are optimally separated on an anti-histidine column at a pH of 5.5, i.e., when the target is partially under its protonated form [25].

The addition of relative small amounts of organic modifiers in the LC mobile phase is possible and can be accounted to alter the retention time and modify the chiral separation of analytes. At the present time, various solvents have been used including acetonitrile, methanol, ethanol, and 2-propanol, with proportion varying from 0 to 10–25% in relation to the type of organic modifiers and the aptameric CSPs employed [23, 26]. Commonly, the addition of such organic solvents is responsible for a reduction of the retention factors of the analyzed target solutes such as adenosine or aromatic amino acid derivatives, revealing that hydrophobic effects are involved in the analyte interaction with the aptamer CSP. It is also possible that a solvent-dependent change of the active aptamer conformation occurs, governing a reduction of the binding affinity of the solute for the aptamer CSP. The enantioselective properties of the aptamer CSP can be variably affected by the presence of the organic modifiers. In some cases , the α-value is not altered with the mobile phase solvent increasing while in other ones, a weak reduction of the apparent separation factor is reported. Nevertheless, the addition of organic modifiers can be a good strategy to reduce the retention time of highly retained compounds, without altering so much the enantioselective properties of the aptamer CSP [23, 26].

3.3 Role of the Temperature on the Chiral Separation

Another essential parameter that controls and modulates the chiral separation is the column or capillary temperature. As the temperature increases, the target enantiomer binding to the aptamer is commonly very strongly diminished [22–28]. This is exemplified in Fig. 4 by the van't Hoff plot (ln k vs $1/T$) obtained for the L-tyrosinamide enantiomer on the anti-L-tyrosinamide aptamer column. The enthalpic term values (ΔH) values obtained for different target enantiomer–aptamer CSP systems studied are presented in Table 2. These are of very great magnitude, varying from -25 to -140 kJ/mol. Such data can be explained by the fact that the target is encapsulated by the aptamer so that a very tight complex, involving strong attractive interactions and/or a great number of interaction points, is formed. In addition, for the vasopressin binding to the aptamer CSP, a large and negative heat capacity change has been reported upon the target enantiomer binding [21] (Table 2). This is consistent with a complex formation in which several contacts between non-polar groups of two species are engaged. A temperature-dependent change in the aptamer conformation or in the relative population of the conformers could also be involved in the variation of the target binding to the aptamer CS [27]. The decrease in the target enantiomer binding with temperature increasing

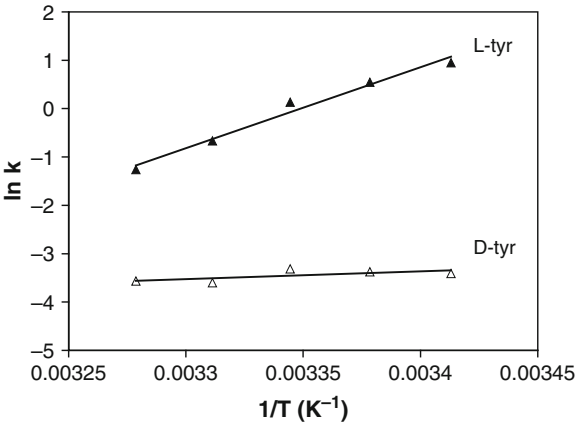


Fig. 4 van't Hoff plots ln k vs $1/T$ for D, L-tyrosinamide (k: retention factor) using the anti-L-tyrosinamide aptamer CSP. Temperature (T) range: 20–32°C. Column: 250 × 0.76 mm; mobile phase: phosphate buffer 20 mM, KCl 25 mM, $MgCl_2$ 1.5 mM, pH 6.0; flow rate: 20 μL/min. Error bars are within the experimental points. Adapted from [22]

is commonly associated with a significant reduction of the apparent selectivity [22–28]. As can be seen in Fig. 5, the adenosine monophosphate (AMP) racemate is very well resolved at 20°C in a partial-filling aptamer-modified micellar electrokinetic chromatography (MEKC) mode while the enantioseparation is greatly reduced at 40°C [28].

Another important aspect is related to the temperature effects on the separation efficiency. As reported above, the slow association–dissociation kinetics for the complex formation is responsible for very poor efficiency performances. As kinetics is highly dependent on the temperature, its modulation is an excellent way to enhance the efficiency of the aptamer separation systems. It has been reported

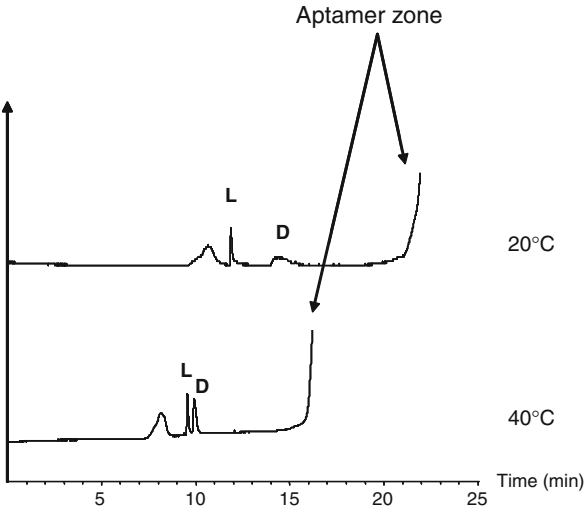


Fig. 5 Effects of the capillary temperature on the adenosine monophosphate (AMP) enantioseparation under aptamer-modified MEKC conditions. Capillary: 50 μm inner diameter PVA-coated silica capillary. Adapted from [28]

that the column/capillary temperature increase is responsible for a strong improvement of the *N* values both in LC and CE, through faster mass transfer kinetics [25, 27, 28]. As a representative example (Fig. 5), the *N* value of the D-AMP target in an aptamer-modified MEKC mode varies from 1100 at 20°C to 21,000 at 40°C [28].

4 Conclusion

The mechanism of the target enantiomer binding to the aptamer CS is quite unusual in the chiral discrimination field. There are some cases of conformational changes of the selector when bound to enantiomers. For example, some proteins such as serum albumins or antibodies are conformationally modified upon enantiomer association [31, 32]. However, these structural changes are commonly subtle and of weak amplitude. In contrast, due to the extreme flexibility and adaptability of the aptamer, the target binding is typically associated to a larger structural transition of the nucleic acid which governs its tight encapsulation. This induced-fit mechanism determines the following chiral separation properties of the aptamer CSs:

– high to very high affinity and enantiospecificity
– poor to very poor efficiency performances
– extreme dependence to the presence of water and cations (especially Mg^{2+})
– very strong effects of the column/capillary temperature

More globally, the strong affinity and selectivity properties of aptamers can found very promising applications in other chiral analysis fields, such as the design of enantioselective assays or sensors. Moreover, very recently, enantioselective aptamer-based affinity probe CE and fluorescence polarization assays have been successfully designed for the rapid detection of enantiomeric impurities [33–35].

References

1. Francotte ER (2001) Enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers. J Chromatogr A 906:379–397
2. Ansell RJ (2005) Molecularly imprinted polymers for the enantioseparation of chiral drugs. Adv Drug Deliv Rev 57:1809–1835
3. Nilsson J, Spégel P, Nilsson S (2004) Molecularly imprinted polymer formats for capillary electrochromatography. J Chromatogr B 804:3–12
4. Hofstetter H, Hofstetter O (2005) Antibodies as tailor-made chiral selectors for detection and separation of stereoisomers. Trends Anal Chem 10:869–879
5. Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. Nature 346:818–822
6. Jayasena SD (1999) Aptamers: an emerging class of molecules that rival antibodies in diagnostics. Clin Chem 45:1628–1650

7. Tombelli S, Mascini M (2009) Aptamers as molecular tools for bioanalytical methods. Curr Opin Mol Ther 11:179–188
8. Müller M, Weigand JE, Weichenrieder O et al (2006) Thermodynamic characterization of an engineered tetracycline-binding riboswitch. Nucleic Acids Res 34:2607–2617
9. Hermann T, Patel DJ (2001) Adaptive recognition by nucleic acid aptamers. Science 287:820–825
10. Latham MP, Zimmermann GR, Pardi A (2009) NMR chemical exchange as a probe for ligand-binding kinetics in a theophylline-binding RNA aptamer. J Am Chem Soc 131:5052–5053
11. Schneider C, Sühnel J (1999) A molecular dynamics simulation of the flavin mononucleotide-RNA aptamer complex. Biopolymers 50:287–302
12. Bishop GR, Ren J, Polander BC et al (2007) Energetic basis of molecular recognition in a DNA aptamer. Biophys Chem 126:165–175
13. Lin PH, Yen SL, Lin MS et al (2008) Microcalorimetrics studies of the thermodynamics and binding mechanism between L-tyrosinamide and aptamer. J Phys Chem B 112:6665–6673
14. Jenison RD, Gill SC, Pardi A et al (1994) High-resolution molecular discrimination by RNA. Science 263:1425–1429
15. Klussmann S, Nolte A, Bald R et al (1996) Mirror-image RNA that binds D-adenosine. Nat Biotechnol 14:1112–1115
16. Shoji A, Kuwahara M, Ozaki H et al (2007) Modified DNA aptamer that binds the (R)-isomer of a thalidomide derivative with high enantioselectivity. J Am Chem Soc 129:1456–1464
17. Geiger A, Burgstaller P, von der Eltz H et al (1996) RNA aptamers that bind L-arginine with sub-micromolar dissociation constants and high enantioselectivity. Nucleic Acids Res 24:1029–1036
18. Majerfeld I, Puthenvedu D, Yarus M (2005) RNA affinity for molecular L-histidine; genetic code origins. J Mol Evol 61:226–235
19. Majerfeld I, Yarus M (2005) A diminutive and specific RNA binding site for L-tryptophan. Nucleic Acids Res 33:5482–5493
20. Williams KP, Liu XH, Schumacher TN et al (1997) Bioactive and nuclease-resistant L-DNA ligand of vasopressin. Proc Natl Acad Sci USA 94:11285–11290
21. Michaud E, Jourdan EA, Villet A et al (2003) A DNA aptamer as a new target-specific chiral selector for HPLC. J Am Chem Soc 125:8672–8679
22. Michaud M, Jourdan E, Ravelet C et al (2004) Immobilized DNA aptamers as target-specific chiral stationary phases for resolution of nucleoside and amino acid derivative enantiomers. Anal Chem 76:1015–1020
23. Ruta J, Ravelet C, Désiré J et al (2008) Covalently bonded DNA aptamer chiral stationary phase for the chromatographic resolution of adenosine. Anal Bioanal Chem 390:1051–1057
24. Brumbt A, Ravelet C, Grosset C et al (2005) Chiral stationary phase based on a biostable L-RNA aptamer. Anal Chem 77:1993–1998
25. Ruta J, Grosset C, Ravelet C et al (2007) Chiral resolution of histidine using an anti-D-histidine L-RNA aptamer microbore column. J Chromatogr B 845:186–190
26. Ravelet C, Boulkedid R, Ravel A et al (2005) A L-RNA aptamer chiral stationary phase for resolution of target and related compounds. J Chromatogr A 1076:62–70
27. Ruta J, Ravelet C, Grosset C et al (2006) Enantiomeric separation using an L-RNA aptamer as chiral additive in partial-filling capillary electrophoresis. Anal Chem 78:3032–3039
28. Ruta J, Perrier S, Ravelet C et al (2009) Aptamer-modified micellar electrokinetic chromatography for the enantioseparation of nucleotides. Anal Chem 81:1169–1176
29. Brion P, Westhof E (1997) Hierarchy and dynamics of RNA folding. Annu Rev Biophys Biomol Struct 26:113–137
30. Draper DE, Grilley D, Soto AM (2005) Ions and RNA folding. Annu Rev Biophys Biomol Struct 34:221–243
31. Tittelbach V, Gilpin RK (1995) Species dependency of the liquid chromatographic properties of silica-immobilized serum albumins. Anal Chem 67:44–47

32. Hofstetter O, Lindstrom H, Hofstetter H (2004) Effect of the mobile phase on antibody-based enantiomer separations of amino acids in high-performance liquid chromatography. J Chromatogr A 1049:85–95
33. Ruta J, Ravelet C, Baussanne I et al (2007) Aptamer-based enantioselective competitive binding assay for the trace enantiomer detection. Anal Chem 79:4716–4719
34. Ruta J, Ravelet C, Baussane I et al (2008) Competitive affinity capillary electrophoresis assay based on a "hybrid" pre-incubation/on-capillary mixing format using an enantioselective aptamer as affinity ligand. J Sep Sci 31:2239–2243
35. Ruta J, Perrier S, Ravelet C et al (2009) Noncompetitive fluorescence polarization aptamer-based assay for small molecule detection. Anal Chem 81:7468–7473

Chiral Ionic Liquids in Chromatographic Separation and Spectroscopic Discrimination

Min Li, David K. Bwambok, Sayo O. Fakayode and Isiah M. Warner

Contents

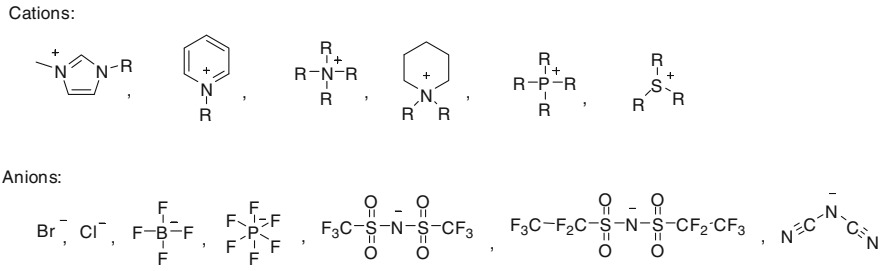
Abstract Chiral ionic liquids (CILs) are a subclass of ionic liquids (ILs) in which the cation, anion, or both may be chiral. The chirality can be central, axial, or planar. CILs possess a number of unique advantageous properties which are inherited from ionic liquids including negligible vapor pressure, wide liquidus temperature range, high thermal stability, and high tunability. Due to their dual functionalities as chiral selectors and chiral solvents simultaneously, CILs recently have been widely used both in enantiomeric chromatographic separation and in chiral spectroscopic discrimination. In this chapter, the various applications of CILs in chiral chromatographic separations such as GC, HPLC, CE, and MEKC are reviewed. The applications of CILs in enantiomeric spectroscopic discrimination using techniques such as NMR, fluorescence, and NIR are described. In addition, chiral recognition and separation mechanism using the CILs as chiral selectors or chiral solvents is also discussed.

I.M. Warner (✉)
Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA
e-mail: iwarner@lsu.edu

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7_11, © Springer-Verlag Berlin Heidelberg 2010

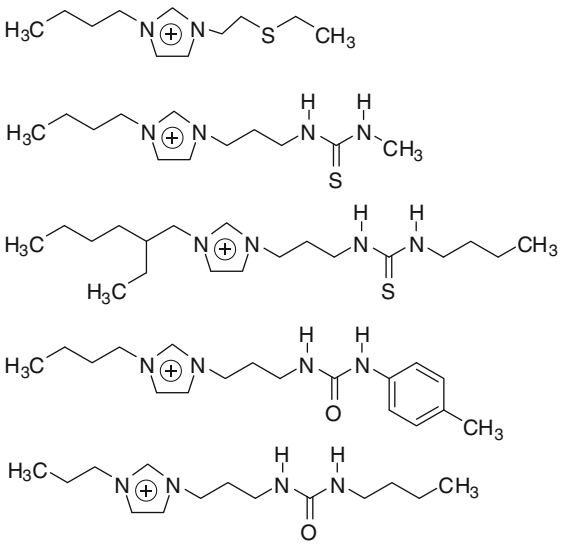
1 Introduction

Currently, one of the major fields of interests for further and extensive studies in chemistry is the development of novel solvents with reduced toxicity, biodegradability, environmentally benign capability, and high biocompatibility [1]. In this regard, much attention has been devoted to ionic liquids (ILs) [2, 3]. ILs are compounds entirely composed of ions and may be liquid at or close to room temperature [4, 5]. This definition that still holds today was given by Paul Walden in 1914 after discovering the first ionic liquid, ethylammonium nitrate with a melting point of 12°C [6, 7]. An arbitrary temperature limit of 100°C has been used to distinguish ILs from inorganic molten salts which generally have high melting points [3]. The low melting point of ILs has been attributed to low lattice energy due to asymmetry of the component cations and anions. This asymmetry leads to frustrated crystal packing [8]. After being latent for sometime, research activity on room temperature ionic liquids (RTILs) was renewed with the discovery of alkylpyridinium and 1,3-dialkylimidazolium haloaluminate salts [9]. In 1975, the first electrochemical study on these new liquid salts such as [ethylpyridinium bromide]-[$AlCl_3$] was reported [10]. The Lewis acidity of these ILs could be tuned by varying the molar ratio of the two ionic components. However, these haloaluminate ionic liquids are extremely sensitive to hydrolysis by atmospheric moisture and require handling strictly under anhydrous conditions. This problem was alleviated with the discovery of imidazolium ILs containing tetrafluoroborate and hexafluorophosphate anions that do not impose such special handling requirements [11, 12]. This has led to extensive research and preparation of a large variety of ILs by choosing various combinations of cations and anions (Scheme 1) [3, 13]. Such ILs are generally regarded as non-functionalized ILs.



Scheme 1 Representative cations and anions used in the synthesis of ILs

The greater than exponential development in the ionic liquid research has generated several research divisions. One of the new directions of ionic liquid research is the preparation of novel functionalized ILs or task-specific ILs and the exploration of their properties and applications such as in metal extraction (Scheme 2) [14, 15]. Among the various research areas in the field of novel functionalized ILs, the synthesis and application of chiral ionic liquid s (CILs) [16] has attracted considerable



Scheme 2 Molecular structures of task-specific IL cations used for metal extraction

interest in both organic and analytical chemistry. Other functionalized ILs of interest include magnetic or metal containing ILs [17] and protic ILs [18]. These functionalized ILs have found wide applications such as in catalysis [19], organic synthesis [20], separation [21], electrochemistry [22], and energy storage [23].

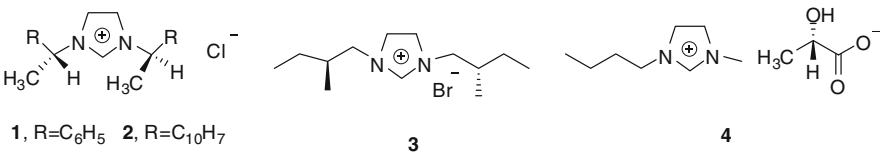
2 Chiral Ionic Liquids (CILs)

2.1 Introduction to CILs

CILs are a subclass of ILs in which the cation or the anion (or both) may be chiral. The chirality can be either central, axial, or planar. It is well established that chirality plays an important role in chemistry. Over the last few years, research for new chiral selectors, solvents, and materials based on CILs has become a topic of increasing interest. A growing number of CILs have been designed, synthesized, and utilized for potential applications in chiral discrimination and separation [24], asymmetric catalysis and synthesis [25], as well as optical resolution of racemates [26]. Because of their high-resolution abilities and liquidus properties, CILs can be used as either chiral agents in regular solvent, or chiral solvents, or both simultaneously. With the rapid development of CILs, these new chiral solvents have the potential to play an important role in enantioselective organic chemistry, chiral separation chemistry, and chiral materials chemistry. Thus, their role in these fields is expected to expand tremendously.

2.2 Evolution of CILs

CILs are a recent development as compared to achiral ILs. During the synthesis of the *N*-heterocyclic carbene complexes based on imidazolium precursors, Herrmann and coworkers [27] prepared two chiral imidazolium chloride salts as synthesis intermediates, **1** and **2** (Scheme 3). Imidazolium salts have been widely used as cations to prepare ILs. However, the two chiral imidazolium chloride salts were not referred to as CILs in Herrmann's work [27]. To our knowledge, Howarth et al. [16] synthesized the first example of a CIL in 1997 with a chiral dialkylimidazolium cation, **3** (Scheme 3). This homochiral and moisture stable imidazolium bromide salt was used as the Lewis acid catalyst in the Diels–Alder reaction, affording low enantiomeric excess (ee less than 5%) of the product. [16]. In 1999, Seddon and coworkers reported the synthesis of CILs containing chiral anions for the first time [28]. The CIL, **4**, was prepared by an anion-exchange reaction between 1-methyl-3-butylimidazolium chloride ([BMIM][Cl]) and sodium lactate (or *S*-2-hydroxypropionate) in acetone (Scheme 3). This CIL was also used as the chiral solvent in Diels–Alder reaction although no chiral induction was obtained [28]. This may suggest that the presence of a CIL does not necessarily guarantee chiral induction or chiral recognition. Simple CILs having limited functional groups may not be able to offer enough enantiomeric interaction sites with the reagents to induce chirality transfer. The molecular structures of CILs play a fundamental role in effecting their chiral induction or discrimination abilities. Therefore, in the later work, more sophisticated CILs possessing multiple functional groups were designed and synthesized with an aim toward fully exploiting CILs. For instance, CILs have been designed and prepared from chiral building blocks obtained from the chiral pool such as amino acids [29]. Amino acids naturally contain a number of functional groups such as hydroxyl, amine, thiol, thioether, carboxylic, and amide. These functional groups adjacent to the stereogenic center can offer multiple chiral interaction sites with the reaction reagents or analytes.



Scheme 3 Molecular structures of examples of earlier CILs

2.3 Classification of CILs

The syntheses of a variety of CILs and their applications have been extensively studied. A number of outstanding reviews provide a summary of the synthesis and application of different CILs [30–37]. Basically, as functionalized ILs, CILs have

been used in enantioselective transformation reactions [38], chiral recognition [39], gas chromatography stationary phases [40], and as chiral mobile phase additives in CE [41]. As indicated earlier, CILs contain either a chiral cation, chiral anion, or both ions may be chiral. Various CILs with chiral cations have been synthesized including chiral imidazolium [42], ammonium [43], pyridinium [44], and azolinium [45]. Representative examples of chiral anions used in the synthesis of CILs include amino acid [46], lactic acid [47], borate [48], and camphorsulfonate [49]. CILs with axial [50] and planar chirality [51] have also been prepared. These various versatile-structured CILs confirmed one of the unique and highly desirable properties of ILs, namely high tunability. Basically, ILs can be designed and tailored to specific applications simply by changing the cation, anion, or their combination. Therefore, CILs could be referred to as chiral "designer solvents" [52]. In this chapter, after a brief summary of the application of CILs in asymmetric synthesis and catalysis, the focus is mainly on the various applications of CILs in enantiomeric chromatographic separation and chiral spectroscopic discrimination. Particularly, the chiral separation or discrimination mechanisms are also discussed.

3 Applications of Chiral Ionic Liquids

3.1 CILs in Asymmetric Catalysis and Synthesis

One of the most important areas of research with CILs is their use in asymmetric catalysis and synthesis. This chapter is focused on the applications of CILs in enantiomeric chromatographic separation and spectroscopic discrimination so it is not possible to study exhaustively the wealth of publications produced in this essential field [53–63].

3.2 CILs in Enantiomeric Chromatographic Separation

Enantiomeric separation of chiral molecules is very important since different enantiomers of a racemic drug may display very different medicinal properties [64]. For example, one enantiomer of a chiral drug may have the desired medicinal properties, while the other enantiomer may be harmful. The three common methods for enantiomeric separation include gas chromatography (GC) [65], capillary electrophoresis (CE) [66, 67] including micellar electrokinetic chromatography (MEKC) [68], and high performance liquid chromatography (HPLC) [69]. In addition, fluorescence [70] and mass spectroscopy [71] are also used in the chiral discrimination and analysis. There are various chiral selectors that can be used for enantiomeric separation, such as cyclodextrins, polysaccharides, molecular micelles, macrocyclic antibiotics, aptamers, proteins, and crown ethers [72–75]. However, the use of many current chiral selectors is often limited due to their low solubility, difficult syntheses, instability at high temperature, as well as high cost. In

addition, many currently available chiral selectors require the use of another solvent and sometimes more than one solvent system if the analyte and the chiral selector are not soluble in the same solvent [24]. The possibility of using CILs simultaneously as solvent and chiral selector may overcome this challenge. Therefore, CILs recently have been used as chiral selectors in a number of enantiomeric separations.

3.2.1 CILs in Gas Chromatography (GC) Separation

The low vapor pressure and high thermal stability of CILs render them suitable for enantioseparations in gas chromatography (GC). Recently, CILs have been used as chiral stationary phases (CSPs) in GC [40]. Armstrong and coworkers carried out enantiomeric separation of chiral alcohols and diols, chiral sulfoxides, some chiral epoxides and acetamides using a CIL based on ephedrinium salt. Using an ephedrinium CIL (**4**) as the CSP, enantiomeric separation of alcohols and diols was achieved (Fig. 1). The presence of both enantiomeric forms of ephedrine makes it possible to produce CSPs of opposite stereochemistry, which could reverse the enantiomeric elution order of the analytes. This offers an additional advantage that may not be easily achieved with common and widely used chiral selectors in GC such as the cyclodextrins. However, there was a decrease in enantiomeric recognition ability of the CSP after a week which the authors attributed to dehydration-induced

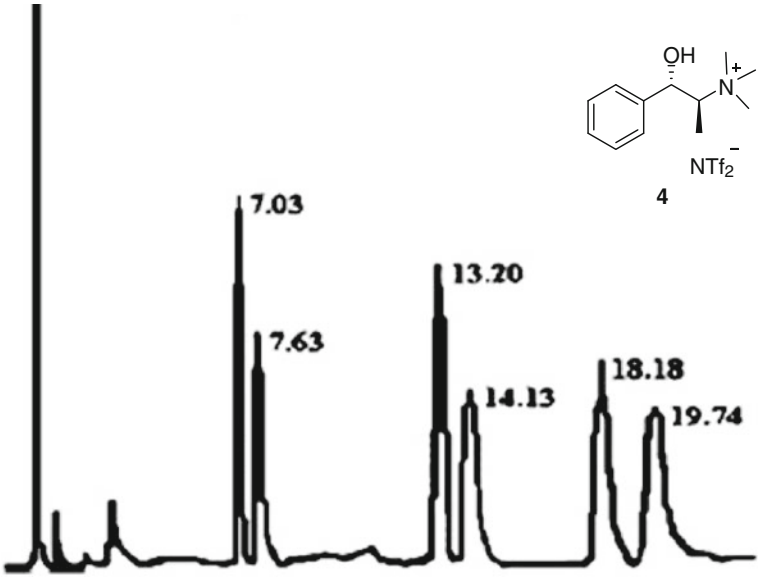


Fig. 1 GC chromatogram showing the enantiomeric separation of 2-phenethyl alcohol, 1-phenyl-1-butanol and *trans*-1,2-cyclohexenediol with a fused-silica capillary column coated with (1*S*,2*R*)-(+)-*N*,*N*-dimethylephedrinium bis(trifluoromethylsulfonyl)amide (4). Adapted from [40]

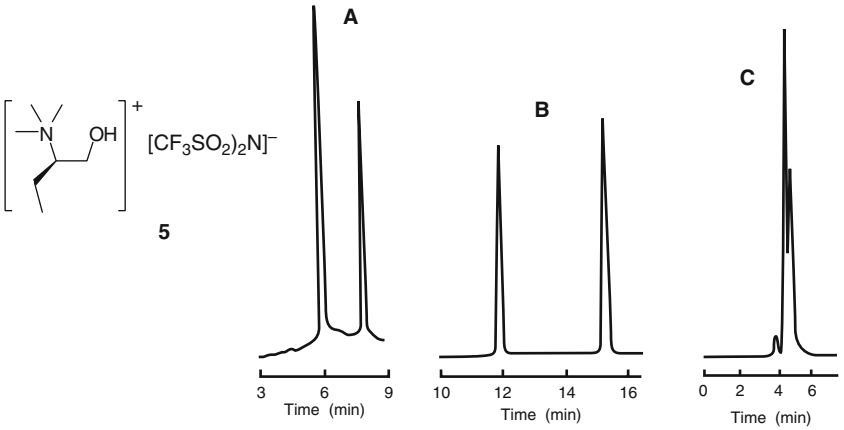


Fig. 2 The chromatograms of enantioseparation using (*R*)-*N,N,N*-trimethyl-2-aminobutanol-bis(trifluoromethanesulfon)imidate CIL **5** as chiral selector. (**a**) propranolol in HPCE, 10 mmol/L CIL **5**; voltage, 16 kV, with anodic detection at 254 nm; (**b**) enantioseparation of 2,2'-diamino-1,1'-binaphthalene in HPLC; eluent: H_2O-CH_3CN (6:4, v/v) containing 10 mmol/L of chiral selector **5**, detection, 254 nm; (**c**) enantioseparation of citronella in GC on **5**, split ratio: 80:1, FID. Adapted from [76]

racemization. This suggests that there is still a need to explore other CILs for their potential as stable CSPs in GC that are not vulnerable to such thermal racemization.

The CIL, (*R*)-*N,N,N*-trimethyl-2-aminobutanol-bis(trifluoromethanesulfon) imide (**5**), was also found to be a good chiral selector for a variety of compounds in GC (Fig. 2). For instance, this CIL afforded good resolution in the enantioseparation of citronellal (Fig. 2C) [76].

3.2.2 CILs in Capillary Electrophoresis (CE) and Micellar Electrokinetic Chromatography (MEKC) Separation

The conductivity of ILs offers the possibility of using them as electrolytes in CE. Enantiomeric separation using CILs in CE is often a desirable alternative, especially when there is a trace amount of sample available and short analysis time is required. Recently, Tran and Mejac demonstrated the separation of various pharmaceutical products using a CIL, *S*-[3-(chloro-2-hydroxypropyl) trimethylammonium] [bis(trifluoromethylsulfonyl)imide] (*S*-$[CHTA]^+[Tf_2N]^-$) (**6**), as co-electrolyte and chiral selector in the presence of chiral additives such as sodium cholate (Fig. 3) [41]. The chiral buffer was also capable of enantiomeric recognition of ibuprofen (Fig. 3). It is worth noting that no resolution was achieved when additives were not used. The authors suggest that the additives provide the three-point interaction required for chiral recognition. This clearly suggests that there is room to explore potential CILs that could offer chiral resolution without the need for other chiral additives.

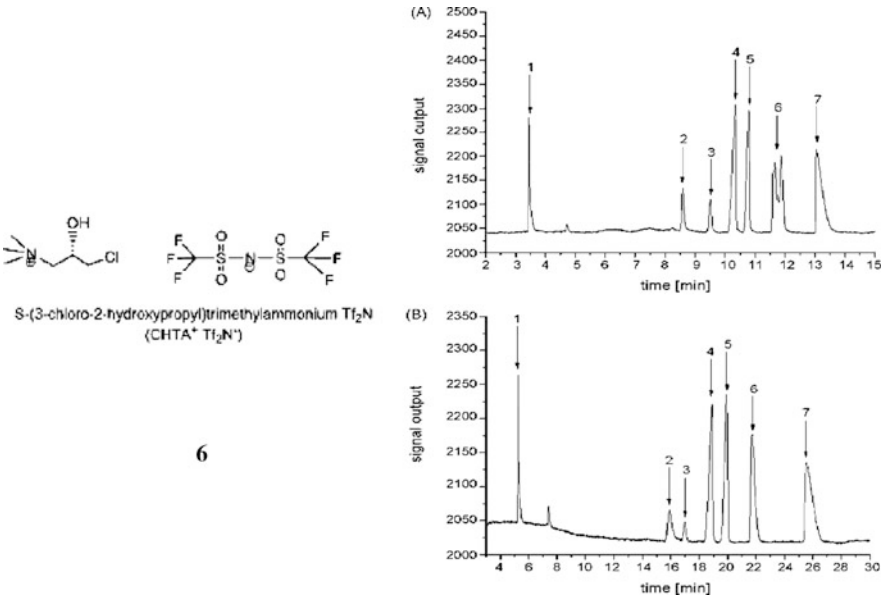


Fig. 3 Electropherograms of a sample containing a mixture of seven compounds. Bare fused-silica capillary 50 cm (effective length, 37 cm)×50 μm I.D. electrolyte: 20 mM [*S*-CHTA]$^+$[Tf_2N]$^-$ **6**, 30 mM sodium cholate; (1) Atenolol, (2) propranolol, (3) warfarin, (4) indoprofen, (5) ketoprofen, (6) ibuprofen and (7) flurbiprofen. Applied voltage: (**a**) 25 kV and with *RS*-ibuprofen; (**b**) 18 kV and with *S*-ibuprofen. Adapted from [41]

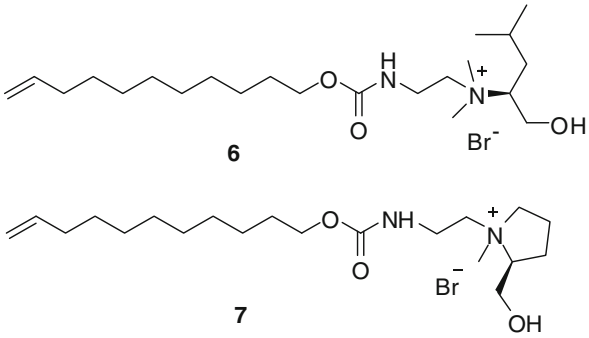
Two CILs [(ethyl and phenyl choline cations with bis (trifluoromethylsulfonyl)imide) anion] have been evaluated by Francois et al. as chiral selectors for enantiomeric separation of arylpropionic acids by CE [77]. No direct enantioselectivity was observed for these two CILs, except in the presence of β-cyclodextrins, suggesting that a synergistic effect of the two selectors is responsible for increased resolution and separation efficiency. The authors demonstrated an influence of the β-cyclodextrin on the competition between the analyte and the IL cation with respect to β-cyclodextrin complexation. However, the presence of the phenyl group in the IL cation appeared to be less important in enhancing the synergistic effects. This indicates that specific ion-pairing interactions could be involved [77].

The synthesis of a new CIL [S-(−)-2-hydroxymethyl-1,1-dimethylpyrrolidinium tetrafluoroborate] derived from L-proline alcohol have been reported by Maier et al. [78]. This CIL was found to be an effective additive to acidic background electrolytes affording the separation of a mixture of five tricyclic antidepressants using capillary zone electrophoresis (CZE). The addition of the CIL to acidic background electrolytes leads to suppression of the magnitude of electroosmotic flow (EOF) and gradually reversed the direction of the EOF. Baseline separation of the five model analytes was achieved. It was observed that the proline-derived CIL offers relatively smaller anodic EOF compared to cationic surfactants that are mostly used for

generating anodic EOF in CE. In this work, the CIL was used as the mobile phase additive. However, the analytes separated were achiral.

The application of novel IL-type like surfactants and their polymers for chiral separation of acidic analytes in MEKC was reported in 2006 by Rizvi and Shamsi [79]. These were derived from the monomers and polymers of undecenoxycarbonyl-L-leucinol bromide (L-UCLB, **6**) which is an IL at room temperature, and undecenoxy carbonyl-L-pyrolidinol bromide (L-UCPB **7**), that forms a greasy solid with a melting point of 30–35°C (Scheme 4). Chiral separation was suggested to be strongly dependent on the presence of opposite charge as well as the structural compatibility between the chiral selector and the analyte. An example of such is shown where an acidic analyte (rac)-α-bromophenylacetic acid could be separated using the monomers and the polymers of both CILs at 25 mM surfactant concentration (Fig. 4) [79].

Scheme 4 Molecular structures of cationic CIL monomers



It is worth noting that the cyclic nitrogen of compound **7** has four different alkyl groups after the quaternization reaction; therefore, an additional nitrogen stereogenic center and epimeric compounds could form accordingly. This phenomenon was not mentioned in the work [79]. According to this work [79], however, this extra stereogenic center did not significantly influence the chiral separation ability of the CIL-modified surfactant polymer, as two acidic analytes were successfully separated in the MEKC mode.

Yuan et al. found that the CIL, (*R*)-*N,N,N*-trimethyl-2-aminobutanol-bis (trifluoromethanesulfonyl) imide (**5**), is an excellent chiral selector that could be used in CE for enatioseparation of various compounds (Fig. 2) [76]. For instance, this CIL afforded the enantioseparation of propranolol which is a commonly used beta blocker (Fig. 2A) [76].

3.2.3 CILs in Liquid Chromatography (LC) Separation

In liquid chromatography (LC), the low vapor pressure of CILs is not a crucial requirement. However, CILs may provide the desired selectivity and solubility for enantiomeric separations. The possibility of replacing organic solvents by ILs in

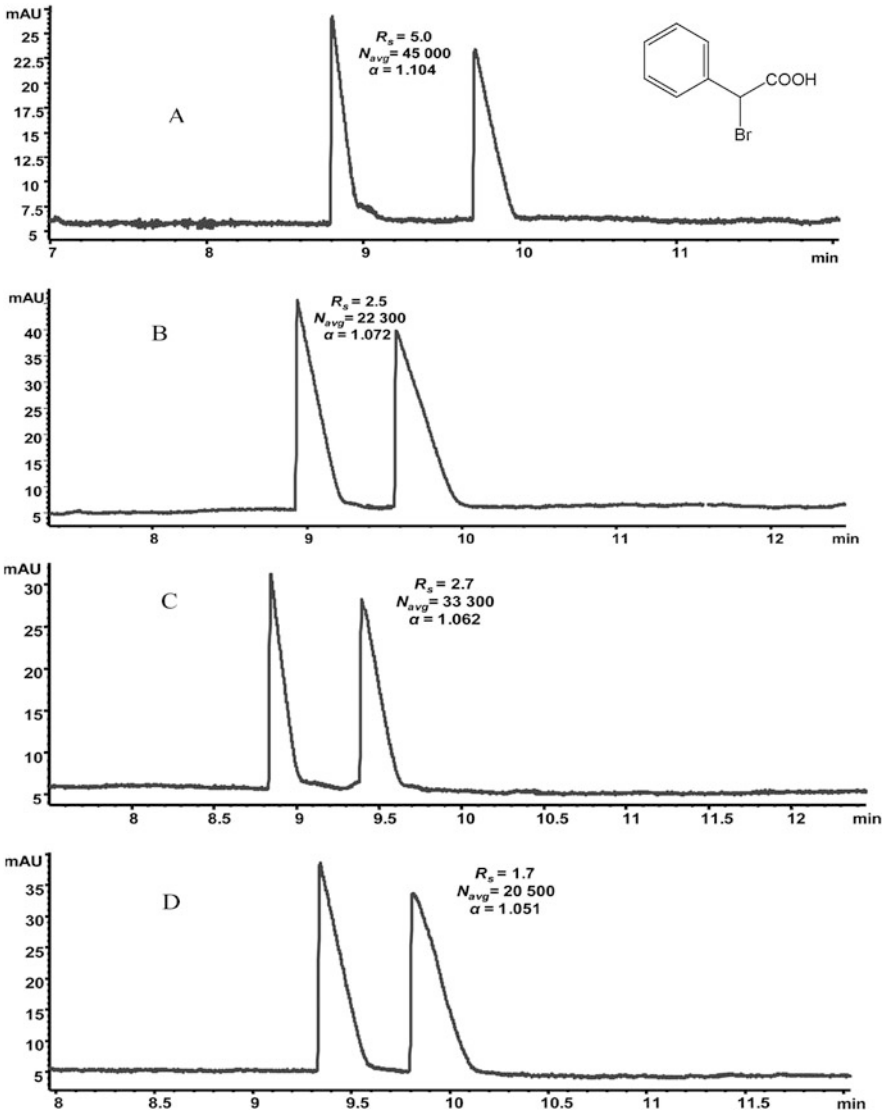


Fig. 4 Comparison of 25 mM L-UCPB (**a**), poly-L-UCPB (**b**), 25 mM L-UCLB (**c**), and poly-LUCLB (**d**) for enantioseparation of (±)-alpha-bromophenylacetic acid [(±)-(α-BP-AA), 2.5 mg/mL in MeOH/H_2O]. MEKC conditions: 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.5, pressure injection 50 mbar 5 s, −20 kV, 20°C, UV detection at 214 nm. Adapted from [79]

LC is limited by their rather high viscosities. In addition, the use of ILs on silica stationary phases often produces interactions between IL cations and the anionic silanol stationary phase, which lead to peak tailing and longer retention times. To circumvent the aforementioned problems, CILs may be used as additives or silanol blocking agents, affording enantiomeric separations [80].

It was found that the CIL, (*R*)-*N,N,N*-trimethyl-2-aminobutanol-bis (trifluoromethanesulfonyl)imide (**5**), was a good chiral selector that can be used in HPLC for enantioseparation of compounds such as alcohol, amine, and amino acids. The presence of this CIL in the mobile phase led to the enantioseparation of 2,2'-diamino-1,1'-binaphthalene (Fig. 2B) [76]. Clearly, these limited studies using CILs in HPLC suggest that more CILs with multiple functional groups need to be explored for enantioseparation of various chiral molecules in HPLC.

3.3 Applications of Chiral Ionic Liquids in Enantiomeric Spectroscopic Discrimination

Chiral chromatographic separation techniques such as GC, HPLC, and CE provide the real separation of enantiomers. By "real," one means that the two enantiomers of the racemates can actually be separated and obtained in individual containers. Particularly for chiral preparative HPLC, both the optically pure enantiomers can be obtained after the chiral chromatographic separation. However, in spectroscopic techniques, there is no real separation of enantiomers. Nonetheless, chiral spectroscopic techniques are still very important and useful resources for chiral technology in that they can rapidly and accurately determine the enantiopurity of chiral compounds. In addition, they can offer important information regarding the structure–property relationship and differentiation mechanism during chiral interaction and recognition. Recently, CILs have been used as the chiral selectors in spectroscopic techniques such as nuclear magnetic resonance (NMR), fluorescence, and near infrared (NIR).

3.3.1 Chiral Discrimination Using CILs in NMR Spectroscopy

Chiroptical methods have been used previously to assess the enantiomeric purity of a chiral compound [81]. With this method, the optical rotation of the sample is determined by use of a polarimeter under defined conditions including temperature, solvent, and concentration at a given wavelength of the incident plane-polarized light. The optical purity of the sample can be obtained by comparing the rotation of the sample with the known enantiopure sample of the same compounds. As a general characterization technique, the optical rotation values of CILs have been determined using a polarimeter in a number of organic solvents [29, 39, 56].

One of the most common methods employed for analysis of chiral compounds is NMR spectroscopy [82, 83]. Enantiomers cannot be discriminated in an achiral medium because the resonances of enantiotopic nuclei are isochronous. However, diastereoisomers may be distinguished as the nuclei resonances are anisochronous. In NMR, nuclei can be classified as isochronous or anisochronous. Where diastereotopic protons show the same chemical shift, they are said to be equivalent or isochronous, and where they have different chemical shifts, the protons are described as anisochronous [84]. As long as there is a large enough

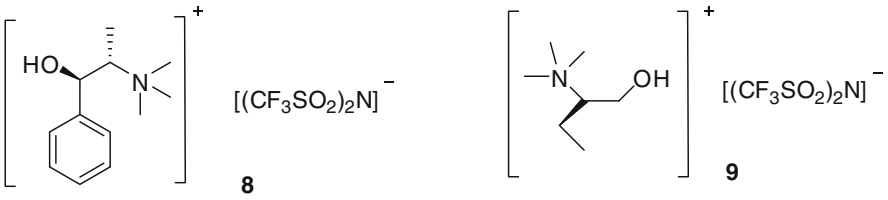
nonequivalent chemical shift to give baseline resolution of the appropriate signals, then integration gives a direct measure of diastereoisomeric composition [85]. Traditionally, three types of chiral auxiliary [86] which converts the mixtures of enantiomers into diastereomeric mixtures are widely used, i.e., chiral lanthanide shift reagents [87], chiral solvating agents [88], and chiral derivatizing agents [89].

Recently, CILs have been widely used as the chiral solvating agents in NMR studies. In these applications, CILs can be dissolved in deuterated NMR solvents and used as the chiral solvating agent. They can form diastereomeric associates in situ with substrate enantiomers via rapidly reversible equilibriums in competition with the bulk solvent. This method is quick and simple to perform with no problems of kinetic resolution or sample racemization which may occur when chiral derivatizing agents are used. Considering the solvent used, nonpolar solvents such as deuterated chloroform, benzene, and carbon tetrachloride tend to maximize the observed anisochrony between the diastereoisomeric complexes while more polar solvents such as deuterated acetonitrile, dimethyl sulfoxide may solvate the solute and decrease peak splitting [85].

In most CILs, the cations are chiral. Therefore, a chiral anionic compound would be very useful to investigate the chiral discrimination mechanism between CILs and the chiral anionic compounds through a possible ion pairing interaction. This makes the Mosher's acid, α-methoxy-α-(trifluoromethyl)phenyl acetic acid, a perfect choice as a chiral anionic probe compound. Mosher's acid was first introduced as a chiral derivatizing agent by Mosher in 1969 [90, 91]. The absence of α-hydrogen in the carboxylic group of Mosher's acid alleviates the problem of racemization during the derivatization. It is now commercially available in enantiopure form, either as the acid or as the acid chloride. In addition, both the ^{1}H NMR and the ^{19}F NMR can be employed to investigate the diastereomeric interactions since Mosher's acid contains both hydrogen and fluorine nuclei. This section summarizes the studies of chiral discrimination abilities of various CILs using NMR spectroscopy. In addition, the possible enantiomeric discrimination mechanisms are discussed.

To our knowledge, the first example of ^{19}F NMR investigation of CILs was reported in 2002 by Wasserscheid and co-workers [92]. The synthesis of CILs with chiral cations derived directly from the chiral pool is described. The three-step synthesis involving a Leuckart–Wallach reaction, alkylation with Me_2SO_4, and anion-exchange readily afforded a new CIL (**8**) from the alkaloid ephedrine in 80% overall yield (Scheme 5). Compound **8** has a melting point of 54°C and is stable up to 150°C under high vacuum conditions. The quest for CILs that are liquids at room temperature has resulted in the synthesis of CIL **9**. Compound **9** is a liquid at room temperature (melting point, −18°C) and has a high thermal stability.

The utility of these CILs for enantioselective reactions and enantiomeric separations was evaluated. The authors investigated the diastereomeric interactions between the substrate and the CIL using ^{19}F NMR spectroscopy. Undoubtedly, these interactions would be essential for all kinds of chirality transfer from the CIL onto the substrate in asymmetric induction. The diastereomeric interactions were probed by ^{19}F NMR spectroscopy using a racemic mixture of sodium Mosher's salt



Scheme 5 Molecular structures of cationic CIL ammoniums NTf_2

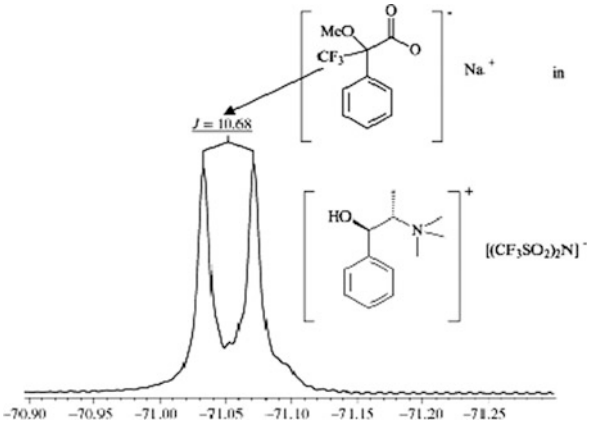


Fig. 5 ^{19}F NMR of racemic sodium Mosher's salt in CIL **8**. Adapted from [92]

as substrate and ephedrine-based CIL, **8**, as the chiral selector. An example of the ^{19}F NMR spectra is shown in Fig. 5.

The splitting of the signal related to the CF_3-group of the racemic sodium Mosher's salt clearly demonstrates that the substrate has been dissolved in a chiral environment. Moreover, the extent of peak splitting can be assigned to the strength of the diastereomeric interactions. In other words, under identical experimental conditions, the greater splitting indicates stronger chiral discrimination abilities of the CILs used as chiral selectors. It is also noteworthy that the chemical shift difference for the two diastereomeric CF_3-groups largely depends on the concentration of the CILs in the deuterated solvent. Generally, the higher the CIL concentration is, the greater the splitting of ^{19}F NMR signal of the CF_3 becomes. Moreover, it has been demonstrated that the addition of water to the chiral ionic liquid solution increases the extent of signal splitting (Fig. 6).

A novel class of chiral thiazolinium-based CILs derived from amino alcohols was reported in 2003 by Gaumont et al. [93]. Chiral recognition abilities of these thiazolinium-based CILs were investigated by determining the interactions between compound **10** and racemic Mosher's acid silver salt. The formation of diastereomeric complexes was confirmed by ^{19}F NMR (Fig. 7). Interaction between thiazolinium cation and Mosher's acid anion induces a downfield shift of the fluorine atom signal in the ^{19}F NMR spectrum (1 ppm). Moreover, the interaction

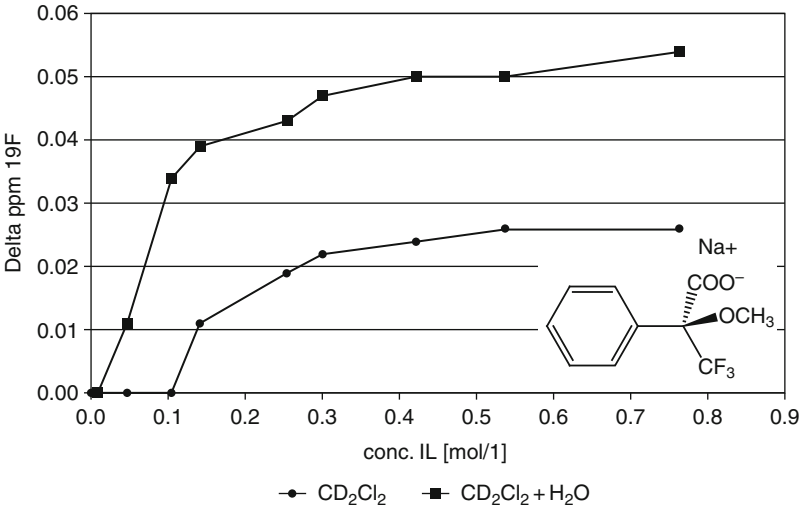


Fig. 6 ^{19}F NMR of racemic sodium Mosher's salt in CIL **8** – concentration dependence and influence of added water. Adapted from [92]

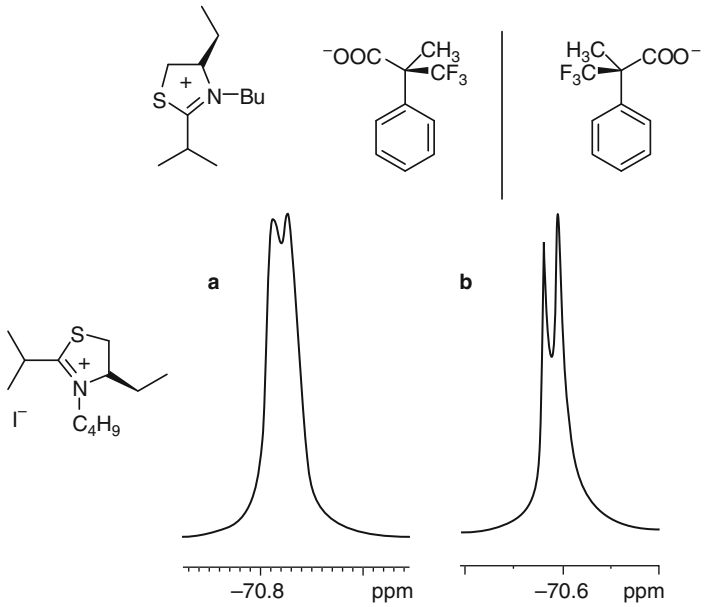


Fig. 7 ^{19}F NMR spectrum of water saturated racemic Mosher's salt with thiazolinium salt **10** in C_6D_6 (**a**, 1 equiv.; **b**, 5 equiv. to Mosher's salt). Adapted from [93]

causes a splitting of the fluorine signal of the Mosher's acid salt, clearly illustrating the formation of diastereomeric complexes. It was also found that the chemical shift distance between the two CF_3 groups depends on the concentration of the CILs in the NMR solvent. Similar to that observed for ammonium salts [92], addition of water to the chiral thiazolinium salt enhanced the extent of signal splitting.

In 2004, Guillemin et al. reported even greater splitting up to 63 Hz in ^{19}F NMR and 60 Hz in ^{1}H NMR spectroscopy using the valine-derived CILs (**11** and **12**) [94]. They modified the ^{19}F NMR protocol developed by Wassercheid and coworkers [92] by using the potassium Mosher's salt instead of the sodium salt and recorded NMR spectra in deuterated acetone. This protocol gives a better difference in the chemical shift difference ($\Delta\delta = 10$–20 Hz) for both methoxy and CF_3 groups of the two enantiomers of Mosher's salt in ^{1}H and ^{19}F NMR, respectively. This result can be explained by the tightness of the anion pair between cation (sodium or potassium) and Mosher acid anion, which is decreased by a bulky counter cation like potassium compared to sodium, leading to increased diastereomeric interactions between CIL and Mosher's acid anion. To confirm this, they added the crown ether 18C6 to trap the potassium cation and obtained even stronger interactions, affording a doubling of the chemical shifts difference ($\Delta\delta = 20 - 30$ Hz) observed in both ^{1}H and ^{19}F NMR spectroscopy. The increase of chiral discrimination obtained in this work could again suggest that the ion pairing interaction between CIL cation and the anionic Mosher's salt plays an important role in the chiral discrimination.

Guillemin and coworkers further investigated the possible enantiomeric discrimination mechanism using the new valine-based CILs. The role of the side chain on the splitting was first investigated. The introduction of a polar group, such as a hydroxyethyl chain (compound **11**), considerably increases the chemical shift difference. Up to 54.7 and 40.4 Hz between the two enantiomers were obtained in ^{1}H and ^{19}F NMR spectroscopy, respectively (Fig. 8a and b). The authors postulated that diastereomeric interactions were favored by hydrogen bonding between the hydroxyl group and the anionic substrate, leading to a folding up of the hydroxyl chain toward the cation. In the control tests, the poor splitting observed with the hydroxyoctyl chain showed that this folding up conformation was favored only for C_2 or C_3 hydroxyalkyl chain. To prove this folding up conformation, a few drops of water were added to the NMR solvent, destroying the hydrogen bonding and leading to diminished chemical shift difference. This is contradictory to the previous findings by Wasserscheid in which the addition of water was found to significantly increase the extent of splittings of the CF_3 signals [92].

In addition, a bulky substituent on the *ortho* position of the aromatic ring seems to be responsible for chiral discrimination (Fig. 9). Indeed, replacement of the initial bulky 2-*tert*-butyl group by a 2-methyl group considerably decreased the extent of splitting of the CF_3 signals. In the structure–interaction relationships, a polar group on the lateral side chain and a bulky *ortho* substituent on the aromatic ring were complementary and necessary to obtain high diastereomeric interactions between the chiral cation and the anionic substrate. This might suggest that steric interactions could provide a positive influence on chiral recognition as one point of the three-point interaction which is needed to obtain chiral recognition.

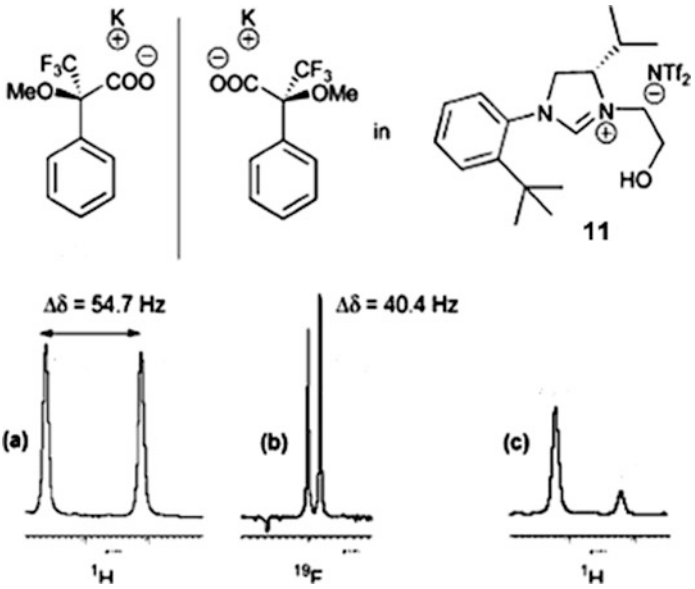


Fig. 8 ^{1}H (**a**) and ^{19}F (**b**) NMR spectra (400 and 376 MHz, respectively, CD_2Cl_2) of (*rac*)-Mosher salt in imidazolinium CIL **11** in presence of crown ether 18C6. (**c**) ^{1}H NMR spectrum of an enantioenriched sample of Mosher salt (60% ee). Adapted from [94]

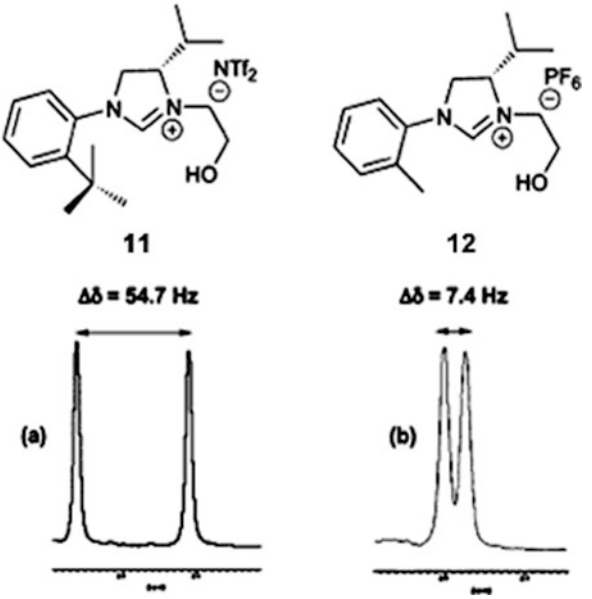
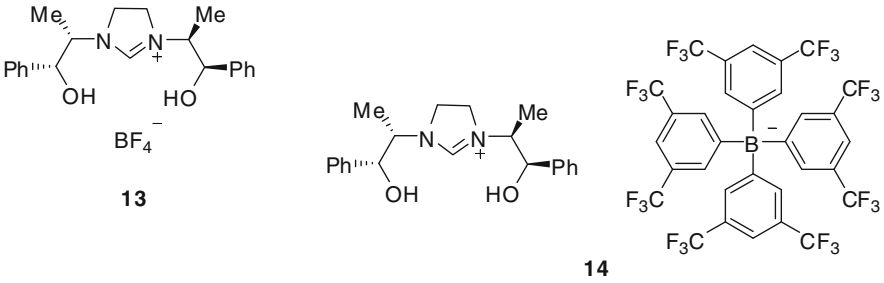


Fig. 9 Influence of the aryl substituent on the chiral discrimination: ^{1}H spectra (400 MHz, CD_2Cl_2) of (*rac*)-Mosher salt in imidazolinium **11** (**a**) and **12** (**b**) in presence of crown ether $18C_6$. Adapted from [94]

In another study, the presence of a second hydroxyl group in the cation structure further improved diastereomeric interaction as shown by the bidentate imidazolium CILs reported by Wilhelm et al. [95]. When enantiopure CIL **13** with a BF_4^- counteranion was used in combination with Mosher's salt, no signal splitting was obtained in either the ^{1}H NMR or the ^{19}F NMR spectra (Scheme 6). However, when the counteranion was changed to NTf_2^-, a significant increase in the splitting to 12 Hz in the ^{1}H NMR and 118 Hz in the ^{19}F NMR was observed. This result clearly suggests that the counteranions used for CILs play an important role in chiral discrimination as shown in the spectra (Fig. 10).

The splitting of the signal could not be improved by use of an excess of the imidazolinium salt. However, with a change in the counteranion from BF_4^- to



Scheme 6 Molecular structures of bidentate CILs with different anions

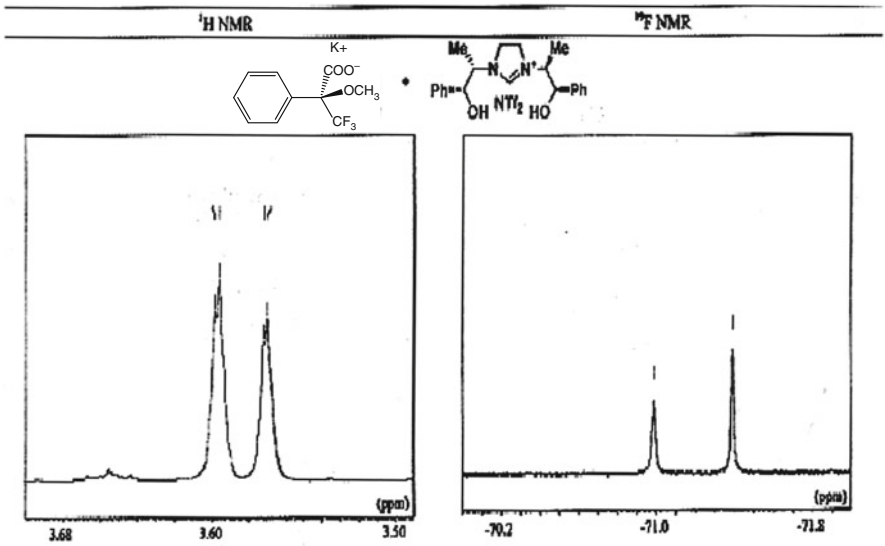


Fig. 10 ^{1}H NMR and ^{19}F NMR spectra of potassium Mosher's salt in the presence of CIL **14**. Adapted from [95]

$B[C_6H_3(CF_3)_2]_4^-$ (**14**), the splitting was increased to 24 and 151 Hz in the ^1H and ^{19}F NMR spectra, respectively [95]. Although only the cation is chiral, the achiral counteranion of the CILs may also significantly influence on the enantiomeric recognition in some cases. The anions' influence could result from either their steric size or hydrophobicity.

The use of isomannide as a bio-renewable substrate for synthesis of novel carbohydrate-based chiral ammonium ILs has been described by Malhotra et al. [96]. The chiral recognition ability of these novel mono-ammonium CILs was studied by investigating the diastereomeric interaction between the CILs and racemic Mosher's acid silver salt. Iodide salt **15** was dissolved in deuterated acetonitrile and the chiral recognition was probed by using ^{19}F NMR spectroscopy (Fig. 11, spectrum a). When the same NMR solution was mixed with 3.5 equiv of CIL **16**, a remarkable increase in chemical shift difference ($\Delta\delta$) of the CF_3 signal was observed (Fig. 11, spectrum b). This result again suggests that the achiral anion, Tf_2N^-, may play an important role in chiral discrimination induced by the CILs. The effect of anions on the diastereomeric interactions between CILs and Mosher's salt was further investigated. A similar ^{19}F NMR experiments in the presence of all other CILs with Tf_2N^- as anions was conducted. The results are compared with those of bis-ammonium CILs and summarized in Table 1. These findings prove that both the cation and the anion have an effect on the chiral discrimination abilities of these CILs, as indicated by differences in the magnitude of $\Delta\delta$ values by variations of the ions.

Malhotra and coworkers also observed that as the concentration of CIL **16** increased, the chemical shift differences ($\Delta\delta$ values) also increased (Fig. 12). A significant increase of 6.1 Hz in the $\Delta\delta$ value was obtained upon increasing the concentration of CIL **16** from 0 to 0.81 mol/L [96].

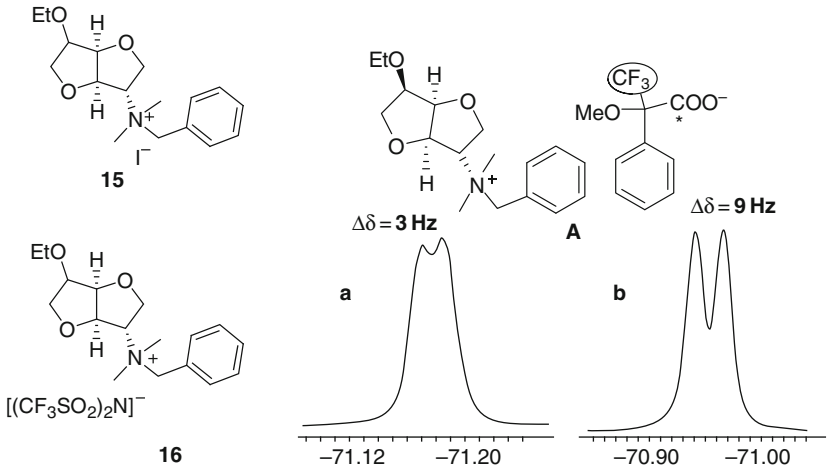


Fig. 11 ^{19}F NMR signal of CF_3 of Mosher's salt in the presence of 3.5 equiv of CIL **15** (**a**) and **16** (**b**). Adapted from [96]

Table 1 Enhanced $\Delta\delta$ values of CF_3 signals of racemic Mosher's acid in the presence of mono- (3.5 equiv) and bis-ammonium (4.0 equiv) CILs – the influence of anions. Data from [96]

Entry	Anion	Mono-ammonium $\Delta\delta$ (Hz)	Bis-ammonium $\Delta\delta$ (Hz)
1	[I]	7	9
2	[Tf_2N]	9	23
3	[PF_6]	8	21
4	[TFA]	5	8
5	BF_4]	5	17
6	[TfO]	9	15

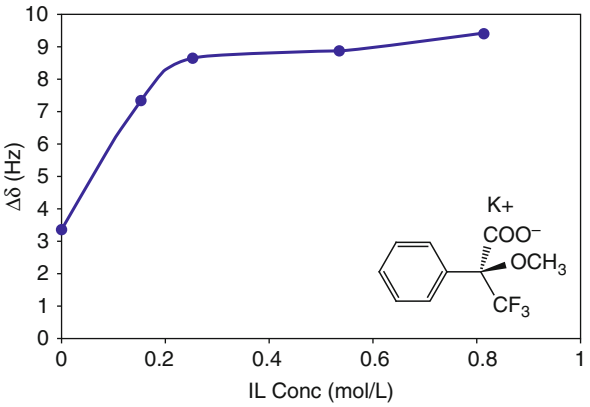


Fig. 12 Concentration effect of CIL **16** on the $\Delta\delta$ values of CF_3 signal of racemic Mosher's salt. Adapted from [96]

The synthesis of novel CILs with a spiro skeleton has been reported by Sasai and coworkers [97]. The influence of *N*-substituents and the counteranions on their chiral discrimination abilities was investigated. In addition, the diastereomeric interaction between the novel spiro imidazolium-based CIL **17** and (*S*)-Mosher's potassium salt was examined. The ^{1}H NMR spectrum of racemic spiro imidazolium salt **17** exhibited two pairs of counteranion from bromide to a chiral anion. The counteranion was changed in situ by treating racemic **17** with the potassium salt of Mosher's acid in the presence of 18-crown-6. The ^{1}H NMR spectrum exhibited excellent splitting in each pair of doublets. These results demonstrate spiro imidazolium salts are potentially useful in chiral molecular recognition (Fig. 13).

The examples of planar CILs, cyclophane-type imidazolium salts, were reported by Saigo et al. [51, 98]. Its potential use in chiral recognition was demonstrated by ^{1}H NMR studies. Besides the Mosher's acid salt which is widely used, enantiopure camphorsulfonate was also used to investigate chiral recognition between a racemic CIL and the enantiopure analyte. As an initial study on the chiral recognition ability of the cyclophane-type ionic liquid **18**, the diastereomeric interaction

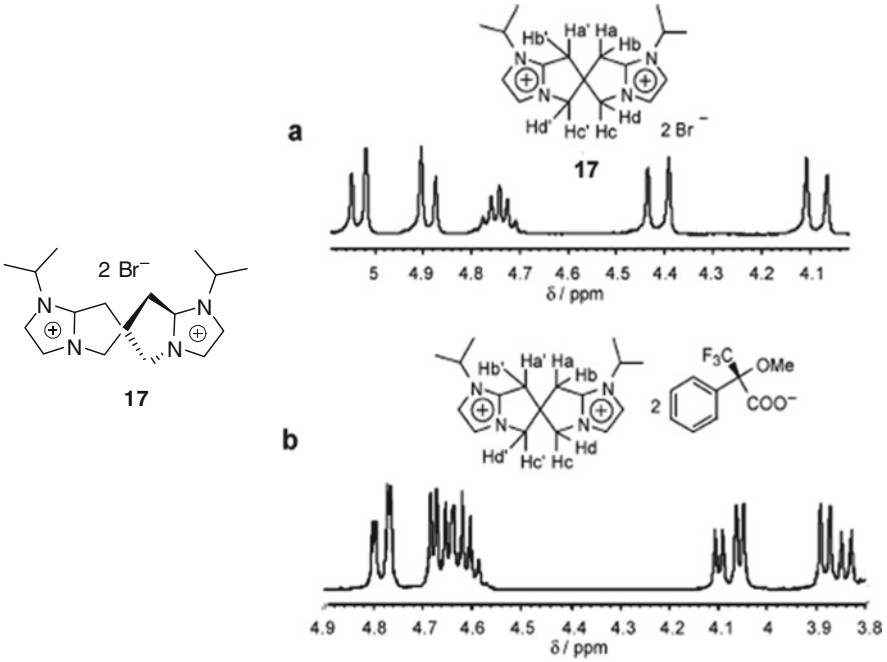


Fig. 13 (**a**) ^{1}H NMR spectrum of **17** in 20% DMSO-d_6-$CDCl_3$. (**b**) ^{1}H NMR spectrum of **17** and potassium salt of (*S*)-Mosher's acid in 20% DMSO-d_6-$CDCl_3$ in the presence of 18-crown-6. Adapted from [97]

between the cation part of **18** and chiral anion, camphorsulfonate, was detected by ^{1}H NMR spectrum in $CDCl_3$. The spectrum presented a pair of doublet signals for the C(5)-H of imidazolium (Fig. 14a). Since the salts with achiral cations gave a doublet for the C(5)-H, the resultant split signals were likely to be assigned to the C(5)-H signals of both diastereomers. This assignment was confirmed with a control experiment using an acyclic analogue, 2,4-dimethyl-1,3-dipentylimidazolium (1*S*)-(+)-10-camphorsulfonate, for which no splitting of the corresponding signal was observed (Fig. 14b).

Warner et al. described the synthesis of CILs based on amino acid esters. These CILs were derived from L- or D-alanine *tert*-butyl ester hydrochloride (**19** and **20**) [24]. The synthesis was accomplished via an anion metathesis reaction between commercially available L or D-alanine *tert*-butyl ester hydrochloride using a variety of counterions such as lithium bis(trifluoromethane) sulfonimide, silver nitrate, silver lactate, and silver tetrafluoroborate. The CILs demonstrated enantiomeric recognition ability as evidenced by splitting of racemic Mosher's sodium salt signal using ^{19}F NMR (Fig. 15). The diastereomeric interactions lead to a shift in the ^{19}F NMR signal of the racemic Mosher's sodium salt. In addition, the ^{19}F NMR signal of the racemic substrate was split by both enantiomeric forms of the ionic liquid demonstrating their enantiomeric recognition

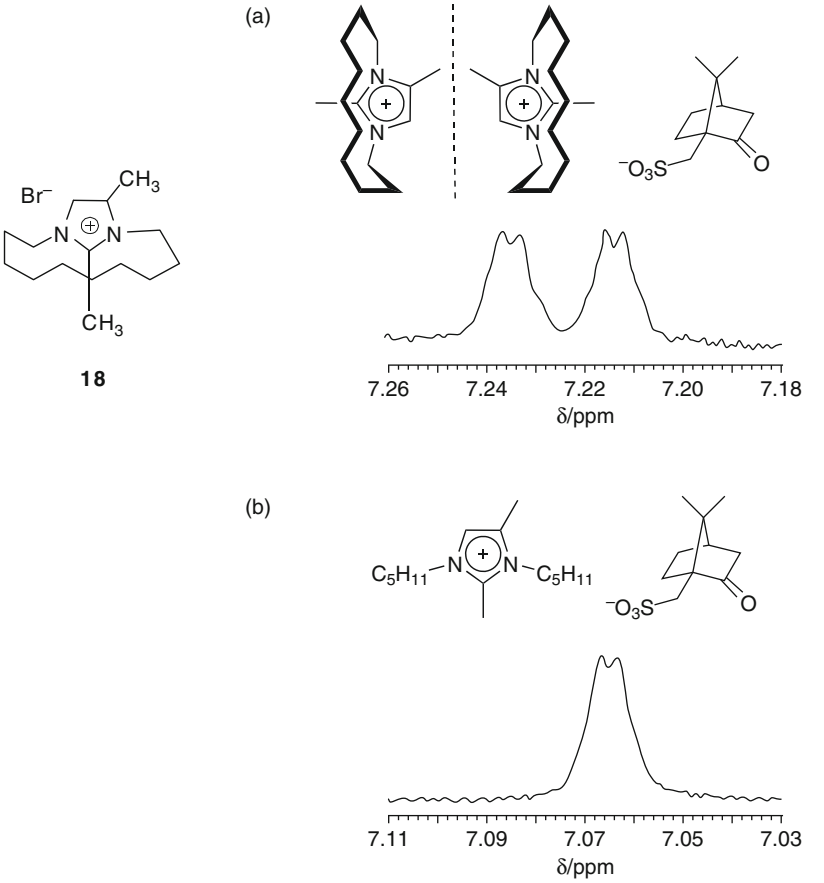


Fig. 14 Partial ^{1}H NMR spectra for the imidazolium C(5)-H **18** in presence of enantiopure camphorsulfonate in $CDCl_3$ recorded at 20°C. Adapted from [51]

A two step synthesis of new enantiopure CILs, (*S*)-(−)- and (*R*)-(+)-*N*-ethyl-1-phenylethylammonium bis(trifluoromethylsulfonylimide), in high yield from the readily available optically active (*S*)-(−)- and (*R*)-(+)-1-phenylethylamine has been reported by Wallace et al. [99]. The CIL, **21**, showed diastereomeric interactions with racemic Mosher's salt as demonstrated by ^{19}F NMR spectral studies of mixtures of **21** and racemic Mosher's acid as substrate. For example, for a mixture of **21** (0.70 M) with potassium Mosher's salt (0.085 M) in $CDCl_3$, the signal for the CF_3 group of the racemic Mosher's salt showed a splitting of 5.4 Hz. The extent of the diastereomeric interaction of **21** with racemic Mosher's acid increased in the presence of added water, with the chemical shift difference between the diastereomeric CF_3 signals increasing to 7.8 Hz (Fig. 16).

Wang and coworkers reported the synthesis of CILs with chiral cations from L-proline [100]. Their potential use in chiral recognition was also demonstrated

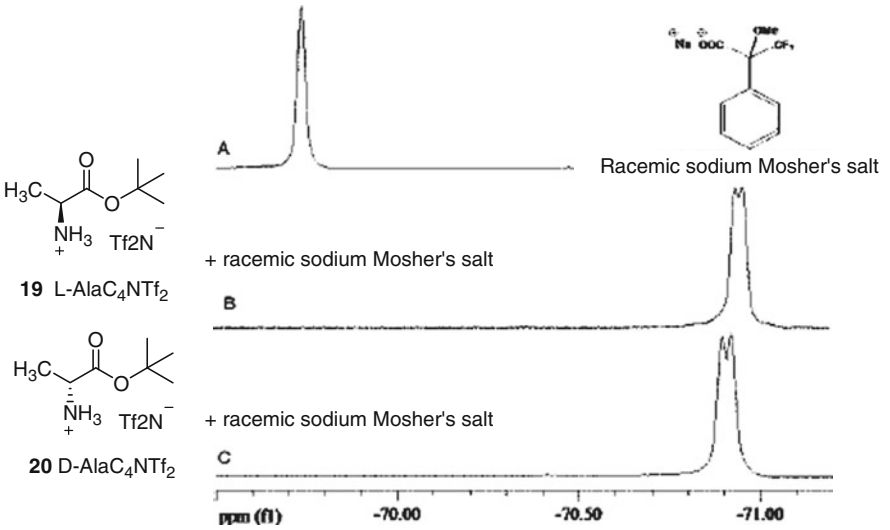
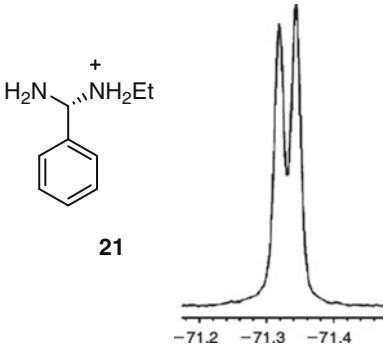


Fig. 15 ^{19}F NMR spectra of (**a**) racemic sodium Mosher's salt; and a mixture of the racemic sodium Mosher's salt with (**b**) **19**: D-AlaC$_4$NTf$_2$ and (**c**) **20**: L-AlaC$_4$NTf$_2$ at room temperature. Adapted from [24]

Fig. 16 ^{19}F NMR spectrum of *rac*-Mosher's acid in CIL **21**. Adapted from [99]



by studying their diastereomeric interactions with racemic Mosher's salt. When racemic Mosher's salt was dissolved in CILs **22** and **23**, diastereomeric complexes were formed between the *N*,*N*-dimethyl-L-proline ethyl ester and racemic Mosher's acid anion. Both racemic Mosher's silver and sodium salts were used. However, no baseline splitting of the CF_3 signal was observed in ^{19}F NMR (Fig. 17). This probably indicates lower chiral recognition abilities for this kind of CILs. The reason for the two peaks of unequal height in Fig. 17b was unknown. It was observed that the concentration of the CILs has a significant influence on the extent of the signal splitting of fluorine in Mosher's salt.

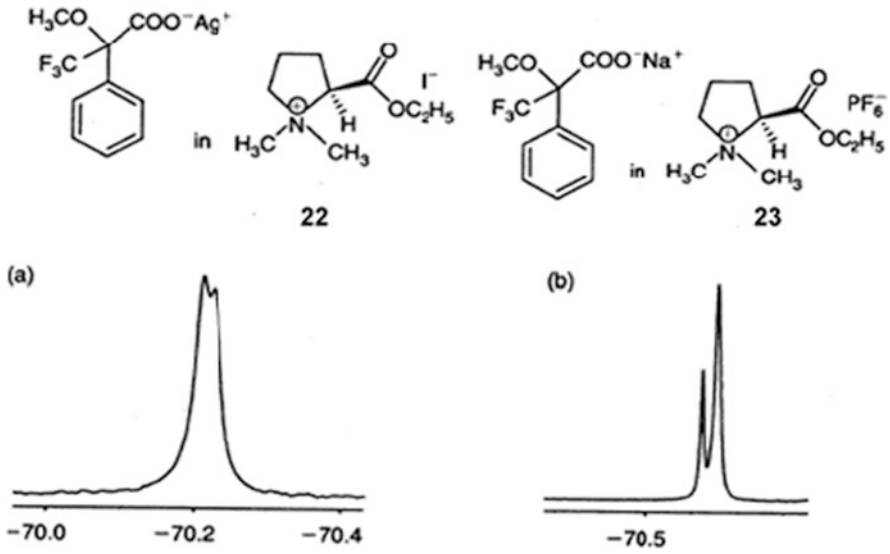


Fig. 17 ^{19}F NMR spectra of (**a**) racemic Mosher's acid silver salt in **22**; (**b**) racemic Mosher's sodium salt in **23**. Adapted from [100]

Tran et al. recently reported the synthesis of a series of novel CILs which have either chiral cation, chiral anion, or both [101]. Cations are an imidazolium group, while anions are based on a borate ion with spiral structure and chiral substituents. In addition, an interesting and novel method was used to investigate the intramolecular chiral recognition from the chiral anions. Chiral recognition between the cations and anions in the CIL themselves was studied. Intramolecular chiral induction from a chiral anion to its cation through ion-pairing effects in a single CIL was observed in the asymmetric catalytic hydrogenation reaction [62]. However, the possible chiral interaction mechanism was not investigated in detail. To study the intramolecular chiral recognition, CILs containing chiral anions and a racemic 1-methyl-3-(2-methyl-butyl) imidazolium cation were synthesized.

The ^{1}H NMR spectra of CIL **24** in two different solvents ($CDCl_3$ and CD_3CN) and three different concentrations are shown in Fig. 18. As illustrated in the spectra, interactions between the chiral anions and racemic cations result in the splitting of the peaks of H6 and H6'. As observed in the previous work [96], the authors also found that signal splittings are dependent not only on the type of solvent but also on the concentration of the CIL. A larger splitting value was found in low polar $CDCl_3$ than that in polar CD_3CN. In addition, increasing the IL concentration from 50 (in $CDCl_3$) to 100 mM also leads to an increase in the splitting. This may suggest that the ion pair formation is stronger in a solvent with lower dielectric constant ($CDCl_3$). The authors postulated that a stronger ion pair formation will bring the anion closer to the cation and make their interaction stronger and, hence, produce larger splitting values. Interestingly, relatively strong enantiomeric recognition toward the racemic

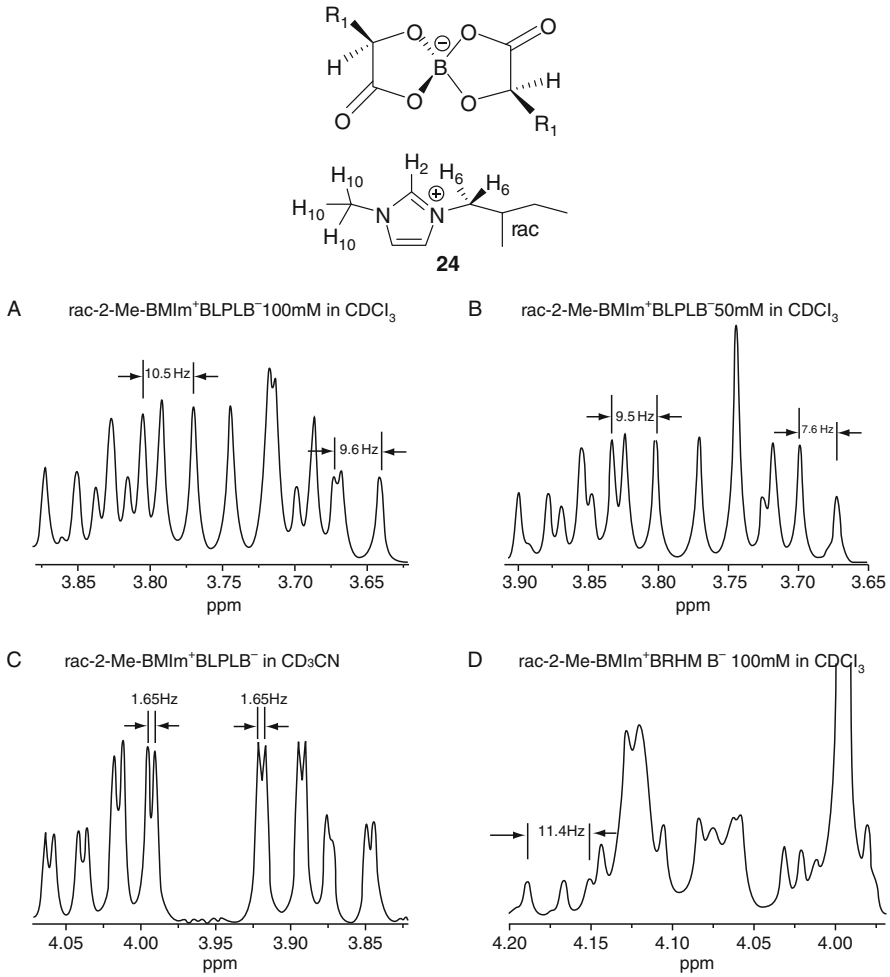


Fig. 18 ^{1}H NMR spectra of CIL **24** in $CDCl_3$ and CD_3CN in different concentrations. Adapted from [101]

cation (1-methyl-3-(2-methyl-butyl) imidazolium) is induced by the chiral anions; however, the reverse is not true. In fact, the chiral cation (*S*)-1-methyl-3-(2-methyl-butyl) imidazolium did not show any appreciable chiral recognition toward racemic anions. Also, better chiral recognition was found for relatively bulky anions. For example, a chiral anion with a phenylmethyl group exhibits stronger chiral recognition compared to that with a phenyl group, whereas an anion with an isobutyl group demonstrated the weakest chiral recognition under the same experimental conditions. This again may suggest that the steric interaction could significantly influence the chiral discrimination.

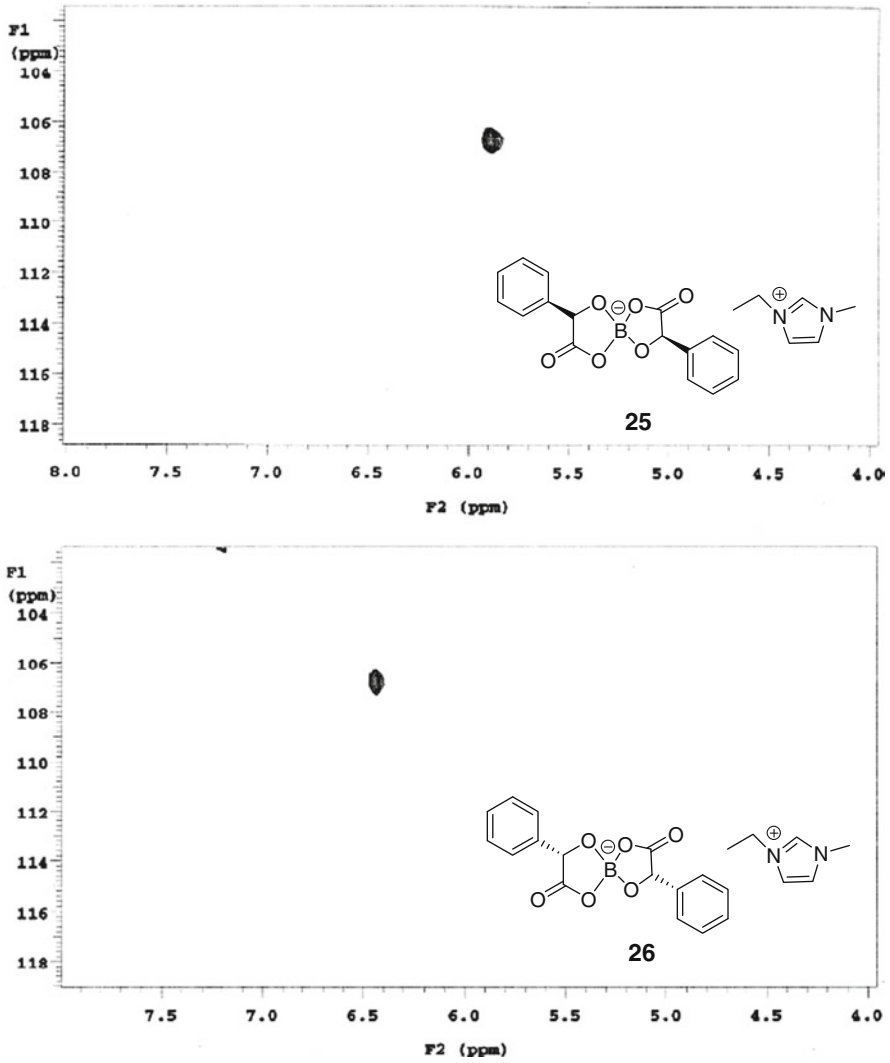


Fig. 19 2D 1H{15N} HSQC NMR spectra of (**a**) a mixture of 40 mM CIL **25** and 40 mM *t*BuCQN·HCl; and (**b**) a mixture of CIL 40 mM **26** and 40 mM *t*BuCQ·HCl. Adapted from [101]

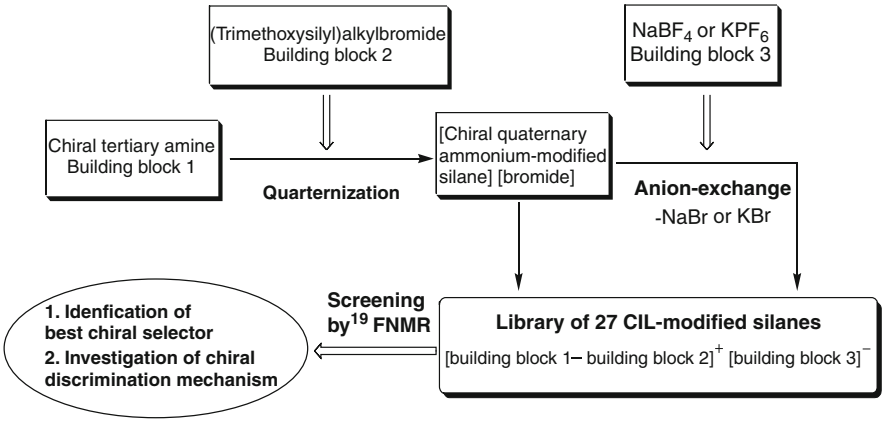
Intermolecular chiral recognition using the quinine carbamate compounds as analytes was also investigated. The results from 2D NMR studies including 1H{15 N} heteronuclear single quantum coherence (HSQC) show that these CILs (**25** and **26**) exhibit intramolecular as well as intermolecular enantiomeric recognition (Fig. 19).

As mentioned before, high tunability is one of the major advantages of chiral ionic liquids. Their structures and/or functionalities can be easily tailored through

judicious selection of their component ions. Thus, taking advantage of this property can easily afford novel chiral molecular structures and chiral materials, as well as new insights into chiral recognition and chiral induction. In this regard, hyphenation between combinatorial and high-throughput screening chemistry and the synthesis and screening of CILs for specific application would be a very desirable approach toward studying chiral interactions.

Using a combinatorial strategy, Warner et al. reported the synthesis of a parallel library of CIL-modified silanes as potential chiral selectors in HPLC (Scheme 7) [102]. The enantiomeric discrimination abilities of the library compounds were screened using ^{19}F NMR spectroscopy (Fig. 20). The screening method allows for rapid identification of the most enantioselective members of the library and simultaneous investigation of their chiral recognition mechanisms. The library compounds were synthesized using standard quaternization and anion-exchange reactions. Three major parameters such as type of chiral cations, anions, and linker chain lengths were included and investigated during the synthesis and screening. ^{19}F NMR screening indicated that the structure of the chiral cation plays an important role in determining chiral recognition abilities. The major classical types of intermolecular interactions including ion-pair, hydrogen bonding, π–π stacking, dipole stacking, and steric interactions were also found to impact chiral discrimination.

Nuclear magnetic resonance (NMR) spectroscopy and chromatography are the most widely used analytical tools for investigation of chiral recognition ability of chiral auxiliaries, including CILs. While NMR and chromatography have been very useful, insightful and remarkable for chiral analysis and enantiomeric discrimination, the high cost of NMR spectrometers, and low sensitivity are challenges often encountered with the use of NMR spectroscopy for chiral analysis. In addition, chromatographic chiral analysis is often too slow compared to spectroscopic analysis. In the case of gas chromatography, the coating procedure of ionic liquid on a capillary column for efficient chiral separation may be a challenge and time consuming,



Scheme 7 Combinatorial approach for the synthesis and screening of CIL-modified silanes. Adapted from [102]

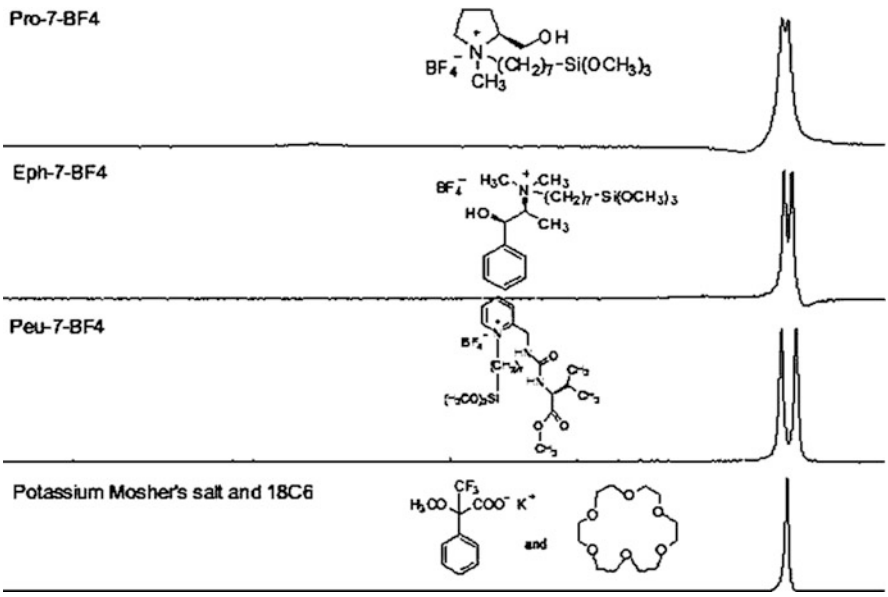


Fig. 20 ^{19}F NMR (CDCl3, 0.5 mL) spectra of potassium Mosher's salt (0.015 mmol) with CIL-modified silane (Pro-7-BF_4, Eph-7-BF_4, or Peu-7-BF_4, 0.075 mmol) as chiral selector in presence of crown ether $18C_6$ (0.015 mmol). The chemical shifts are normalized for easier comparison. Adapted from [102]

making chiral column typically expensive. Slow analysis time, poor sensitivity, and high-cost considerably limit the practical utility and may preclude the use of NMR and chromatography as a high throughput analytical technique for rapid, sensitive, and accurate screening of several hundred of thousand of potential chiral molecules of pharmaceutical interest. Thus, there is need for the development of a more rapid analytical method for chiral analysis.

The chemical and physical properties, including the absorption and emission spectral properties of enantiomers of a chiral molecule, are identical in a non-chiral or isotropic environment. In contrast, enantiomers of chiral molecules have significantly different spectral properties in a chiral (anisotropic) environment as a result of induced diastereomeric complex formation with chiral auxiliary. Differences in the spectral properties of enantiomers in a chiral environment therefore permit the use of absorption and/or emission spectroscopy for effective chiral discrimination of enantiomers. Analytical spectroscopy such as fluorescence, infrared, and ultraviolet-visible are rapid and relatively less expensive compared to NMR spectroscopy and may offer a high throughput chiral analysis at an affordable cost. Additionally, fluorescence is highly sensitive and selective, allowing investigation of chiral molecules at low concentrations. The low cost, coupled with rapid analysis time and high sensitivity of fluorescence, UV-visible, near infrared, FTIR, and circular dichroism spectroscopy, has lately prompted their use as possible alternative analytical tools for chiral analysis. Recently, CILs have been used as the chiral selectors in

spectroscopic techniques such as nuclear magnetic resonance (NMR), fluorescence, and near infrared (NIR).

3.3.2 Chiral Discrimination Using CILs in Fluorescence Spectroscopy

Fluorescence is a spectroscopic technique which has been widely used to study molecular interactions, particularly in biological systems [103]. It is also a useful tool for the study of chiral interaction and discrimination. It can provide useful information about molecular interactions with a high sensitivity. The application of fluorescence to specifically study enantioselective interactions and chiral recognition has been reported [104–113]. Most of these fluorescence studies involve the measurements of fluorescence emission intensities, spectral shifts, and quenching effects.

Life Time Study

Chiral recognition, enantioselectivity, and influence of room temperature CILs (RTIL) synthesized from 1-methyl imidazole and chloromethyl menthyl ether on excited-state photophysics of (*S*)-*N*-methyl-2-pyrrolidinemethyl 2(*S*)-(6-methoxy-2-naphthyl)propionate [(*S*,*S*)-NPX-PYR] was recently investigated using a fluorescence lifetime [114]. The chemical structures of the CILs and chiral analytes used for the lifetime study are shown in Fig. 21, left. The lifetime fluorescence decay of [(*S*,*S*)-NPX-PYR] in RTIL chiral environment is shown in Fig. 21, right.

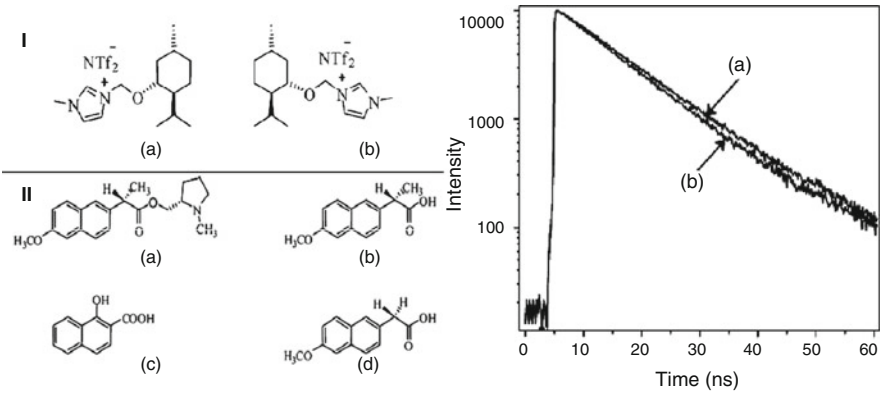


Fig. 21 Left: Chemical structures of: (*I*) (**a**) (–), and (**b**) (+)-RTIL synthesized from (–), and (+)-chloromethyl menthyl ether, respectively. (II) (**a**) (*S*,*S*)-NPX-PYR, (**b**) (*S*)-(+)-6-methoxy-2-naphthylpropionic acid [naproxen, NPX], (**c**) 1-hydroxy 2-naphthoic acid, and (**d**) 6-methoxy-2-naphthalene acetic acid. Right: fluorescence decay traces ($\lambda_{ex} = 266$ nm, $\lambda_{em} \geq 300$ nm) of (*S*,*S*)-NPX-PYR in (**a**) (–)- and (**b**) (+)-CILs. There is an ~10% decrease in lifetime of (*S*,*S*)-NPX-PYR in (+)-CIL compared to that in (–)-CIL. The lifetime of (*S*,*S*)-NPX-PYR in acetonitrile is ~3.1 ns in acetonitrile due to intramolecular electron transfer. In the CILs, the lifetime is significantly increased and there is a difference in lifetime in the two solvents. Adapted from [114]

Table 2 Fluorescence lifetime parameters of (*S*,*S*)-NPX-PYR and related systems. Data from [114]

Ionic liquid selector and selectand system	τ (ns)
(*S*,*S*)-NPX-PYR; (+)-RTIL	10.0±0.3
(*S*,*S*)-NPX-PYR; (−)-RTIL	10.9±0.2
(*S*)-NPX; (+)-RTIL	10.8±0.3
(*S*)-NPX; (−)-RTIL	9.7±0.3
(*S*)-NPX; acetonitrile	7.2± 0.2
(*S*,*S*)-NPX-PYR; acetonitrile	3.1±0.5
1-Hydroxy-2-naphthoic acid; (+)-RTIL	2.4±0.2
1-Hydroxy-2-naphthoic acid; (−)-RTIL	2.4 ±0.2
6-Methoxy-2-naphthalene acetic acid; (+)-RTIL	11.5±0.2
6-Methoxy-2-naphthalene acetic acid; (−)-RTIL	11.5±0.2

The lifetime of [(*S*,*S*)-NPX-PYR] dissolved in pure (+)-RTIL was 10.0±0.3 ns. However, [(*S*,*S*)-NPX-PYR] had a lifetime of 10.9 ± 0.2 ns in the presence of (−)-RTIL. The difference in the fluorescence lifetime of [(*S*,*S*)-NPX-PYR] observed in (+)-RTIL and (−)-RTIL shows a 10% stereodifferentiation.

A similar study conducted using *S*-naproxen as chiral analyte also demonstrates a 10% difference in fluorescence lifetimes of *S*-naproxen in the presence of (+)-RTIL and (−)-RTIL [115]. In contrast, there was no significant difference in the fluorescence lifetime of 1-hydroxy-2-naphthoic acid and 6-methoxy-2-naphthalene acetic acid achiral molecules in (+)-RTIL or (−)-RTIL chiral environment. Table 2 presents the summary of the fluorescence lifetime of investigated chiral and achiral molecules dissolved in pure (+)-RTIL and (−)-RTIL chiral ionic liquid. All the investigated chiral molecules show ~10% differences in fluorescence lifetimes. However, no significant difference in the lifetime of achiral molecules was observed in ionic liquid, demonstrating the chiral recognition ability of RTIL. Unlike the lifetime study performed in conventional solvents, there was no evidence of electron or charge transfer from chiral analytes in a chiral RTIL environment. The observed enantioselectivity was reported to be primarily due to chiral solvation by induced diastereomeric complex formation as a result of chiral solute and solvent interaction.

Steady-State Study

Steady-state fluorescence spectroscopy has also been successfully employed for enantiomeric discrimination of chiral molecules of pharmaceutical products including, propranolol, naproxen, and warfarin using *S*-[(3-chloro-2-hydroxypropyl) trimethylammonium] [bis((tri-fluoromethyl) sulfonyl)amide] (*S*-[CHTA]$^+$ [Tf_2N]$^-$, compound **6**) chiral ionic liquid [26]. Figure 20 shows the fluorescence emission spectra of samples containing 10 μM propranolol, 10 μM naproxen, and 10 μM warfarin dissolved in *S*-[CHTA]$^+$ [Tf_2N]$^-$ ionic liquid. Although each sample contained a fixed 10 μM concentration of chiral analytes, the enantiomeric composition of samples, in term of mole fraction of chiral analyte, varied from 0.0 to 1.0. The

emission spectra of samples obtained varied with enantiomeric mole fraction of chiral analyte in chiral ionic solvent. Variation in enantiomeric compositions of samples is expected to result in the formation of different diastereomeric complexes of chiral guest in chiral ionic liquid solvent. Therefore, difference in emission spectra of samples obtained in Fig. 20 was an indication of induced diastereomeric complex formation, showing chiral discrimination property of *S*-$[CHTA]^+$ $[Tf_2N]^-$.

The application of multivariate analysis involving the use of regression analysis of emission spectra of guest–host complexes for rapid, accurate, and robust determination of enantiomeric excess (ee) of chiral analytes using chiral ionic liquid auxiliary has also been explored. Partial-least-square (PLS), principal component analysis (PCA), and multiple linear regressions (MLR) are the modern regression techniques currently used to correlate changes in spectral data with known analyte concentration [116–122]. In a multivariate regression analysis, a regression model is developed and carefully optimized by selecting the appropriate wavelength regions where the spectra vary most with analyte concentration. The developed model can then be used to subsequently predict the analyte concentration of future samples solely from their spectral data. The accuracy of a regression model to correctly predict the future analyte concentration is often evaluated using the absolute error, percent relative error, or root-mean square-percent relative error. The general applications of multivariate analysis in analytical spectroscopy and detailed description of multivariate regression modeling of spectral data of guest–host complexes for rapid determination of enantiomeric composition of samples have been previously reported elsewhere [123–126].

The emission spectra in Fig. 22 were subjected to PLS regression analysis and were subsequently used to predict the enantiomeric excess (ee) of propranolol of independent samples [127]. The summary of the actual ee and predicted ee by regression model from the validation study performed for propranolol are shown in Table 3. The performance of the model to accurately predict the ee of samples was evaluated using percent relative error of prediction. Overall, the models were able to correctly predict the ee of propranolol with low percent relative error. Similar validation study conducted for naproxen and warfarin also demonstrate accurate predictions of ee of naproxen and warfarin samples by the developed regression model. In addition, using this technique, ee as low as 0.30% can be accurately determined. Determination of sample ee using this approach is highly promising and offers many advantages compared to the conventional method of chiral analysis. First, the method is rapid, reducing the analysis time. Second, the technique does not require prior separation of chiral analyte, eliminating the need of expensive chiral column. Furthermore, the use of a polarized light typically used in chiroptical analytical spectroscopy such as circular dichroism and polarimetry is not a requirement, making chiral analysis less expensive.

In a related study, the use of a steady-state fluorescence spectroscopy for investigation of chiral recognition ability of amino acid-based L-alanine *tert* butyl ester bis (trifluoromethane) sulfonamide (L-$AlaC_4NTf_2$) chiral ionic liquids was recently demonstrated [24]. In this study, L-$AlaC_4NTf_2$ was used as a solvent and chiral auxiliary for enantiomeric discrimination of warfarin, naproxen, and

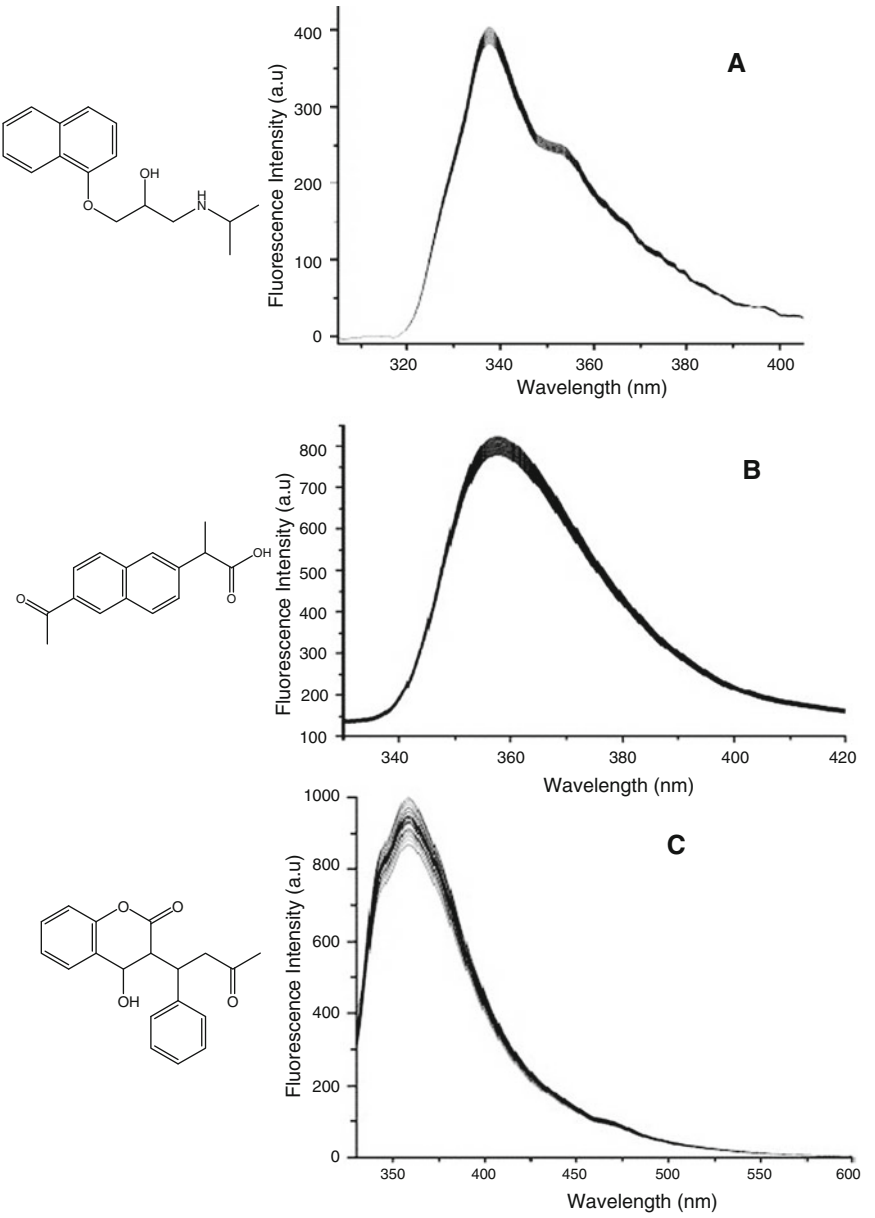


Fig. 22 Emission spectra of calibration samples of varying enantiomeric composition in the presence of *S*-[CHTA]. **a**. 10 μM propranolol, $\lambda_{ex} = 280$ nm; **b**. 10 μM naproxen, $\lambda_{ex} = 280$ nm; **c**. 10 μM warfarin, $\lambda_{ex} = 320$ nm. Adapted from [127]

Table 3 Actual and calculated enantiomeric excess (ee%) of solution of 10 μM propranolol in *S*-CHTA$^+$ Tf_2N^- IL. Data from [127]

Sample	*R*-Propranolol mole fraction	*S*-Propranolol mole fraction	Actual ee%[a]	Calculated ee%	Relative error[b] (%)
1	0.050	0.950	−90.0	94.19	4.66
2	0.250	0.750	−50.0	47.85	4.31
3	0.440	0.560	−12.0	12.77	6.42
4	0.5015	0.4985	0.3	0.32	5.88
5	0.503	0.497	0.6	0.64	7.13
6	0.510	0.490	2.0	2.14	7.09
7	0.535	0.465	7.0	6.37	8.94
8	0.725	0.275	45.0	47.95	6.56
9	0.900	0.100	80.0	75.30	5.87
10	0.985	0.015	97.0	93.18	3.94

[a] ee% = [(*R*-propranolol − *S*-propranolol)/(*R*-propranolol + *S*-propranolol)] × 100.
[b] Relative error = (actual value − calculated value) × 100.

2,2,2-trifluoroanthrylethanol (TFAE). Figure 23A1, B1, and C1 shows the fluorescence emission spectra of warfarin, naproxen, and TFAE enantiomers dissolved in L-AlaC_4NTf_2 ionic liquid, respectively. Obviously, enantiomers of chiral guests displayed significant different emission spectral intensities in the presence of L-AlaC_4NTf_2 ionic liquid. However, no noticeable difference in the emission spectra of enantiomers was observed in a non-chiral solvent, demonstrating chiral discrimination ability of L-AlaC_4NTf_2 CIL.

Multivariate analysis involving the use of mean-centered plot was also employed to analyze the emission spectra of warfarin, naproxen, and TFAE enantiomers dissolved in L-AlaC_4NTf_2 CIL (Fig. 23A2,B2,C2). Interestingly, enantiomers of a given chiral analyte show equal magnitude but opposite direction in the mean centered plot. The observed opposite mean centered plots of enantiomers in a chiral environment suggests the possible binding of enantiomers of chiral analytes in opposite orientations with L-AlaC_4NTf_2 CIL. This further demonstrates chiral recognition ability of L-AlaC_4NTf_2.

Warner et al. reported the first examples of magnetic chiral ionic liquids (MCILs) derived from amino acids [128]. The MCILs simultaneously contain chiral and magnetic properties. Steady-state fluorescence spectroscopy was used to investigate the chiral recognition of fluorescent analytes in ethanol solutions using MCIL, L-AlaOMeFeCl_4, as chiral selector (Fig. 24). With (*R*)- and (*S*)-2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE) as analytes, the emission intensity difference between (*R*)- and (*S*)-TFAE confirmed the chiral discrimination ability of L-AlaOMeFeCl_4. Similarly, with L-AlaOMeFeCl_4 as chiral selector, the chiral discrimination of 1,10-binaphthyl-2,20-diamine (BNA) and 2-(6-methoxy-2-naphthyl)propionic acid (naproxen) were also achieved.

So far most of the enantiomeric discrimination studies using CILs as chiral selectors have focused on non-fluorescent CILs and fluorescent analytes. Considering

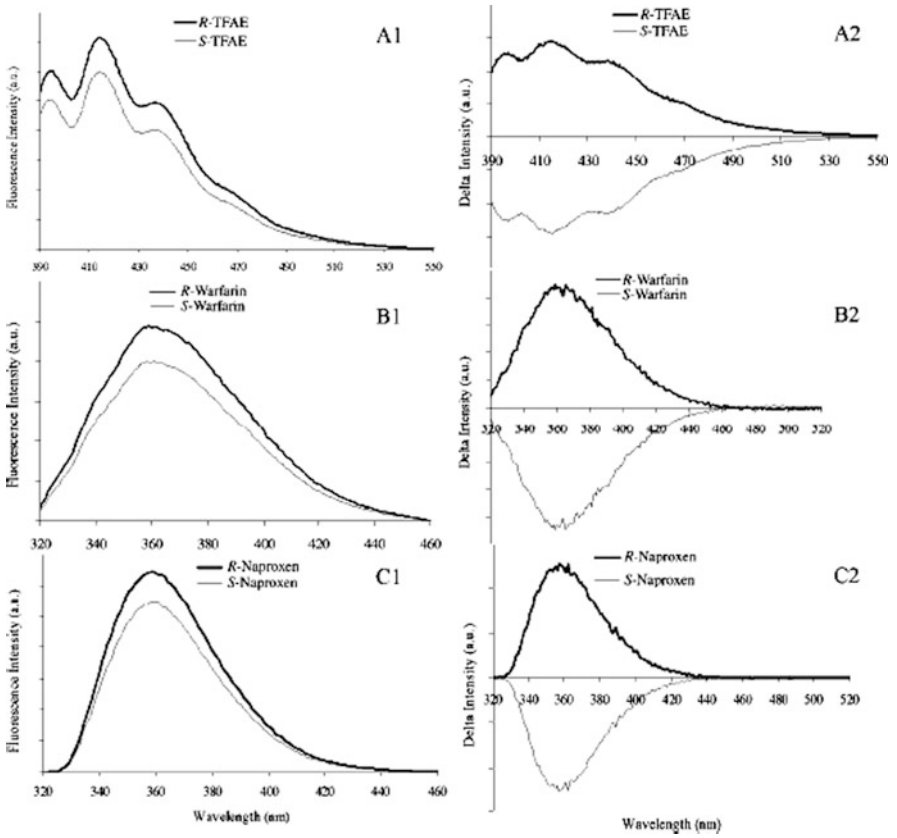


Fig. 23 Fluorescence emission and mean centered spectral plots of 10 μM *R*- and *S*- (**a**) trifluoroanthryl ethanol (TFAE), (**b**) warfarin, and (**c**) naproxen enantiomers in the presence of L-AlaC_4NTf_2 CIL. The emission spectra of TFAE, warfarin, and naproxen were monitored at excitation wavelength of 365, 306, and 280 nm, respectively, at room temperature. Adapted from [24]

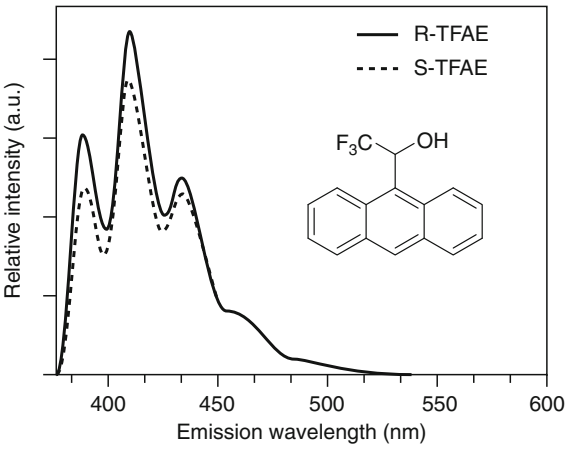


Fig. 24 Fluorescence emission spectral plots of (*R*)- and (*S*)-TFAE enantiomers using L-AlaOMe$FeCl_4$ as chiral selector. The emission spectra of TFAE were collected at an excitation wavelength of 365 nm at room temperature. Adapted from [128]

that some analytes have reduced fluorescence emission, there is need for a fluorescent CIL that can be used for enantiomeric recognition of nonfluorescent analytes. Fluorescent sensors have been used for chiral recognition for various nonfluorescent compounds by monitoring the change in fluorescence properties due to a binding event. Therefore, the use of CILs for enantiomeric recognition of both nonfluorescent and fluorescent analytes is a possible future research area.

3.3.3 Chiral Discrimination Using CILs in Near Infrared (NIR) Spectroscopy

The practical utility of near infrared spectroscopy (NIR) for chiral discrimination of atenolol using *S*-[(3-chloro-2-hydroxypropyl) trimethylammonium] [bis((trifluoromethyl)sulfonyl)amide] (*S*-$[CHTA]^+$ $[Tf_2N]^-$) chiral ionic liquids has been demonstrated [129]. In addition to fast analysis time and inexpensive instrumentation, the use of NIR spectroscopy for chiral discrimination is particularly appealing. Since almost all organic molecules and most inorganic molecules are known to have NIR absorption of C–H, O–H, N–H, or C=O overtone and combination transition, NIR spectroscopy can potentially be used as a universal technique for chiral discrimination of guest–host complexes. Second, NIR spectroscopy is a noninvasive and a nondestructive technique, with potential online chiral screening and detection in a drug manufacturing industry.

Figure 25 shows the NIR spectra of 60 mM atenolol of varying enantiomeric composition dissolved in pure *S*-$[CHTA]^+$ $[TF_2N]^-$ ionic liquid (compound **6**). As noted earlier in the fluorescence study, the NIR spectra of samples also varies with enantiomeric composition of atenolol in a chiral environment, demonstrating induced diastereomeric complex formations and chiral discrimination of *S*-$[CHTA]^+$ $[TF_2N]^-$ for atenolol enantiomers. The summary of the predicted ee and actual ee of atenolol in the validation study from the regression model using NIR spectra of guest–host complexes shown in Fig. 23 is presented in Table 4. Once again, the regression analysis of NIR spectra demonstrates accurate prediction of ee of atenolol

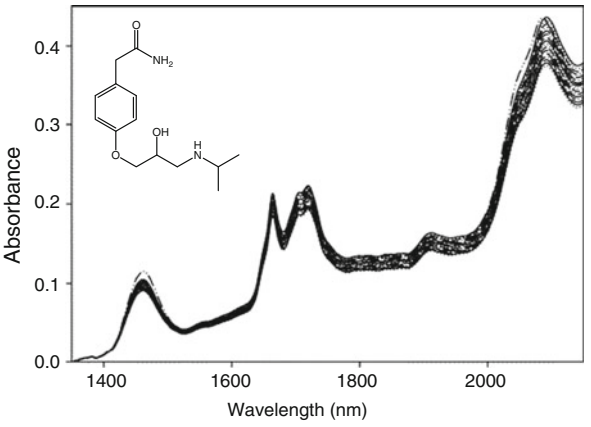


Fig. 25 NIR spectra of the pure (*S*)-$CHTA^+Tf_2N^-$ and 17 solutions of atenolol with the same total concentration of 60 mM but different enantiomeric compositions. Adapted from [129]

Table 4 Actual and calculated enantiomeric excess (ee%) of a solution of 10 mM atenolol in (*S*)-CHTA$^+$Tf$_2$N$^-$ ionic liquid. Data from [129]

	Mole fraction		ee,%		
Sample	(*R*)-Atenolol	(*S*)-Atenolol	Actual	Calculated	Relative error
1	0.050	0.950	−90.00	−82.17	8.70
2	0.150	0.850	−70.00	−72.95	4.21
3	0.300	0.700	−40.00	−39.34	1.65
4	0.365	0.650	−27.00	−27.69	2.55
5	0.480	0.520	−4.00	−4.25	6.25
6	0.496	0.504	−0.80	−0.74	7.50
7	0.503	0.497	0.60	0.58	3.33
8	0.510	0.490	2.00	2.10	5.00
9	0.800	0.200	60.00	61.86	3.10
10	0.985	0.015	97.00	101.06	4.19

samples with overall low relative error of prediction of 3.33%. Additionally, the ee values as high as −90.0 or +97.0% or as low as 0.6% can be correctly determined using this technique. Moreover, the method is capable of determination of atenolol at 2.66 mg/mL concentration, demonstrating high sensitivity of the technique for chiral analysis.

In conclusion, the use of fluorescence and NIR for rapid chiral discrimination of diverse chiral analytes using chiral ionic liquids as chiral discriminating agents has been demonstrated. In addition, accurate determination of enantiomeric excess of chiral molecules by regression analysis of fluorescence or infrared spectra of guest–host chiral ionic complexes has been explored. Fast analysis time, high sensitivity, and accuracy of the method for ee determination make the use of fluorescence and infrared spectroscopy appealing and a potential future analytical method of chiral analysis.

4 Concluding Remarks

In this chapter, the application of CILs in various fields has been discussed. This is with particular focus on the chiral chromatographic separations and spectroscopic discrimination. Chiral recognition and separation mechanism using CILs so far have not been thoroughly studied. This chapter discusses the enantiomeric discrimination mechanism using CILs as chiral selectors from some preliminary results.

As a subclass of functionalized or task-specific ionic liquids, CILs generally possess a number of unique and beneficial properties attributed to ILs including negligible vapor pressure, high thermal stability, high electro-conductivity, wide electrochemical window, wide liquidus range, and high tunability. In particular, high-tunability is the most desirable property which makes the design and preparation of novel chiral solvent, chiral separation medium, and chiral materials possible.

Indeed, the concept of "tailor-made" chiral solvents is rather new and could afford novel molecular structures and materials, as well as new insights for chiral recognition and chiral transfers. In this regard, a possible future research direction could be hyphenation between the combinatorial and high-throughput screening approach and the design and synthesis of novel CILs. Combinatorial chemistry, a powerful tool in many areas such as drug discovery, materials research, and catalysis, can also be effectively used in the area of chiral recognition to discover new selectors and chiral separation or reaction medium using CILs. The power of combinatorial chemistry resides in both the preparation of large numbers of compounds in short period of time and rapid assay to identify the optimal library members. Therefore, the enormous possibility of structure variations of CILs can be fulfilled using combinatorial chemistry simply by changing the structures of either cation, anion, or both. In addition, for future design and application of CILs in chiral discrimination and separation, it is important to understand how chiral interaction occurs. In other words, the chiral recognition mechanism and the structure–property relationship, and the physicochemical properties of new CILs are very important areas that need further investigations.

Acknowledgments We acknowledge the NSF for financial support during preparation of this manuscript.

References

1. Sinha M, Achenie LEK, Ostrovsky GM (1999) Environmentally benign solvent design by global optimization. Comput Chem Eng 23:1381–1394
2. Welton T (1999) Room-temperature ionic liquids. Solvents for synthesis and catalysis. Chem Rev 99:2071–2083
3. Earle MJ, Seddon KR (2000) Ionic liquids. Green solvents for the future. Pure Appl Chem 72:1391–1398
4. Seddon KR, Stark A, Torres M-J (2000) Influence of chloride, water, and organic solvents on the physical properties of ionic liquids. Pure Appl Chem 72:2275–2287
5. Huddleston JG, Rogers RD (1998) Room temperature ionic liquids as novel media for "clean" liquid-liquid extraction. Chem Commun 1765–1766
6. Walden P (1914) Molecular weights and electrical conductivity of several fused salts. Bull Acad Sci St Petersburg 8:405–422
7. Hapiot P, Corinne L (2008) Electrochemical reactivity in room-temperature ionic liquids. Chem Rev 108:2238–2264
8. Del P, Mario G, Voth GA (2004) On the Structure and dynamics of ionic liquids. J Phys Chem B 108:1744–1752
9. Wilkes JS (2002) A short history of ionic liquids-from molten salts to neoteric solvents. Green Chem 4:73–80
10. Chum HL, Koch VR, Miller LL et al (1975) Electrochemical scrutiny of organometallic iron complexes and hexamethylbenzene in a room temperature molten salt. J Am Chem Soc 97:3264–3265
11. Wilkes JS, Zaworotko MJ (1992) Air and water stable 1-ethyl-3-methylimidazolium based ionic liquids. Chem Commun 965–967
12. Fuller J, Carlin RT, De L, Hugh C, Haworth D (1994) Structure of 1-ethyl-3-methylimidazolium hexafluorophosphate: model for room temperature molten salts. Chem Commun 299–300

13. Wasserscheid P, Welton T (2003) Ionic liquids in synthesis. Wiley-VCH, Weinheim
14. Visser AE, Swatloski RP, Reichert WM et al (2001) Task-specific ionic liquids for the extraction of metal ions from aqueous solutions. Chem Commun 135–136
15. Visser AE, Swatloski RP, Reichert WM (2002) Task-specific ionic liquids incorporating novel cations for the coordination and extraction of Hg^{2+} and Cd^{2+}: synthesis, characterization, and extraction studies. Environ Sci Technol 36:2523–2529
16. Howarth J, Hanlon K, Fayne D et al (1997) Moisture-stable dialkylimidazolium salts as heterogeneous and homogeneous Lewis acids in the Diels-Alder reaction. Tetrahedron Lett 38:3097–3100
17. Hayashi S, Hamaguchi H (2004) Discovery of a magnetic ionic liquid [bmim]$FeCl_4$]. Chem Lett 33:1590–1591
18. Greaves TL, Drummond CJ (2008) Protic ionic liquids: properties and applications. Chem Rev 108:206–237
19. Zhao D, Fei Z, Geldbach TJ et al (2004) Nitrile-functionalized pyridinium ionic liquids: synthesis, characterization, and their application in carbon-carbon coupling reactions. J Am Chem Soc 126:15876–15882
20. Bonnette F, Mincheva Z, Lavastre O (2006) Functionalized ionic liquids as new supports for peptide coupling and traceless catalyzed carbon-carbon coupling reactions. Comb Chem High Throughput Screen 9:229–232
21. Li M, Wang T, Pham PJ et al (2008) Liquid phase extraction and separation of noble organometallic catalysts by functionalized ionic liquids. Sep Sci Technol 43:828–841
22. Donato RK, Migliorini MV, Benvegnu MA et al (2007) The electrochemical properties of a platinum electrode in functionalized room temperature imidazolium ionic liquids. J Solid State Electrochem 11:1481–1487
23. Seki S, Kawano R, Watanabe M (2006) Ionic liquids and clean energy conversion and storage. Kagaku Kogyo 59:760–763
24. Bwambok DK, Marwani HM, Fernand VE et al (2008) Synthesis and characterization of novel chiral ionic liquids and investigation of their enantiomeric recognition properties. Chirality 20:151–158
25. Chen D, Schmitkamp M, Francio G et al (2008) Enantioselective hydrogenation with racemic and enantiopure binap in the presence of a chiral ionic liquid. Angew Chem Int Ed 47:7339–7341
26. Tran CD, Oliveira D (2006) Fluorescence determination of enantiomeric composition of pharmaceuticals via use of ionic liquid that serves as both solvent and chiral selector. Anal Biochem 356:51–58
27. Herrmann WA, Goossen LJ, Koecher C (1996) Heterocyclic carbenes. 9. Chiral heterocyclic carbenes in asymmetric homogeneous catalysis. Angew Chem Int Ed 35:2805–2807
28. Earle MJ, McCormac PB, Seddon KR (1999) Diels-Alder reactions in ionic liquids. Green Chem 1:23–25
29. Bao W, Wang Z, Li Y (2003) Synthesis of chiral ionic liquids from natural amino acids. J Org Chem 68:591–593
30. Baudequin C, Bregeon D, Levillain J et al (2005) Chiral ionic liquids, a renewal for the chemistry of chiral solvents? Design, synthesis and applications for chiral recognition and asymmetric synthesis. Tetrahedron Asymmetry 16:3921–3945
31. Baudequin C, Baudoux J, Levillain J (2003) Ionic liquids and chirality: opportunities and challenges. Tetrahedron Asymmetry 14:3081–3093
32. Patil ML, Sasai H (2008) Recent developments on chiral ionic liquids. Design, synthesis, and applications. Chem Record 8:98–108
33. Bica K, Gaertner P (2008) Metal-containing ionic liquids as efficient catalysts for hydroxymethylation in water. Eur J Org Chem 20:3453–3456
34. Winkel A, Reddy PVG, Wilhelm R (2008) Recent advances in the synthesis and application of chiral ionic liquids. Synthesis 7:999–1016
35. Ding J, Armstrong DW (2005) Chiral ionic liquids. Synthesis and applications. Chirality 17:281–292

36. Chen X, Li X, Hu A et al (2008) Advances in chiral ionic liquids derived from natural amino acids. Tetrahedron Asymmetry 19:1–14
37. Headly AD, Ni B (2007) Chiral imidazolium ionic liquids: their synthesis and influence on the outcome of organic reactions. Aldrichimica Acta 40:107–117
38. Zhou W, Xu Li, Qiu H et al (2008) Synthesis of a novel chiral ionic liquid and its application in enantioselective aldol reactions. Helv Chim Acta 91:53–59
39. Kumar V, Olsen CE, Schaeffer SJC et al (2007) Synthesis and applications of novel bis(ammonium) chiral ionic liquids derived from isomannide. Org Lett 9:3905–3908
40. Ding J, Welton T, Armstrong DW (2004) Chiral ionic liquids as stationary phases in gas chromatography. Anal Chem 76:6819–6822
41. Tran CD, Mejac I (2008) Chiral ionic liquids for enantioseparation of pharmaceutical products by capillary electrophoresis. J Chromatogr A 1204:204–209
42. Lv X, Wang Z, Bao W (2006) CuI catalyzed C-N bond forming reactions between aryl/heteroaryl bromides and imidazoles in [Bmim]BF_4. Tetrahedron 62:4756–4761
43. Thanh GV, Pegot B, Loupy A (2004) Solvent-free microwave-assisted preparation of chiral ionic liquids from (-)-N-methylephedrine. Eur J Org Chem 1112–1116
44. Patrascu C, Sugisaki C, Mingotaud C et al (2004) New pyridinium chiral ionic liquids. Heterocycles 63:2033–2041
45. Malhotra SV, Wang Y (2006) Application of chiral ionic liquids in the copper catalyzed enantioselective 1,4-addition of diethylzinc to enones. Tetrahedron Asymmetry 17: 1032–1035
46. Fukumoto K, Yoshizawa M, Ohno H (2005) Room temperature ionic liquids from 20 natural amino acids. J Am Chem Soc 127:2398–2399
47. Gausepohl R, Buskens P, Kleinen J et al (2006) Highly enantioselective aza-Baylis-Hillman reaction in a chiral reaction medium. Angew Chem Int Ed 45:3689–3692
48. Branco LC, Gois PMP, Lourenco NMT et al (2006) Simple transformation of crystalline chiral natural anions to liquid medium and their use to induce chirality. Chem Commun 2371–2372
49. Yu L, Jin X, Zeng X (2008) Methane interactions with polyaniline/butylmethylimidazolium camphorsulfonate ionic liquid composite. Langmuir 24:11631–11636
50. Baudoux J, Judeinstein P, Cahard D et al (2005) Design and synthesis of novel ionic liquid/liquid crystals (IL_2Cs) with axial chirality. Tetrahedron Lett 46:1137–1140
51. Ishida Y, Miyauchi H, Saigo K (2002) Design and synthesis of a novel imidazolium-based ionic liquid with planar chirality. Chem Commun 2240–2241
52. Freemantle M (1998) Designer solvents – Ionic liquids may boost clean technology development Chem Eng News 76:32
53. Seebach D, Oei HA (1975) Mechanism of electrochemical pinacolization. First asymmetric electrosynthesis in a chiral medium. Angew Chem 87:629–30
54. Antonietti M, Kuang D, Smarsly B (2004) Ionic liquids for the convenient synthesis of functional nanoparticles and other inorganic nanostructures. Angew Chem Int Ed 43:4988–4992
55. Xu W, Cooper EI, Angell CA (2003) Ionic liquids: ion mobilities, glass temperatures, and fragilities. J Phys Chem B 107:6170–6178
56. Jurčik V, Wilhem R (2006) The preparation of new enantiopure imidazolium salts and their evaluation as catalysts and shift reagents. Tetrahedron Asymmetry 17:801–810
57. Pegot B, Vo-Thanh G, Gori D (2004) First application of chiral ionic liquids in asymmetric Baylis-Hillman reaction. Tetrahedron Lett 45:6425–6428
58. Wang Z, Wang Q, Zhang Y (2005) Synthesis of new chiral ionic liquids from natural acids and their applications in enantioselective Michael addition. Tetrahedron Lett 46:4657–4660
59. Ding J, Desikan V, Han X (2005) Use of chiral ionic liquids as solvents for the enantioselective photoisomerization of dibenzobicyclo[2.2.2]octatrienes. Org Lett 7:335–337
60. Kiss L, Kurtan T, Antus S et al (2003) Further insight into the mechanism of Heck oxyarylation in the presence of chiral ligands. ARKIVOC 2003(v):69–76

61. Yadav LDS, Rai A, Rai V et al (2008) Chiral ionic liquid-catalyzed Biginelli reaction: stereoselective synthesis of polyfunctionalized perhydropyrimidines. Tetrahedron 64: 1420–1429
62. Schulz PS, Mueller N, Boesmann A et al (2007) Effective chirality transfer in ionic liquids through ion-pairing effects. Angew Chem Int Ed 46:1293–1295
63. Kitazume T (2001) Optically active ionic liquid. US 2001031875
64. Caldwell J (1996) Importance of stereospecific bioanalytical monitoring in drug development. J Chromatogr A 719:3–13
65. Schurig V (2001) Separation of enantiomers by gas chromatography. J Chromatogr A 906:275–299
66. Vespalec R, Bocek P (1999) Chiral separations in capillary electrophoresis. Electrophoresis 20:2579–2591
67. Rizzi A (2001) Fundamental aspects of chiral separations by capillary electrophoresis. Electrophoresis 22:3079–3106
68. Shamsi SA, Warner IM (1997) Monomeric and polymeric chiral surfactants as pseudostationary phases for chiral separations. Electrophoresis 18:853–872
69. Gübitz G (1990) Separation of drug enantiomers by HPLC using chiral stationary phases – a selective review. Chromatographia 30:555–564
70. Al Rabaa AR, Tfibel F, Merola F et al (1999) Spectroscopic and photophysical study of an anthryl probe: DNA binding and chiral recognition. J Chem Soc Perkin Trans 2: Phys Org Chem 341–352
71. Sawada M (1997) Chiral recognition detected by fast atom bombardment mass spectrometry. Mass Spectrom Rev 16:73–90
72. Fanali S (2000) Enantioselective determination by capillary electrophoresis with cyclodextrins as chiral selectors. J Chromatogr A 875:89–122
73. Desiderio C, Fanali S (1998) Chiral analysis by capillary electrophoresis using antibiotics as chiral selector. J Chromatogr A 807:37–56
74. Ho Hyun M, Sung Jin J, Lee W (1998) Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether. J Chromatogr A 822:155–161
75. Fakayode SO, Williams AA, Busch MA (2006) The use of poly(sodium N-undecanoyl-L-leucylvalinate), poly(sodium N-undecanoyl-L-leucinate) and poly(sodium N-undecanoyl-L-valinate) surfactants as chiral selectors for determination of enantiomeric composition of samples by multivariate regression modeling of fluorescence spectral data. J Fluoresc 16:659–670
76. Yuan LM, Han Y, Zhou Y et al (2006) (R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethane-sulfon)imidate chiral ionic liquid used as chiral selector in HPCE, HPLC, and CGC. Anal Lett 39: 1439–1449
77. Francois Y, Varenne A, Juillerat E (2007) Evaluation of chiral ionic liquids as additives to cyclodextrins for enantiomeric separations by capillary electrophoresis. J Chromatogr A 1155:134–141
78. Maier V, Horáková J, Petr J et al (2006) Using of S-(-)-2-hydroxymethyl-1,1-dimethylpyrrolidinium tetrafluoroborate as additive to the background electrolyte in capillary electrophoresis. J Chromatogr A 1103:337–343
79. Rizvi SAA, Shamsi SA (2006) Synthesis, characterization, and application of chiral ionic liquids and their polymers in micellar electrokinetic chromatography. Anal Chem 78: 7061–7069
80. Berthod A, Ruiz-Angel MJ, Carda-Broch S (2008) Ionic liquids in separation techniques. J Chromatogr A 1184:6–18
81. Rickman BH, Matile S, Nakanishi K et al (1998) A two-step chemical/chiroptical method for determining absolute configurations of α-hydroxy acids. Tetrahedron 54:5041–5064
82. Buckingham AD, Fischer P (2006) Direct chiral discrimination in NMR spectroscopy. Chem Phys 324:111–116

83. Wenzei TJ (2007) Discrimination of chiral compounds using NMR spectroscopy. Wiley, Chicehster
84. Morris DG (2001) Stereochemistry. Royal Society of Chemistry, Great Britain
85. Parker D (1991) NMR determination of enantiomeric purity. Chem Rev 91:1441–1457
86. Roos G (2002) Compendium of chiral auxiliary applications. Academic Press, New York
87. Rinaldi PL (1982) The determination of absolute configuration using nuclear magnetic resonance techniques. Prog Nucl Magn Reson Spectrosc 15:291–352
88. Pirkle WH, Hoover DJ (1982) NMR chiral solvating agents. Top Stereochem 13:263–331
89. Pirkle WH (1966) The nonequivalence of physical properties of enantiomers in optically active solvents. Differences in nuclear magnetic resonance spectra. J Am Chem Soc 88:1837
90. Dale JA, Dull DL, Mosher HS (1969) α-Methoxy-α-trifluoromethylphenylacetic acid, a versatile reagent for the determination of enantiomeric composition of alcohols and amines. J Org Chem 34:2543–2549
91. Sullivan GR, Dale JA, Mosher HS (1973) Correlation of configuration and fluorine-19 chemical shifts of α-methoxy-α-trifluoromethylphenyl acetate derivatives. J Org Chem 38:2143–2147
92. Wasserscheid P, Boesmann A, Bolm C (2002) Synthesis and properties of ionic liquids derived from the "chiral pool". Chem Commun 200–201
93. Levillain J, Dubant G, Abrunhosa I (2003) Synthesis and properties of thiazoline based ionic liquids derived from the chiral pool. Chem Commun 2914–2915
94. Clavier H, Boulanger L, Audic N (2004) Design and synthesis of imidazolinium salts derived from (L)-valine. Investigation of their potential in chiral molecular recognition. Chem Commun 1224–1225
95. Jurčík V, Gilani M, Wilhelm R (2006) Easily accessible chiral imidazolinium salts bearing two hydroxy-containing substituents as shift reagents and carbene precursors. Eur J Org Chem 5103–5109
96. Kumar V, Pei C, Olsen CE et al (2008) Novel carbohydrate-based chiral ammonium ionic liquids derived from isomannide. Tetrahedron Asymmetry 19:664–671
97. Patil ML, Rao CVL, Yonezawa K (2006) Design and synthesis of novel chiral spiro ionic liquids. Org Lett 8:227–230
98. Yasuhiro I, Daisuke S, Hiroyuki M et al (2004) Design and synthesis of novel imidazolium-based ionic liquids with a pseudo crown-ether moiety: diastereomeric interaction of a racemic ionic liquid with enantiopure europium complexes. Tetrahedron Lett 45: 9455–9459
99. Ashraf SA, Pornputtkul Y, Kane-Maguire LAP (2007) Facile synthesis of a chiral ionic liquid derived from 1-phenylethylamine. Aust J Chem 60:64–67
100. Gao H-S, Hu Z-G, Wang J-J et al (2008) Synthesis and properties of novel chiral ionic liquids from L-proline. Aust J Chem 61:521–525
101. Yu S, Lindeman S, Tran CD (2008) Chiral ionic liquids: synthesis, properties, and enantiomeric recognition. J Org Chem 73:2576–2591
102. Li M, Gardella J, Bwambok DK (2009) A combinatorial approach to enantiomeric discrimination: synthesis and ^{19}F NMR screening of a chiral ionic liquid – modified silane library. J Comb Chem 11:1105–1114
103. Rawitch AB, Weber G (1972) Reversible association of lysozyme and thyroglobulin. Cooperative binding by near-neighbor interactions. J Bio Chem 247:680–685
104. James TD, Sandanayake KRAS, Shinkai S (1995) Chiral discrimination of monosaccharides using a fluorescent molecular sensor. Nature 374:345–347
105. Corradini R, Sartor G, Marchelli R et al (1992) Enantioselective fluorescence quenching by a chiral copper(II) complex in ligand exchange equilibria. J Chem Soc Perkin Trans 2 Phys Org Chem 11:1979–83
106. Lin J, Hu Q, Xu M et al (2002) A practical enantioselective fluorescent sensor for mandelic acid. J Am Chem Soc 124:2088–2089

107. Pischel U, Abad S, Miranda MA (2003) Stereoselective fluorescence quenching by photoinduced electron transfer in naphthalene-amine dyads. Chem Commun 1088–1089
108. Xu M H, Lin J, Hu Q et al (2002) Fluorescent sensors for the enantioselective recognition of mandelic acid: signal amplification by dendritic branching. J Am Chem Soc 124: 14239–14246
109. Liu T, Chen Y, Zhang K et al (2001) Enantiomeric recognition of chiral 3,3-bridged-1,1'-binaphthol dimer toward α-phenylethylamine and α-amino acid ester. Chirality 13:595–600
110. Beer G, Daub J, Rurack K (2001) Chiral discrimination with a fluorescent boron-dipyrromethene dye. Chem Commun 1138–1139
111. Abe Y, Shoji T, Matsubara M et al (2000) Chiral discrimination of N-carbazole-carbonyl derivatives of α-amino acids with a short linear alkyl side chain by bovine serum albumin. Chirality 12:565–567
112. Pagliari S, Corradini R, Galaverna G et al (2000) Enantioselective sensing of amino acids by copper(II) complexes of phenylalanine-based fluorescent β-cyclodextrins. Tetrahedron Lett 41:3691–3695
113. McCarroll ME, Billiot FH, Warner IM (2001) Fluorescence anisotropy as a measure of chiral recognition. J Am Chem Soc 123:3173–3174
114. Adhikary R, Bose S, Mukherjee P et al (2008) Influence of chiral ionic liquids on the excited-state properties of naproxen analogs. J Phys Chem B 112:7555–7559
115. Matthias O (1999) Chemometrics. Wiley-VCH, Weinheim
116. Beebe KR, Pell RJ, Seasholtz MB (1998) Chemometrics – a practical guide. Wiley-Interscience, New York
117. Massart DL, Vandeginste BGM, Buydens LM et al (1997) Handbook of chemometrics and qualimetrics, part A. Elsevier, Amsterdam
118. Vandeginste BG, Massart MD, Buydens MC et al (1998) Handbook of chemometrics and qualimetrics, part B. Elsevier, Amsterdam
119. Martens H, Naes T (1989) Multivariate calibration. Wiley, Chicehster
120. Edmund RM (1991) Factor analysis in chemistry. Wiley-Interscience, New York
121. Kramer R (1998) Chemometric techniques for quantitative analysis. Marcel Dekker, New York
122. Muhammad AS, Deborah LI, Bruce RK (1986) Chemometrics. Wiley-Interscience, New York
123. Busch KW, Busch MA (2006) Chiral analysis by regression modeling of spectral data in chiral analysis. In: Kenneth WB, Marianna AB (eds) Elsevier, Amsterdam, pp 363–396
124. Busch KW, Swamidoss IM, Fakayode SO et al (2003) Determination of the enantiomeric composition of guest molecules by chemometric analysis of the UV-visible spectra of cyclodextrin guest-host complexes. J Am Chem Soc 125:1690–1691
125. Busch KW, Swamidoss IM, Fakayode SO et al (2004) Determination of the enantiomeric composition of some molecules of pharmaceutical interest by chemometric analysis of the UV spectra of cyclodextrin guest-host complexes. Anal Chim Acta 525:53–62
126. Fakayode SO, Swamidoss IM, Busch MA et al (2005) Determination of the enantiomeric composition of some molecules of pharmaceutical interest by chemometric analysis of the UV spectra of guest-host complexes formed with modified cyclodextrins. Talanta 65: 838–845
127. Tran CD, Oliveira D (2006) Fluorescence determination of enantiomeric composition of pharmaceuticals via use of ionic liquid that serves as both solvent and chiral selector. Anal Biochem 356:51–58
128. Li M, De Rooy SL, Bwambok DK et al (2009) Magnetic chiral ionic liquids derived from amino acids. Chem Commun 6922–6924
129. Tran CD, Oliveira D, Yu S (2006) Chiral ionic liquid that functions as both solvent and chiral selector for the determination of enantiomeric compositions of pharmaceutical products. Anal Chem 78:1349–1356

Index

A. Berthod (ed.), *Chiral Recognition in Separation Methods*,
DOI 10.1007/978-3-642-12445-7, © Springer-Verlag Berlin Heidelberg 2010