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Chiral Separations by Liquid Chromatography

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CIBA-GEIGY Corporation

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Foreword

THE ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset, but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the editors with the assistance of the Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

Preface

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MOLECULES RELATING TO EACH OTHER as an object and its nonsuperimposable mirror image are enantiomers, or chiral pairs (from the Greek word *cheiro*, meaning hand); they are like a pair of hands. Stereoisomers (isomeric molecules with identical constitution but with a different spatial arrangement of atoms) are classified by symmetry as either enantiomers or diastereoisomers. A pair of enantiomers is possible for all molecules containing a single chiral carbon atom (one with four different groups attached). Although a molecule may have only one enantiomer, it may have several diastereoisomers. However, two stereoisomers cannot be both enantiomers and diastereoisomers of each other simultaneously. Stereoisomers can result from a variety of other sources, such as helicity (e.g., helix nature of tertiary structure of proteins), planar chirality, axial chirality, torsonial chirality, and topological asymmetry.

Accurate assessment of the enantiomeric purity of substances is critical because isomeric impurities may have unwanted toxicological, pharmacological, or other effects. Such impurities may be carried through a synthesis and preferentially react at one or more steps and may yield an undesirable level of another impurity. Frequently, one isomer of a series may produce a desired effect, but another may be inactive or even produce some undesired effect. Large differences in activity between enantiomers demonstrate the need to evaluate accurately isomeric purity of pharmaceutical, agricultural, or other chemical entities.

The importance of determining the stereoisomeric composition of chemical compounds, especially those of pharmaceutical importance, cannot be overemphasized. Dextromethorphan provides a dramatic example because it is an over-the-counter antitussive, whereas levomethorphan, its stereoisomer, is a controlled narcotic. Nearly 60% of the most frequently prescribed drugs in the United States possess one or more asymmetric centers in the drug molecule. The differences in the physiological effects between enantiomers of these racemic drugs have not yet been examined in many cases, probably because of the difficulty of obtaining both enantiomers in optically pure forms. To ensure the safety and effect of currently used and newly developed drugs, it is important to isolate the enantiomers and to examine each one separately. Furthermore, it is necessary to monitor the stereochemical composition of drugs during manufacture and storage and during pharmacological, metabolic, and toxicological studies.

Chromatographic methods, such as thin-layer chromatography, gas–liquid chromatography, and high-pressure liquid chromatography (HPLC), offer distinct advantages over classic techniques in the separation and analysis of stereoisomers, particularly for the more difficult class, enantiomers. Less than 20 years ago, systematic research was initiated for the design of chiral stationary phases to separate enantiomers by gas chromatography. Since then, scientists have examined molecular design and preparation of the chiral-phase systems for liquid chromatography. More recently, chemists have directed their efforts to finding new types of chiral stationary and mobile phases on the basis of the stereochemical viewpoint and on the technical evolution of modern liquid chromatography.

Most of the discussion in this book is on HPLC because it offers the greatest promise. HPLC is now one of the most powerful separation techniques, so resolution of enantiomers by HPLC is expected to progress rapidly with the availability of efficient chiral stationary phases. Large-scale preparative liquid-chromatographic systems have already been put on the market as process units for isolating and purifying chemicals and natural products. Chiral HPLC is ideally suited for large-scale preparation of optical isomers.

The chromatographic separation of enantiomers can be achieved by various methods; however, it is always necessary to use some kind of chiral discriminator or selector. A chiral additive in the mobile phase and a chiral stationary phase are two types of selectors. Another possibility is precolumn derivatization of the sample with chiral reagents to produce diastereomeric molecules that can be separated by nonchiral chromatographic methods.

This book includes significant discussion of these topics and brings some unity to this broad field. It will be a reference on state-of-the-art information for researchers working on problems involving chirality.

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Chapter 1

Chiral Separations

An Overview

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Optically active compounds have attracted great attention because living systems are chiral. Proteins, nucleic acids, and polysaccharides possess chiral characteristic structures that are related closely to their functions. Because of chirality, living organisms usually show different biological responses to one of a pair of enantiomers (optical isomers) in drugs, pesticides, or waste compounds. For example, sodium L-(+)glutamate tastes good, whereas its mirror image D-(-)glutamate tastes bitter or flat, depending on the taster (1).

Molecules that relate to each other as an object and its mirror image that is not superimposable are enantiomers or chiral (from the Greek word, cheiro, meaning hand); they are like a pair of hands. Stereoisomers are isomeric molecules with identical constitution but a different spatial arrangement of atoms. The symmetry factor classifies stereoisomers as either enantiomers, as defined above, or diastereoisomers. A pair of enantiomers is possible for all molecules containing a single chiral carbon atom (one with four different groups attached). Diastereoisomers or diastereomers, are basically stereoisomers that are not enantiomers of each other. Although a molecule may have only one enantiomer, it may have several diastereoisomers. However, two stereoisomers cannot be both enantiomers and diastereoisomers of each other simultaneously. Stereoisomerism can result from a variety of sources, including the single chiral carbon (or chiral center), for example, a chiral atom that is a tetrahedral atom with four different substituents. It is not necessary for a molecule to have a chiral carbon in order to exist in enantiomeric forms, but it is necessary that the molecule as a whole be chiral. Detailed discussion on these topics may be found in several books and review articles (2-9).

Enantiomers have identical physical properties except for a plus or minus sign of the optical rotation. A racemate, a mixture consisting of equal amounts of enantiomers is obtained experimentally by chemi-

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cal reactions carried out in an achiral environment. Therefore, the separation of an enantiomeric mixture, or optical resolution, is necessary for yielding optically pure species.

It should be noted that the R,S convention for nomenclature is not necessarily connected with the direction of optical rotation, i.e., the rotation that solutions of certain molecules impart to the plane of polarized light. This clearly indicates that a molecule's chirality, evidenced by the direction or magnitude of specific rotation, must be experimentally determined (although some empirical correlations exist) regardless of its absolute configuration.

Accurate assessment of the isomeric purity of substances is critical since isomeric impurities may have unwanted toxicologic, pharmacologic, or other effects. Such impurities may be carried through a synthesis and preferentially react at one or more steps and yield an undesirable level of another impurity. Frequently one isomer of a series may produce a desired effect, while another may be inactive or even produce some undesired effect. Large differences in activity between stereoisomers point out the need to accurately assess isomeric purity of pharmaceutical, agricultural, or other chemical entities. Often these differences exist between enantiomers, the most difficult stereoisomers to separate. Some examples of activity differences between stereoisomers are noted in Table 1.1.

Many factors may be responsible for the extent of interactions of stereoisomeric molecules in any environment: electrostatic forces; inductive effects; dipole-dipole interactions; ion-dipole interactions; hydrogen bonding; hydrophobic bonding; resonance interactions/stabilization; van der Waals forces; structural rigidity/conformational flexibility; steric interference, that is, size, orientation, and spacing of groups; solubilities; pKa differences--extent of ionization; partition coefficient differences; ligand formation; and temperature. The nature and effects of some of these factors as they influence chromatography of stereoisomers is of great importance.

The importance of determining the stereoisomeric composition of chemical compounds, especially those of pharmaceutical importance, cannot be overemphasized (1). Dextromethorphan provides a dramatic example in that it is an over-the-counter antitussive, whereas levomethorphan, its stereoisomer, is a controlled narcotic. Likewise, it has been reported that the teratogenic activity of thalidomide may reside exclusively in the (S)-enantiomer (10). Less dramatic examples abound; 12 of the 20 most prescribed drugs in the USA and 114 of the top 200 possess one or more asymmetric centers in the drug molecule (11). About half of the 2050 drugs listed in the U.S. Pharmacopeial Dictionary of Drug Names contain at least one asymmetric center, and 400 of them have been used in racemic or diastereomeric forms (12). The differences in the physiologic properties between enantiomers of these racemic drugs have not yet been examined in many cases, probably because of difficulties of obtaining both enantiomers in optically pure forms. Some enantiomers may exhibit potentially different pharmacologic activities, and the patient may be taking a useless, or even undesirable, enantiomer when ingesting a racemic

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Table 1.1 Activities of Some Stereoisomers (6)

Compound	Stereoisomers and Activities
Amphetamine	d-Isomer is a potent CNS stimulant, while ℓ-isomer has little, if any, effect
Diethylstilbestrol (DES)	trans-Isomer is much more estrogenic than cis-
Ascorbic acid	(+) Isomer is a good antiascorbutic, while (-) has no such properties
Propoxyphene	α-ℓ is an active antitussive, α-d is a potent analgesic, but β-d and β-ℓ are substantially inactive
Epinephrine	(-) Isomer is more than 10 times more active a vasoconstrictor than (+)
Synephrine	(-) Isomer has 60 times the pressor activity than (+)
Quinine/Quinidine	Quinidine is (+) enantiomer, with cardiac suppressant effects; quinine is (-) enantiomer with other medicinal uses
Bermethrin	d-Isomers of this insecticide are much more toxic than ℓ
Propranolol	Racemic propranolol is administered but only S-(-)isomer has desired β-adrenergic blocking activity
Warfarin	Racemic warfarin is administered but S-(-) isomer is 5 times more potent as a blood anti-coagulant than R-(+) isomer

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mixture. To ensure the safety and effect of currently used and newly developing drugs, it is important to isolate and examine both enantiomers separately. Furthermore, it is necessary, in at least three situations, to measure and control the stereochemical composition of drugs. Each situation presents a specific technical problem: (a) during manufacture, where problems of physical, preparative scale separations may be involved; (b) during quality control (or regulatory analysis), where analytical questions of purity and stability predominate; (c) during pharmacologic studies of plasma disposition and drug efficacy, where ultratrace methods may be required (4).

1.1 Chromatographic Methods

Optical resolution of racemates is one way to obtain pure isomers. Since Pasteur reported the first example of optical resolution in 1848, more than 7000 compounds have been resolved, mainly by fractional crystallization of diastereomeric salts. Resolution by entrainment or with enzyme or bacteria has also been applied to a large-scale separation of some racemates, for example, amino acids.

Chromatographic methods are considered most useful for optical resolution. A historical account of chiral separations by chromatography is given in Table 1.2. Derivatization of a given enantiomeric mixture with a chiral reagent, leading to a pair of diastereomers, (indirect method) allows separation of samples by chromatography. On the other hand, using the chiral stationary or mobile-phase systems in chromatography (direct method) is an alternative procedure that has recently come into use. This approach has been examined rather extensively by many research scientists. Early successful results did not attract much interest; the technique remained relatively dormant and little was done to develop this approach into a generally applicable method. Less than 20 years ago, systematic research was initiated for the design of chiral stationary phases functioning to separate enantiomers by gas chromatography. Molecular design and preparation of the chiral phase systems for liquid chromatography have been examined since then. More recently, efforts have been directed to finding new types of chiral stationary and mobile phases on the basis of the stereochemical viewpoint and the technical evolution of modern liquid chromatography.

Since HPLC is now one of the most powerful separation techniques, resolution of enantiomers by HPLC is expected to move rapidly if an efficient chiral stationary phase (CSP) is available. Large-scale, preparative liquid chromatography systems have already been put on the market as process units for isolating and purifying chemicals and natural products. Chiral HPLC is ideally suited for large-scale preparation of optical isomers.

It is now recognized that chromatographic methods (TLC, GLC, and HPLC) offer distinct advantages over classic techniques in the separation and analysis of stereoisomers, particularly for the more difficult class, enantiomers (2,3,13-16). Most of the discussion in this book is on HPLC, as it offers the greatest promise. Chromatographic methods show promise for moderate-scale separations of syn-

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Table 1.2 Historical Account of Chiral Separations by Chromatography

Year	Event
1939	Henderson and Rule: resolution of a racemic camphor derivative by chromatography on lactose
1952	Dalgliesh: postulation of the three-point rule in the paper chromatography of amino acids
1966	Gil-Av et al.: direct resolution of enantiomers by GC
1971	Davankov and Rogozhin: Introduction of chiral ligand exchange chromatography
1972	Wulff and Sarhan: preparation of enzyme analogue polymers for chiral LC
1973	Hesse and Hagel: preparation of cellulose triacetate for chiral resolution
1973	Stewart and Doherty: use of agarose-bonded bovine serum albumin (BSA) for chiral resolution.
1974	Blaschke: synthesis of chiral polymers from optically active monomers, for chiral LC
1975	Cram and co-workers: development of host-guest chromatography using chiral crown ethers
1979	Pirkle and House: synthesis of first silica-bonded CSP and application in chiral LC
1979	Okamoto et al.: synthesis of helical polymers for chiral LC
1982	Allenmark et al.: use of agarose-bonded BSA in chiral LC
1983	Hermansson: use of silica-bonded α_1-acid glycoprotein for chiral resolution
1984	Armstrong and DeMond: preparation of silica-bonded cyclodextrins

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thetic intermediates as well as for final products. For large-scale separations and in consideration of the cost of plant-scale resolution processes, the sorption methods offer substantial increases in efficiency over recrystallization techniques.

Of various stereoisomers, diastereomers specifically are inherently easier to separate because they already possess differences in physical properties. In recent years many significant advances have occurred which allow the chromatographic resolution of enantiomers. There are basically two approaches to the separation of an enantiomeric pair by chromatography. In the indirect approach, the enantiomers may be converted into covalent, diastereomeric compounds by a reaction with a chiral reagent, and these diastereomers are typically separated on a routine, achiral stationary phase. In the direct approach, several variations can be tried: (a) the enantiomers or their derivatives are passed through a column containing a chiral stationary phase, or (b) the derivatives are passed through an achiral column using a chiral solvent or, more commonly, a mobile phase that contains a chiral additive. In either variation of the second case, one depends on differential, transient diastereomer formations between the solutes and the selector to effect the observed separation.

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1.2 Modes of Separation

The chromatographic separation of enantiomers can be achieved by various methods; however, it is always necessary to use some kind of chiral discriminator or selector (7,17). Two different types of selectors can be distinguished: a chiral additive in the mobile phase (see Section 1.2.2) or a chiral stationary phase (Section 1.2.3). Another possibility is precolumn derivatization (Section 1.2.1) of the sample with chiral reagents to produce diastereomeric molecules which can be separated by non-chiral chromatographic methods.

The mechanism of separation is dependent on the mode of separation used. Some discussion on the mechanism of separation is provided for each mode of separation; however, it should be recognized that detailed mechanisms for chiral separations have not been worked out. The proposals made by certain scientists appear attractive; however, vigorous differences prevail, so an attempt has been made not to highlight a single proposal. Various types of columns used for chiral separations are given in Table 1.3. Detailed below are various approaches that can be used for chiral separations.

1.2.1 Chromatography of Diastereomeric Derivatives

This is the oldest and most widely used chromatographic approach to the resolution of enantiomers (8). The precolumn derivatization of an optically active solute with another optically active molecule depends on the ability to derivatize the target molecule. A large number of functional groups and derivatives have been investigated including amino groups (derivatized to amides, carbamates, ureas, thioureas, and sulfonamides), hydroxyl groups (esters, carbonates,

and carbamates), carboxy groups (esters and amides), epoxides (isothiocyanate), olefins (chiral platinum complexes), and thiols (thioesters).

This method has been used with a wide variety of HPLC columns and mobile phases, including normal- and reversed-phase approaches. At present there is no definitive way of determining which chromatographic approach will work. The advantages of derivatization are:

1. The methodology has been extensively studied, making the application relatively easy and accessible.

2. It is possible to use readily available, standard HPLC supports and mobile phases.

3. Detectability can be improved by appropriate selection of a derivatizing agent with a strong chromophore or fluorophore.

The main limitations are as follows:

1. The synthesis of the diastereomeric derivatives requires the initial isolation of the compounds of interest prior to their derivatization. This hinders the development of an automated procedure for large numbers of samples.

2. The application to routine assays often is limited by enantiomeric contamination of the derivatizing agent, which can lead to inaccurate determinations. The problem of enantiomeric contamination of the derivatizing agent has been encountered in a number of studies. Silber and Riegelman (18), for example, used (-)-N-trifluoroacetyl-l-prolyl chloride (TPC) in the determination of the enantiomeric composition of propranolol in biological samples. They found that commercial TPC was contaminated with 4 to 15% of the (+)-enantiomer and that the reagent rapidly racemized during storage.

3. Enantiomers can have different rates of reaction and/or equilibrium constants when they react with another chiral molecule. As a result, two diastereomeric products may be generated in proportions different from the starting enantiomeric composition (19).

1.2.2 Enantiomeric Resolution Using Chiral Mobile-phase Additives

The resolution of enantiomeric compounds has been accomplished through the formation of diastereomeric complexes with a chiral molecule(s) added to the mobile phase. The chiral resolution is due to differences in the stabilities of the diastereomeric complexes, solvation in the mobile phase, or binding of the complexes to the solid support. A general overview of this method has been published by Lindner and Pettersson (20).

There are three major approaches to the formation of diastereomeric complexes: transition metal ion complexes (ligand exchange), ion

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Table 1.3 Commercial Chiral Columns

Name	Chemical Name
Apex prepsil L-ualylphenylurea	(S)-valyl-phenylurea
Bakerbond chiral covalent DNBLeu	(S)-DNB-Leucin (covalent)
Bakerbond chiral covalent DNBPG	(R)-DNB-Phenylglycine (covalent)
Bakerbond chiral ionic DNBPG	(R)-DNB-Phenylglycine (ionic)
Cellulose CEL-AC-40 XF	Cellulose-triacetate
Chiral-AGP	α_1-Acid-glycoprotein
Chiralcel CA-1	Cellulose-triacetate
Chiralcel OA	Cellulose-triacetate
Chiralcel OB	Cellulose-tribenzoate
Chiralcel OC	Cellulose-trisphenylcarbamate
Chiralcel OO	Cellulose-tris-3,5-dimethylphenylcarbamate
Chiralcel OF	Cellulose-tris-4-chlorphenylcarbamate
Chiralcel OG	Cellulose-tris-4-toluylcarbamate
Chiralcel OJ	Cellulose-tris-4-toluylate
Chiralcel OK	Cellulose-tricinnamate
Chiralcel WE	N-(2-Hydroxy-1,2-diphenylethyl)glycine-copper
Chiralpak OT(+)	Poly(triphenylmethyl-methacrylate)
Chiralpak OP(+)	Poly(2-pyridyl-diphenylmethyl-methacrylate)
Chiralpak WH	Proline copper
Chiralpak UM	Amino acid copper
Chiral D-DL=Daltosil 100	(R)-DNB-Leucine (covalent)
Chiral L-DL=Daltosil 100	(S)-DNB-Leucine (covalent)
Chiral D-DPG=Si100	(R)-DNB-Phenylglycine (covalent)
Chiral L-DPG=Daltosil 100	(S)-DNB-Phenylglycine (covalent)
Chiral hypra-Cu=Daltosil 100	Hydroxyproline copper
Chiral proCu=Si100	Proline copper
Chiral valCu=Si100	Valine copper
Chiral protein 1	Beef serum albumin
Chiral protein 2	Human serum albumin
ChiraSpher	Poly-n-acryloyl-(S)-phenylalaninethylester
ChiRSil I	(R)-ONB-Phenylglycine (ionic)
Chi-RoSil	(R)-DNB-Phenylglycine (ionic)
Covalent L-leucine	(S)-DNB-Leucin (covalent)
Covalent D-naphthylalanine	(R)-Naphthylalanine
Covalent L-naphthylalanine	(S)-Naphthylalanine
Covalent D,L-naphthylalanine	(R,S)-Naphthylalanine
Covalent D-phenyl glycine	(R)-DMB-Phenylglycine (covalent)
Covalent L-phenyl glycine	(S)-DMB-Phenylglycine (covalent)
Covalent D,L-phenyl glycine	(R,S)-DNB-Phenylglycine (covalent)

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for HPLC*

Type	Particle Size	Source
Brush	8	Jones
Brush	5	Baker
Brush	3,5,10	Baker
Brush	5	Baker
Helix	7	Macherey-Nagel
Protein	5	ChromTech
Helix	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Ligand exchange	10	Daicel
Helix	10	Daicel
Helix	10	Daicel
Ligand exchange	10	Daicel
Ligand exchange	10	Daicel
Brush	4	Serva
Brush	4	Serva
Brush	3,5	Serva
Brush	4	Serva
Ligand exchange	4	Serva
Ligand exchange	5	Serva
Ligand exchange	5	Serva
Protein		SFCC
Protein		SFCC
Helix	5	Merck
Brush	5,10	RSL
Brush	5	RSL
Brush	5	Regis, Alltech
Brush	5	" "
Brush	5	" "
Brush	5	" "
Brush	5,10	" "
Brush	5,10	" "
Brush	5	" "

Continued on next page

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Table 1.3 Commercial Chiral Columns for HPLC*

Name	Chemical Name
Crownpak CR	Crown ether
Cyclobond I	β-Cyclodextrin
Cyclobond II	γ-Cyclodextrin
Cyclobond III	α-Cyclodextrin
Cyclobond I-acetylated	Acetylated β-cyclodextrin
Cyclobond III-acetylated	Acetylated-cyclodextrin
α-Cyclodextrin=Daltosil 100	α-Cyclodextrin
β-Cyclodextrin=Daltosil 100	β-Cyclodextrin
EnantioPac	α_1-Acid-glycoprotein
ES D-DMB-LEU	(R)-DNB-Leucine (covalent
ES L-DNB-LEU	(S)-DNB-Leucine (covalent)
ES D-DNB-PHGLY	(R)-DNB-Phenylglycine (covalent)
ES L-DNB-PHGLY	(S)-DNB-Phenylglycine (covalent)
ES R-PU	(R)-Phenylethylurea
ES S-PU	(S)-Phenylethylurea
Grom-chiral-(R)-DNBPG-C	(R)-DNB-Phenylglycine (covalent)
Grom-chiral-(R)-DNBPG-I	(R)-DNB-Phenylglycine (ionic)
Grom-chiral-(S)-DNBPG-C	(S)-DNB-Phenylglycine (covalent)
Grom-chiral-(S)-DNBPG-I	(S)-DNB-Phenylglycine (ionic)
Grom-chiral-(R)-DNBL-C	(R)-DNB-Leucine (covalent)
Grom-chiral-(R)-DNBL-I	(R)-DMB-Leucine (ionic)
Grom-chiral-(S)-DNBL-C	(S)-DNB-Leucine (covalent)
Grom-chiral-(S)-DNBL-I	(S)-DNB-Leucine (ionic)
Grom-chiral-beta-CD	β-Cyclodextrin
Grom-chiral-HP	Hydroxyproline copper
Grom-chiral-P	Prolinamide
Grom-chiral-PC	Proline copper
Grom-chiral-PC	Proline copper
Grom-chiral-U	(R)-N-α-Phenylethylurea
Grom-chiral-UC	Ualin copper
Ionic L-leucine	(S)-DMB-leucine (ionic)
Ionic D-phenyl glycine	(R)-DMB-phenylglycine (ionic)
MCI gel CRSIOW	C_{18}-Silica gel with N,N-dioctyl-(S)-alanine
Nucleosil chiral-1	Hydroxyproline copper
Nucleosil chiral-2	
Optimer P1	Aromatic amide
Optimer L1	Aliphatic amino acid copper
Optimer L2	Aromatic amino acid copper
Resolvosil	Beef serum albumin

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(continued)

Type	Particle Size	Source
Cavity	10	Daicel
Cavity	5	Astec
Cavity	5	Astec
Cavity	5	Astec
Cavity	5	Astec
Cavity	5	Astec
Cavity	4	Serva
Cavity	4	Serva
Protein	10	Pharmacia
Brush	5	ES
Brush	5	ES
Brush	5	ES
Brush	5	ES
Brush	5	ES
Brush	5	ES
Brush	5	Grom
Brush	5	Grom
Brush	5	Grom
Brush	5	Grom
Brush	5	Grom
Brush	5	Grom
Brush	5	Grom
Brush	5	Grom
Cavity	5	Grom
Ligand exchange	5	Grom
Ligand exchange	5	Grom
Ligand exchange	5	Grom
Ligand exchange	5	Grom
Brush	5	Grom
Ligand exchange	5	Grom
Brush	5	Regis, Alltech
Brush	5	Regis, Alltech
Ligand exchange		Mitsubishi
Ligand exchange	5	Macherey-Nagel
Brush	5	Macherey-Nagel
Brush	4	Toyo Soda
Ligand exchange	5	Toyo Soda
Ligand exchange	5	Toyo Soda
Protein	7	Macherey-Nagel

Continued on next page

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Table 1.3 Commercial Chiral Columns for HPLC*

Name	Chemical Name
Spherisorb chiral 1	(R)-N-α-Phenylethylurea
Spherisorb chiral 2	(R)-Naphthylethylurea
Sumichiral OA-1000, OA	α-Naphthylethylamide
Sumichiral OA-2000	(R)-DMB-Phenylglycine (ionic)
Sumichiral OA-2000A	(R)-DNB-Phenylglycine (covalent)
Sumichiral OA-2100	Chlorphenyl-isovaleroyl-phenylglycine
Sumichiral OA-2200	Chrysanthemoyl-phenylglycine
Sumichiral OA-3000	tert, Butylaminocarbonyl-valine
Sumichiral OA-4000	(S),(S)-α-Naphthylethyl-aminocarbonyl-valine
Sumichiral OA-4100	(R),(R)-α-Naphthylethyl-aminocarbonyl-valine
Supelcosil LC-(R)-naphthylurea	(R)-Naphthylethylurea
Supelcosil LC-(R)-urea	(R)-Phenylethylurea
Triacetylcellulose	Cellulose-triacetate
Trichsep-100	Cellulase
TSKgel Enantio Li	Aliphatic amino acid copper
TSKgel Enatio L2	Aromatic amino acid copper
TSKgel Enantio P1	(S)-Aromatic amide
Ultron OVM	Ovomucoid
VMC-Pak K	Polymer with (R)-naphthylethylamine

*Adapted from V. Meyer

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(continued)

Type	Particle Size	Source
Brush	5	PhaseSep
Brush	5	PhaseSep
Brush	5,10	Sumika
Brush	5,10	Sumika
Brush	5,10	Sumika
Brush	5,10	Sumika
Brush	5,10	Sumika
Brush	5,10	Sumika
Brush	5,10	Sumika
Brush	5,10	Sumika
Brush	5	Supelco
Brush	5	Supelco
Helix	10	Merck
Protein	10	Sonsep
Ligand exchange	5	TosoHaas
Ligand exchange	5	TosoHaas
Brush	4	TosoHaas
Protein		Shinwa
Brush	5	YMC

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pairs, and inclusion complexes. Each method is based on the formation of reversible complexes and uses an achiral chromatographic packing.

A. Ligand Exchange

Chiral ligand exchange is an excellent method for the resolution of amino acids and amino-acid-like compounds. The molecules need not be derivatized, and the aqueous mobile phases are compatible with automated column-switching techniques.

A number of chiral molecules have been resolved by ligand-exchange chromatography. However, the resolution is possible for only those molecules that are able to form coordination complexes with transition metal ions. This method is most often utilized with free and derivatized amino acids and similar compounds. There has been some success with other classes of compounds including carboxylic acids, amino alcohols (as Schiff bases), barbiturates, hydantoins, and succinimides (20). The mobile phases employed with chiral ligand exchange are aqueous, with the metal ions and selector ligands added as modifiers.

Chiral ligand-exchange chromatography is based on the formation of diastereomeric complexes involving a transition metal ion (M), a single enantiomer of a chiral molecule (L), and the racemic solute (d and l). The diastereomeric mixed chelate complexes formed in this system are represented by the following formulas: L-M-d and L-M-l. The most common transition metal ion used in these separations is Cu^{+2}, and the selector ligands are usually amino acids such as L-proline. The chromatography is most often carried out using an achiral HPLC packing (such as C-18) with these compounds added to the mobile phase.

The efficiency and selectivity of a chiral ligand-exchange system can be improved by binding the selector ligand to the stationary phase. Some examples of this approach are the L-proline-containing stationary phase, an L-(+)-tartaric-acid-modified silica reported by Kicinski and Kettrup, and a chiral phase composed of a C-18 column dynamically coated with (R,R)-tartaric acid mono-n-octylamide (7).

The major disadvantage of chiral ligand exchange is the small number of compounds that can be resolved by this approach. Many of the cationic and anionic molecules of pharmacologic interest have not been resolved by this method.

B. Ion Pairing

Ion-pair chromatography is a liquid chromatographic method commonly used with charged solutes. The method is based on the formation of a "neutral complex" (ion pair, SC) between a charged solute (S^+) and a counterion of opposite charge (C^-).

When both the solute and the counterion are optically active, diastereoisomeric ion pairs are formed. These ion pairs often can be

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separated by differences in their solvation in the mobile phase or in their binding to the stationary phase. A number of different counterions have been employed in this approach, including (+)-10-camphorsulfonic acid, quinine, quinidine, cinchonidine, (+)-di-n-butyltartrate, and the protein albumin. This method has been reviewed recently by Pettersson and Schill (21).

The solutes resolved by chiral ion-pairing chromatography have included amino alcohols such as alprenolol, carboxylic acids such as tropic acid and naproxen, and amino acids such as tryptophan.

The composition of the mobile phase depends on the chiral agent used. When a chiral counterion is used, a mobile phase of low polarity such as methylene chloride is used to promote a high degree of ion-pair formation. The retention of the solute can be decreased by increasing the concentration of the counterion or by the addition of a polar modifier such as 1-pentanol. The latter approach usually results in a decrease in the stereoselectivity. The water content of the mobile phase also appears to be important, and a water content of 80 to 90 ppm has been recommended.

With serum albumin as the chiral agent, aqueous mobile phases containing phosphate buffers are used. The retention and stereoselectivity can be altered by changing the pH. Both aqueous and nonaqueous mobile phases can be used when (+)-di-n-butyltartrate is the chiral modifier. In some cases it appears that the modifier is retained by the stationary phase when the column is equilibrated with an aqueous mobile phase. The system then can be used with an organic mobile phase (21).

The chiral ion-pairing systems are not stable. The chromatography can be affected by the water content of the mobile phase, temperature, pH, and a number of other variables. This makes the routine applications difficult. In addition, the counterions often absorb in the UV region, reducing the sensitivity of the system; indirect photometric detection (22) or other detection methods must be used.

C. Inclusion

Cyclodextrins are cyclic oligosaccharides composed of d-α-glucose units linked through the 1,4 position. The three most common forms of this molecule are α-,β-, and γ-cyclodextrin, which contain 6, 7, and 8 glucose units, respectively. Because of the d-α-glucose units, cyclodextrin has a stereospecific, doughnut-shaped structure. The interior cavity is relatively hydrophobic and a variety of water-soluble and insoluble compounds can fit into it, forming inclusion complexes. If these compounds are chiral, diastereoisomeric inclusion complexes are formed.

β-Cyclodextrin has been used by Sybilska et al (23) as a chiral mobile-phase additive in the resolution of mephenytoin, methylphenobarbital, and hexobarbital. They attribute the observed resolution to two different mechanisms. The resolution of mephenytoin is due to a difference in the absorption of the diastereoisomeric complexes on

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the achiral C-18 support. For methylphenobarbital and hexobarbital, the relative stabilities of the diastereoisomeric complexes are responsible for the resolution of these compounds.

In addition to the compounds listed above, mobile phases modified with β-cyclodextrin can resolve mandelic acid and some of its derivatives (24,25). Aqueous mobile phases modified with a buffer such as sodium acetate are commonly utilized. Alcoholic modifiers such as ethanol can be added to the mobile phase to reduce retention.

Automation is possible for the direct measurement of biological samples. Sybilska et al (23) have used it for preparative separations of mephenytoin, methylphenobarbital, and hexobarbital.

The applications of this approach seem limited. For example, unlike hexobarbital and methylphenobarbital, the chiral barbiturates secobarbital, pentobarbital, and thiopental are not resolved when chromatographed with a β-cyclodextrin-containing mobile phase (21). Other structural limitations of resolution involving cyclodextrin inclusion complexes are discussed in Section 1.2.3.

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1.2.3 Enantiomeric Resolution Using Chiral Stationary Phases

Enantiomers can be resolved by the formation of diastereomeric complexes between the solute and a chiral molecule that is bound to the stationary phase. The stationary phase is called a CSP, and the use of these phases is the fastest-growing area of chiral separations. The first commercially available HPLC-CSP was introduced by Pirkle in 1981 (26). Currently a large number of chiral phases are commercially available.

The separation of enantiomeric compounds on CSP is due to differences in energy between temporary diastereomeric complexes formed between the solute isomers and the CSP; the larger the difference, the greater the separation. The observed retention and efficiency of a CSP is the total of all the interactions between the solutes and the CSP, including achiral interactions.

Since there are so many HPLC-CSPs available to the chromatographer, it is difficult to determine which is most suitable to solve a particular problem. This difficulty can be partially overcome by grouping the CSPs for chiral separations according to a common characteristic. The first step, that is, the formation of the solute-CSP complexes, is more readily adaptable to the development of a classification system. Using this as a criterion for the division of CSPs into groups, the current commercially available CSPs can be divided into five categories (7).

Type 1 - The solute-CSP complexes are formed by attractive interactions, hydrogen bonding, π-π interactions, dipole stacking, etc., between the solute and CSP.

Type 2 - The primary mechanism for the formation of the solute-CSP complex is through attractive interactions but where inclusion complexes also play an important role.

Type 3 - The solute enters into chiral cavities within the CSP to form inclusion complexes.

Type 4 - The solute is part of a diastereoisomeric metal complex (chiral ligand-exchange chromatography).

Type 5 - The CSP is a protein and the solute-CSP complexes are based on combinations of hydrophobic and polar interactions.

HPLC resolutions of enantiomers induced by molecular associations, in which the main driving force is the action of weak hydrogen bonds, have been described (27-28). The hydrogen-bond association potential was first demonstrated through the optical resolution of racemic N-acylated amino acid esters using a chiral stationary phase (N-acyl-L-valylamino) propyl silica gel. Following this preliminary study, application was made of the chiral mobile phase additive (CMPA1) on which the fundamental structure of the chiral graft of CSP is reproduced, to resolve the above enantiomers in liquid-solid chromatography. The addition of N-acetyl-L-valine tert-butylamide to the nonaqueous mobile phase solvent of a silica gel column successfully brought about this optical resolution. Two types of chiral additives derived from a chiral skeleton (R,R)-tartaric acid were found capable of resolving various kinds of enantiomers, such as dialkyl tartrate and dialkyl tartramide. Of these two, the latter having an isopropyl substituent (CMPA2), led to a wide range of resolution of enantiomers of the following categories: α- and β-hydroxycarboxylic acid, β-hydroxy ketone, β-amino alcohol, α-amino acid, α-hydroxy ketoxime derivatives, and bi-β-naphthol. This occurred when the enantiomers, except β-hydroxy ketones, α-hydroxy ketoximes, 1,2-diols, and bi-β-naphthol, were derivatized to respond to the hydrogen bonding sites of the additive molecules.

Cyclodextrin chiral phases have been shown to be widely applicable for the separation of enantiomers, diastereoisomers, structural isomers, and routine compounds (Table 1.4). The efficiency and selectivity of the β-cyclodextrin column have been improved. In addition, the mechanism of separation on cyclodextrin bonded media, solvent effects, temperature effects, and structural effects on chiral separations have been investigated (29).

It is widely believed that an inclusion complex should be formed for chiral recognition to be possible (30). This has been verified by performing a normal-phase separation, for example, using a hexanol: 2-propanol mobile phase, on a β-cyclodextrin column. The hydrophobic solvent occupies the cyclodextrin cavity and the enantiomeric solute is restricted to the outside surface of the cyclodextrin cavity. No enantiomeric resolutions have been achieved in this mode as yet, although excellent routine separations are common. Apparently, the inclusion complex formed should be relatively "tight fit" for the hydrophobic species in the cyclodextrin cavity (29,31). For example, β-cyclodextrin seems to exhibit better enantioselectivity for molecules the size of biphenyl or naphthalene than it does for smaller molecules (29). Smaller molecules are not tightly held and appear to move in a manner where they feel the same average environment. It

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Table 1.4 A Brief Summary of Stereoisomeric Separations of Different Classes of Compounds. One Specific Example is Given for Each Class (29)

Enantiomeric Compounds[a]	k'	α	R_s	Mobile Phase[b]
Dansyl amino acids				
(l)-norleucine	1.90	1.26	2.30	50:50
(d)-norleucine	2.40			
β-Naphthyl amino acid derivatives				
l-alanine β-naphthylamide	5.1	1.20	2.00	50:50
d-alanine β-naphthylamide	6.1			
Barbiturates				
(-)mephobarbital[c]	14.8	1.14	1.6	20:80
(+)mephobarbital	16.9			
Metallocenes				
(-)s-(1-ferrocenylethyl)-thiophenol	3.1	1.39	2.27	90:10
(+)s-(1-ferrocenylethyl)-thiophenol	4.3			
Carboxylic acids				
α-methoxy-α-trifluoromethyl-	7.5	1.31	0.6	50:50
phenyl acetic acid[c]	9.8			
Miscellaneous				
(-)DIOP[d]	10.56	1.12	1.2	48:52
(+)DIOP	11.84			

[a] A 10 cm β-cyclodextrin column was used.

[b] Numbers represent the volume percent of methanol to water. The flow rate was 1.0 mL/min.

[c] 25 cm β-cyclodextrin in column.

[d] 2,3,0-isopropylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane.

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also appears that the chiral center must be near the cavity entrance or have a substituent oriented in a specific position so that it would be able to form at least one strong interaction with the groups present at the cavity entrance. When an enantiomer is able to fulfill the above conditions, the possibility for chiral recognition is good. Enantioselectivity appears to be due to a combination of cyclodextrin's gross geometry, which allows inclusion complex formation and the chirality of the number 2 and 3 glucose carbons at the entrance of the cavity.

It is necessary for a solute to interact with the mouth of the cyclodextrin cavity in order to observe enantioselectivity (30,32-35). Extensive use of CD-bonded phases makes it apparent that small changes in the structure of either the cyclodextrin or the chiral solute, can in some cases cause large differences in enantioselectivity. Norgestrel is an example where the chiral center (the number 17 carbon) and its substituents are spatially too far from the mouth of the CD cavity to interact with the 2-hydroxyl groups. Derivatizing the hydroxyl group effectively changes the enantioselectivity of the stationary phase and enhances chiral recognition. Conversely, if the chiral center of the solute is hidden between large bulky substituents, one can alter the structure of the solute to enhance chiral recognition. This has been demonstrated with a series of metallocene compounds (30). Resolution of (±) α-ferrocenylbenzylalcohol is not possible since the hydroxyl substituent attached to the chiral carbon is apparently hidden between the bulky ferrocene and phenyl groups. By replacing the hydroxyl group with thioethanol, the length of the hydroxy-substituent on the chiral carbon was extended beyond the bulky groups and good resolution was observed. Another example of how small changes in a solute's structure can effect selectivity is the compound binaphthyl crown-5 (36). This crown ether was baseline-separated on a 25-cm β-cyclodextrin column. But when one of the crown oxygen atoms was replaced with a nitrogen atom (binaphthyl mono-azo-crown-5), resolution was no longer observed. It is apparent that the ability to make small changes in either the cyclodextrin or enantiomer structure provides an additional powerful tool to resolve enantiomeric mixtures.

A wide range of solvents can be used with cyclodextrin-bonded phases depending on the particular application. By using mobile-phase mixtures such as hexanol:2-propanol, the cyclodextrin stationary phase is made to function as a normal phase. Separations tend to be analogous to those of a diol column because solutes adsorb to the hydroxyls on the outside of the cyclodextrin while the hydrophobic solvent occupies the cavity. Inclusion complexes usually are formed only in the presence of water and certain organic modifiers such as dimethyl sulfoxide, dimethyl formamide, acetonitrile, and alcohols (29,32). Since the interaction of solutes with cyclodextrin is greatest in water, retention can be increased by increasing the water concentration in the mobile phase. While broad peaks and tailing are artifacts often associated with long retention times, they can be minimized by the use of buffers. Buffers such as 0.1 to 1% triethylammonium acetate (TEAA), pH = 4.1, sharpen eluting peaks and increase resolution and efficiency. For the derivatives of amino acids and

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peptides, use of the buffer TEAA (1%, pH = 4.1) instead of water, has produced up to fourfold increases in efficiency. Other buffers such as ammonium acetate and phosphate buffers that are compatible with cyclodextrin bonded phases, may also be used.

It should be noted that the elution order of most compounds in the reversed-phase mode on β-cyclodextrin media can be different from that using traditional reversed-phase columns. This is indicative of the fact that the retention mechanisms are not the same. For example, at all solvent compositions measured, the arene tricarbonylchromium complex of benzene is retained much longer on β-CD than on C-18 while the benzene free-ligand is retained much longer on ODS than β-CD (37).

Mobile-phase composition affects enantiomeric separations. For example, as the methanol concentration is decreased, resolution and retention time increase. Therefore, the higher the concentration of the organic modifier, the easier it is for a solute to be displaced from the cyclodextrin cavity. Acetonitrile and ethanol exhibit a greater affinity for the cyclodextrin cavity than methanol, consequently much lower concentrations of these modifiers are needed to obtain comparable retention times. Furthermore, selectivities of some compounds are very different in $MeOH/H_2O$. While most compounds studied have exhibited a higher degree of selectivity in methanol/water, a few compounds give better separations in acetonitrile/water mobile phases (29). Further studies are currently in progress to understand fully the mechanisms involved.

Temperature changes have a greater effect on the retention of solutes on cyclodextrin bonded phases than on comparable reversed-phase columns. This is because the binding constant of a solute to the cyclodextrin is significantly affected by temperature. As temperature is increased, the binding of the solute to cyclodextrin decreases rapidly. In fact, K_s approaches zero between 60°C and 80°C for most compounds (29).

Chromatographic techniques have been widely used for the separation of various metal complexes and have been recognized by coordination chemists as indispensable for the separation and purification of various kinds of isomers (geometric, diastereomeric, and enantiomeric) of a wide variety of coordination compounds. A vast number of studies have been carried out in this area. Most of them, however, had as their aim the isolation of pure isomers, with a discussion of the correlation between properties and structures, so that their efforts were concentrated mainly on the search for appropriate separation conditions. From the chromatographic viewpoint, the data are diverse and non-systematic.

The addition of chiral N-acetyl-L-valine-*tert*-butylamide to the mobile phase using a silica gel column has resulted in the optical resolution of d- and l-amino acid derivatives of N-acetyl-O-tert.-butyl esters. The enantioselectivity generated by the diastereoisomeric chelate-like solvates is based on intermolecular hydrogen bonds between the chiral additive and amino acid enantiomers. The degree of enantioselection was found to depend markedly on the composition

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of the chloroform-n-hexane mobile phase containing the chiral additive. The chromatographic process responsible for the recognition of the enantiomers is related to the equilibrium relationship in the column, involving the chiral additive and amino acid derivatives to be resolved (38).

Direct optical resolution by HPLC based on the enantioselective properties of a protein, particularly bovine serum albumin (BSA), has been shown to be a very versatile method with many useful analytical applications. Although the mechanism of chiral recognition by the protein is largely unknown, some empirically found correlations between retention behavior and mobile phase composition gave a general idea of the main types of solute-protein interactions involved (39).

Analytical-scale optical resolution of a series of N-(2,4-dinitrophenyl)-and dansyl-d,l-amino acids has been affected by the use of a bovine serum albumin (BSA) silica column (Resolvosil). Decreasing retention was found for both types of amino acid with increasing pH or 1-propanol content of the mobile phase. In the dinitrophenyl series, the aspartic acid derivatives showed very large enantiomeric separation factors compared with the glutamic acid homologue, and an analogous, but less pronounced, effect was found for the phenylglycine-phenylalanine pair. Fluorimetric studies of dansyl-alanine showed that by a simple postcolumn addition of 1-propanol, the fluorescence yield can be increased by a factor of over 20, giving a very low detection limit. The analytical technique is useful for the determination of the bacterial marker compounds, D-alanine and D-glutamic acid, present in cell wall hydrolysates (40).

A commercially available chiral stationary phase containing α-$_1$-acid glycoprotein on silica (EnantioPac, LKB) has been applied to the resolution of a number of pharmacologically important enantiomeric ammonium compounds. The optimization of retention and selectivity by cationic, anionic, and neutral modifiers in the mobile phase was studied. The results suggest that the solutes are retained according to an ion-pair distribution model. Compounds of widely different structures were studied, and high separation factors were achieved for a majority (41). Some examples covering different kinds of hydrogen bonding (HB) groups are given in Table 1.5. The results suggest that the magnitude of the enantiomeric resolution is affected by the strength of the HB substituent.

Previous studies on separation of enantiomeric ions by binding to the HB group and the charged site of achiral reagent indicate that the distance between these binding sites is of vital importance. These relationships could not be confirmed in this study. No chiral resolution is obtained when the asymmetric carbon atom is in a beta-position relative to the amide. Differences in stereoselectivity also appear between diastereoisomers. The R,S;S,R enantiomers of some α,β-amino alcohols, for example, labetalol A and ephedrine, display a higher selectivity than the corresponding R,R;S,S enantiomers, labetalol B and pseudoephedrine. Nadolol displays the same tendency when chromatographed with 0.001M tetrabutylammonium bromide as the

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Table 1.5 Highest Separation Factors with a Selection of Mobile Phases (41)

Mobile Phase: Modifier in 0.02M Phosphate Buffer

Solute	α	Modifier[a]	Solute	α	Modifier[a]
Atropine	1.64	8	Mepivicaine	1.25	3
Bromodiphenhydramine	1.17	5	Methadone	1.59	6
Brompheniramine	1.50	14	Methorphan	2.54	12
Bupivicaine	1.41	3	Methylatropine	1.27	9
Butorphanol[b]	1.99	2	Methylhomatropine	4.2	11
Carbinoxamine	1.33	16	Methylphenidate[b]	1.70	14
Chlorpheniramine	2.26	14	Metoprolol	1.64	4
Clidinium	1.21	1	Nadolol A	3.98	12
Cocaine[b]	1.46	15	Nadolol B	3.03	12
Cyclopentolate	3.86	11	Oxyphencyclimine	1.42	6
Dimethindene	1.53	6	Oxprenolol	1.25	5
Diperodone	1.47	17	Phenmetrazine[b]	1.57	8
Disopyramide	2.70	6	Phenoxybenzamine	1.37	16
Doxylamine	1.37	13	Promethazine	1.25	5
Ephedrine	1.83	7	Pronethalol	1.26	16
Ephedrine, pseudo-	1.34	7	Propoxyphene[b]	2.3	6
Homatropine	1.63	8	Propranolol	1.13	17
Labetalol A	2.10	14	Terbutaline	1.22	11
Labetalol B	1.36	7	Tocainide	1.44	10
Mepensolate	1.32	5	Tridihexethyl	1.64	17

[a]Modifiers: (H_3PO_4 or NaOH added to give the indicated pH)
1 = 0.33M 2-propanol, pH 7.0;
2 = 0.67M 2-propanol, pH 7.0;
3 = 1.33M 2-propanol, pH 7.0;
4 = 0.1M NaCl, pH 7.0;
5 = 0.1M NaCl + 1.74M ethanol, pH 7.0;
6 = 0.1M Nacl + 1.33M 2-propanol, pH 7.0;
7 = 0.05M butyric acid, pH 7.0;
8 = 0.01M octanoic acid, pH 7.0;
9 = 0.25M cyclohexylsulfamic acid, pH 7.0;
10 = 0.001M tetrapropylammonium bromide (TPrABr), pH 6.0;
11 = 0.003M TPrABr, pH 7.0;
12 = 0.001M tetrabutylammonium bromide (TBuABr), pH 6.0;
13 = 0.003M TBuABr, pH 6.0;
14 = 0.003M TBuABr, pH 7.0;
15 = 0.001M dimethyloctylamine (DMOA), pH 7.0;
16 = 0.001M DMOA + 0.17M 2-propanol, pH 7.0;
17 = 0.002M DMOA + 0.33M 2-propanol, pH 7.0;

[b]One of the diastereoisomers (for which there are no generally accepted names).

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modifier. However, when 0.025 M butyric acid is used as the modifier, the R,R;S,S enantiomeric pair, nadolol B, has a higher selectivity, that is, 2.30, compared to 2.16 for nadolol A.

A chiral α-$_1$-acid glycoprotein column (EnantioPac®)has been used for the separation of the enantiomers of some acidic drugs (ibuprofen, ketoprofen, naproxen, 2-phenoxypropionic acid, bendroflumethiazide, ethotoin, and hexobarbital) and basic drugs, such as disopyramide. The column was prepared by immobilization of the human plasma protein α-$_1$-acid glycoprotein on silica particles. The retention and the enantioselectivity of the solutes were easily regulated by the addition of the tertiary amine N,N,-dimethyloctylamine (DMOA) to the mobile phase. DMOA decreased the retention and the enantioselectivity of the weaker acids, whereas the retention and the enantioselectivity of the stronger acids increased drastically with increasing DMOA concentration.

The influence of column temperatures between 25°C and 80°C on the separation factor, separation efficiency, and the resolution has also been evaluated. Stability studies indicated that the α-$_1$-acid glycoprotein column (EnantioPac®) is very stable. It can be used at elevated temperatures and with 2-propanol, and loses <10% of its capacity after 12 months' storage in water-2-propanol mixture (42).

Chromatography with the use of immobilized BSA as a stationary phase and aqueous buffer systems as eluents has proved to be a highly selective method, capable of separating structurally very closely related compounds. Retention can be effectively regulated by changes in at least three independent parameters of the mobile phase, which may be used for an optimization of separation factors. Particularly, the enantioselective properties of the chiral stationary phase have been demonstrated to be useful for the analytical resolution of a variety of racemates into enantiomers. From the variation of the retention behavior with sustituent effects, as well as the mobile-phase composition, some indications regarding the molecular interaction forces regulating the substrate protein equilibria have been obtained (43).

The plasma protein α-$_1$-acid glycoprotein (orosomucoid) has been used as a chiral complexing agent in the mobile phase in combination with a non-chiral diol silica column for the resolution of racemic drugs (neuroleptics and tricyclic antidepressants). The retention behavior of the solutes has been investigated and a model for the retention has been developed. From the retention data, it has been possible to calculate the affinity constants for the binding of the enantiomers of promethazine, alimenazine, and trimipramine to the protein. The capacity factors of the solutes were regulated by the protein concentration or by adding a tertiary amine to the mobile phase. Increasing the protein concentration had little influence on the separation factor but decreased the capacity factors. The addition of the tertiary amine to the mobile phase affected the stereoselectivity, and hydrophobic amines caused drastic effects (44).

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α-$_1$-Acid glycoprotein (orosomucoid) was immobilized on silica microparticles and used as a chiral phase for liquid chromatographic resolutions of enantiomers of racemic drugs. The capacity factors of the solutes were easily regulated by changing the pH, or by adding 1-propanol to the mobile phase. The separation factor increased for some enantiomeric pairs, whereas it decreased for others with increasing pH of the mobile phase. Addition of 1-propanol to the mobile phase decreased the separation factor. Separation factors between 1.1 and 3.0 were obtained. Eight racemic drugs or drug metabolites were resolved (45).

It is well known from the literature that albumin shows high stereoselectivity against a variety of chiral organic compounds, for example, drugs. Albumin-silica columns were scaled up to study albumin usefulness for preparative purposes. By adsorbing albumin onto a prepacked silica column using a phosphate buffer at pH 5.0, columns containing 0.1 g albumin per g silica were easily made. Because of the high molecular weight of the chiral component and the limited number of binding sites of each molecule, the capacity of the column, an important factor, was investigated. Leaching of albumin from the column limits the use for preparative separations, and the contamination of albumin in compounds isolated from collected fractions was studied as a function of amount of organic modifier in the mobile phase. The study emphasized the ease of preparation of a chiral column and the limitations of using albumin as an adsorbed stationary phase (46).

By immobilization of a protein to a suitable support, its stereodifferentiating binding properties can be used for direct liquid chromatographic separation of enantiomers (47). Quite different racemic organic compounds undergo optical resolution on columns utilizing immobilized BSA or human serum albumin (HSA) and an aqueous buffer as a mobile phase. A BSA-silica column for analytical HPLC, permitting fast separations, has been developed.

Capacity ratios, k', and enantiomeric separation factors, α, were strongly influenced by three different mobile-phase parameters, viz. pH, ionic strength, and organic solvent modifier content (1-propanol). Hydrophobic substituents gave increased retention of a compound; for example, k'-values of N-2-naphtoyl derivatives of amino acids are much larger than of the N-benzoyl derivatives. Other substituent effects were reflected in k' and α in an unpredictable manner. The steric bulk of the substituent affects the enantioselection, as reflected in the α=7.5 found for R_1=t-Bu.

From the applications point of view, the method is well suited to stereochemical bioanalytical problems requiring a highly sensitive analytical technique for determination of enantiomeric composition. It has been shown that stereoselective kinetic processes, such as microbial degradation of N-benzoyl-d,l-alanine and α-chymotrypsin-catalyzed hydrolysis of N-acetyl-d,l-tryptophan ethyl ester, can be investigated by direct chromatographic studies of the product formation as a function of time.

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A new, commercially available glycoprotein CSP, based on immobilized ovomucoid (Ultron), has been evaluated and compared to the second-generation acid glycoprotein CSP (Chiral AGP) for the direct resolution of enantiomers of several commercially available racemic drugs and seven proprietary development drug candidates (48). The experimental protocol utilized simple mobile phases with optimization schemes varying only in phosphate buffer concentration, pH, and the uncharged solvent modifiers. Because of concerns for consistent column performance, cationic and anionic modifiers were not used to achieve separations. Stability was monitored by comparing separation factors, resolution, and peak widths following 200 injections made during the course of methods development. In general, separations using the ovomucoid column were more efficient and stable than those using the acid glycoprotein phase, with beta blockers as a class being the exception. Coupling achiral-chiral separations using the ovomucoid column achieved detection limits suitable for pharmacokinetic monitoring.

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Chapter 2

Analytical Criteria for Chiral High-Pressure Liquid Chromatography

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The determination of enantiomeric composition via chiral stationary phases (CSPs) has evolved over the past decade to a point where this analytical methodology now has the characteristics of a mature technology. High selectivities and efficiencies combine to produce resolutions more than adequate to meet most analytical challenges. Less measurable, but equally significant, are gains in ruggedness, versatility, and procedural simplicity. Examples from the author's laboratory involving the chromatographic resolution of chiral pharmaceuticals illustrate these advances. The analyses of amphetamine, methamphetamine, tryptophan and ibuprofen are discussed in detail; all are assayed with high efficiency and selectivity on a 1-(1-naphthyl)ethyl urea CSP, following procedurally convenient derivatization with achiral reagents. Determinations of trace enantiomeric contamination at 1%, 0.1% and below are achieved with high precision. Guidelines for system suitability criteria for chiral HPLC are proposed.

The explosive growth of methodology for chiral separations in the past decade, especially in the area of development of HPLC chiral stationary phases (CSPs), has been well documented (*1*, *2*). This growth has been accompanied by a gradual shift in perceptions among practitioners concerning what is both possible and practical in this field. The direct separation of enantiomers, once considered rare except in special cases, is now a realistic objective, not only at the research level, but also in the working analytical laboratory, and this is true for the vast majority of chiral compounds of interest. Indeed, often a variety of available CSPs (and of techniques appropriate for a given CSP) are effective, so that the chemist has the luxury of selecting the approach most suited to the analytical objective.

Thus, the focus of the chemist's efforts in the analytical laboratory has shifted from that of merely attaining observable separation to that of achieving optimum resolution, and of applying the resulting methodology to practical problems in chiral

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analysis. Further, the chemist can now realistically select methodology on the basis of the analytical objective; for example, addressing considerations of whether mere isomeric identification is the goal, or whether more accurate and precise measurements of trace and ultra-trace isomeric contamination are desired.

This chapter presents an overview of how the enantiomeric analysis of chiral drug substances via HPLC chiral stationary phases has evolved in the author's drug analysis research laboratory over the past decade, becoming in the process a powerful technique which meets all the analytical criteria which are routinely required of other, non-chiral, methodologies. Specifically, the analyses of the chiral pharmaceutical substances amphetamine, methamphetamine, tryptophan and ibuprofen are discussed in detail, from the viewpoint of the criteria required to establish suitability of the methods for quantitative determination of chiral composition.

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Methodology Considerations.

Choice of CSP. In this laboratory, most of the work has been with a class of CSP variously know as Pirkle-type, brush-type, or synthetic multiple-interaction (SMI) CSPs; the SMI designation will be employed in this chapter, since it is the most descriptive for members of this class, as well as the least exclusive (*3*). These CSPs typically consist of relatively simple chiral molecules, with one or two chiral centers, usually covalently bonded to silica through a methylene chain.

It is characteristic of SMI CSPs that the chiral moiety is rationally selected, or synthesized, with a view to providing discrete solute-CSP interactions, of three types: (1) pi-pi aromatic interaction, (2) hydrogen-bond and/or dipolar interaction and (3) van der Waals attraction and/or steric repulsion. It is frequently hypothesized, in accord with the original suggestion of Dalgliesh (*4*), that three interactions are required for effective enantiodiscrimination. Regardless of the merits of this hypothesis, it has proved to be a useful starting point for efforts to explain the separation process, and as a guide for the design of effective CSPs.

Although this laboratory has investigated many SMI CSPs during the course of research in chiral pharmaceutical analysis, and although more than 100 such CSPs have been described in the literature, with approximately fifteen currently available commercially; nevertheless, for the purpose of discussing the evolution of analytical criteria, it is useful to restrict detailed discussion to two. These are: (1) the covalent 3,5-dinitrobenzoylphenylglycine (DNBPG) CSP (1) of Pirkle (*5*), which was one of the first, and which remains the most thoroughly studied and applied CSP of this type, and; (2) the 1-(1-naphthyl)ethyl urea (NEU) CSP (2) of Oi (*6*), which this laboratory has found to afford superior analytical performance in virtually every application.

$(O_2N)_2C_6H_3$-CO-NH-CH(C_6H_5)-CO-NH-$(CH_2)_3$-Si- (1)

CH_3-CH($C_{10}H_7$)-NH-CO-NH-$(CH_2)_3$-Si- (2)

Both of these CSPs are available commercially, and in both (R)- and (S)-configurations; selection of a particular configuration permits control of enantiomeric elution order of chiral solutes.

The NEU CSP can also be readily prepared by a simple and convenient *in-situ* technique (*7*); this is useful in obtaining customized column lengths and/or silica particle sizes for particular analytical applications.

Among the reasons for selecting this class of CSP are: ruggedness (columns frequently last five years or more); high efficiency (8,000-40,000 plates/m is the commonly observed range, depending in part on solute type and on mobile phase composition); good selectivity (when appropriate interacting groups are present or introduced -- see discussion below); and excellent concentration tolerance (important for quantitative trace analysis).

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Need for Derivatization. Although many chiral molecules can be resolved on SMI CSPs without derivatization (*3*), frequently it is desirable or necessary to modify the molecule. Most commonly, a pi-basic (e.g., naphthyl), or pi-acidic (e.g., 3,5-dinitrophenyl) aromatic group is introduced, for the purpose of enhancing interaction with the complementary aromatic group on the CSP. In addition, such derivatization also provides a useful degree of detection enhancement, particularly in the case of trace analysis.

The derivatizing agent in these applications is normally achiral; therefore, there is no possible analytical error from enantiomeric contamination of the reagent, as is often a serious limitation for diastereomeric methods of chiral analysis. However, it is important to ascertain that derivatization proceeds without measurable racemization at the existing chiral center or centers of the analyte.

Chiral Analysis of Amphetamine

Amphetamine (3) is a chiral pharmaceutical which is manufactured and dispensed in a variety of isomeric compositions. The analysis of this substance provides a good example of how analytical objectives and criteria have evolved over the past decade. Amphetamine itself does not have the structural features conducive to direct resolution on SMI CSPs; chromatography of amide derivatives (readily prepared by

$$C_6H_5\text{-}CH_2\text{-}CH(CH_3)\text{-}NH_2 + Ar\text{-}COCl \longrightarrow C_6H_5\text{-}CH_2\text{-}CH(CH_3)\text{-}NH\text{-}CO\text{-}Ar$$

(3) (4) (5)

standard methods) is more successful. Among the many resolutions achieved in this laboratory during initial studies (*8*), the most suitable for analytical purposes was separation of the 2-naphthoyl amide derivative on the DNBPG CSP (*9*). As shown in Figure 1a, baseline resolution was not quite achieved for the racemic mixture. This is consistent with the calculated resolution factor (R_s) of 1.35 (the generally accepted chromatographic guideline is that an R_s of 1.5 is the minimum requirement for baseline resolution (*10*)).

At a more fundamental level, the failure to obtain baseline resolution is an expected and essentially unavoidable result of two factors: (1) the relatively low

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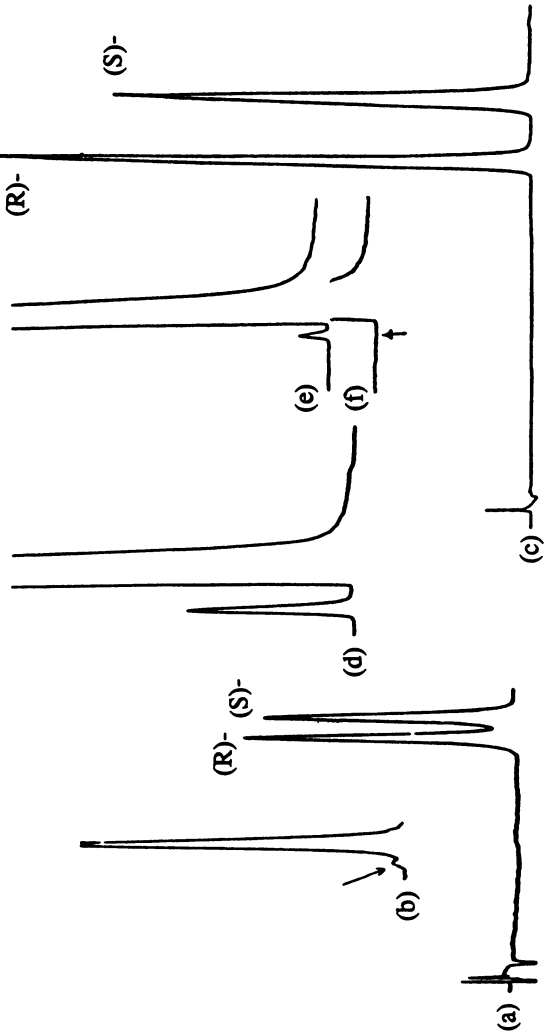

Figure 1. Chiral Resolution of Amphetamine: (a) racemic 2-naphthoyl amide derivative on (R)-DNBPG CSP; (b) same at 1/99 (R)-/(S)- ratio; (c) racemic 3,5-DNB amide derivative on (S)-NEU CSP; (d) and (e) same at 1% and 0.1% (R)- spike of (S)-amphetamine; (f) same, unspiked (S)-amphetamine.

selectivity of 2-(naphthoyl)amphetamine on this CSP (α = 1.09), and (2) the maximum column efficiency associated with commercially available DNBPG CSPs (this will vary with solute type, and with various experimental parameters, but was found to be 4400 plates/25 cm column length, for the example under discussion). From Equation 1, the fundamental relation between R_s and efficiency (N), selectivity (α) and retentivity (k') (*10*), it can be calculated that a plate count of approximately

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{(\alpha-1)}{\alpha} \cdot \frac{k_2'}{(k_2'+1)} \qquad (1)$$

6000 plates/column would be required to achieve baseline resolution within reasonable retention times ($k_2' \leq 20$), in contrast to the experimentally observed value of 4400 plates.

Nevertheless, despite the lack of baseline resolution, the 1984 method (*9*) proved suitable both for enantiomeric identification and for quantitative determination, provided that the proportions of the two isomers were comparable, since mutual peak overlap approximately cancels out in this situation. Suitability of the method for this limited application was demonstrated by an independent collaborative study conducted by Alembik and Wainer (*11*). However, when enantiomeric ratios were 1/99 or less, estimation only was possible. The same collaborative study (*11*) showed poor relative standard deviations (RSDs) between 47 and 71% at the 1% trace level.

Trace Analysis. For trace analysis (1% enantiomeric contamination and below) analytical criteria are more rigorous. Some of the reasons for this are obvious, such as unequal contributions from peak overlap, complications from peak asymmetry, which invalidate computational algorithms, etc. Other factors are more subtle. For example, capacity factors are often observed to be concentration-dependent; the kinetics of mass transfer in chromatographic systems usually result in non-equilibrium conditions. Observations in this laboratory show, in agreement with theory, that capacity factors generally increase with decrease in solute concentration. Experimentally, this increase is often significant.

The fact that capacity factors are concentration-dependent has important practical consequences for trace analysis: the observed selectivity, and therefore the resolution, are less than predicted by results for the racemic mixture. While this is true only for the case where the trace component elutes first, this is, however, the situation which usually occurs by design in a well-chosen chiral chromatographic system. When the trace component elutes last, problems associated with tailing of the preponderant component are often insuperable.

In the case of the naphthoylamide/DNBPG CSP procedure for amphetamine, calculations from empirical results demonstrated that selectivity decreased from 1.09 for the racemate to 1.06 for a 1/99 (R)-/(S)- mixture. The calculated resolution factor correspondingly decreased from 1.35 to 0.92. At this poor resolution (see Figure 1b), quantitative determinations are precluded; the only analytical objectives which can be realized at the 1% level are detection or, at best, estimation.

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System Suitability Criteria. It is necessary to provide for such systematic variations in selectivity during trace analysis, and in addition, to introduce a measure of ruggedness into the analytical methodology, so as to compensate for column variability, especially as the number of commercial sources increases, and also to provide for possible extra-column band-broadening, which may vary among individual HPLC systems. Experience in this laboratory suggests that a minimum resolution factor of 2.0 for the racemic mixture is an appropriate criterion for quantitative applications.

In attempting to meet this criterion, the limiting factor in chiral liquid chromatography will normally be the enantioselectivity of the system, as measured by the separation factor, α. The specific value which will be required in a given case can be calculated from Equation 1. For resolution of amide derivatives of amphetamine on the DNBPG CSP (where N = 4400, and k_2' is experimentally adjusted to approximately 20), this corresponds to a minimum required selectivity, α, of 1.15.

Current Methodology. Alternative methods for chiral resolution of amphetamine on the DNBPG CSP approached, but did not meet, this goal. A variety of other amide derivatives were prepared (*8*), but the results confirmed the importance of the strongly pi-basic naphthyl group as a major factor in chiral recognition. Later, baseline resolution of the racemate was achieved by chromatography of the 2-naphthylcarbamate derivative on the DNBPG CSP (*12*), but selectivity, while improved, was only 1.13. Furthermore, the method was relatively impractical since the required naphthylchloroformate reagent is difficult to prepare.

The current, completely satisfactory, approach developed in this laboratory utilizes instead the NEU CSP, and for appropriate solute:CSP interaction, the 3,5-dinitrobenzoyl amide derivative (the derivatization is quantitative within one minute at room temperature). Figure 1c shows the chromatographic results. The selectivity of 1.19 and plate count of 9800 results in a resolution factor of 4.1, well in excess of the proposed suitability criterion of 2.0.

As a consequence, quantitative trace analysis is readily achieved on this system. Figures 1d and 1e show analysis of 1/99 and 0.1/99.9 enantiomeric mixtures, respectively; Figure 1f shows the result for analysis of standard (S)-amphetamine. Baseline resolution was maintained even at the 0.1% level: it is important to note that the apparent selectivity has decreased, as discussed above, but that the high inherent selectivity compensates for this. The peak shape of the preponderant component also contributes to success of the method: the sharply rising peak front (as opposed to noticeable tail) is expected from the non-equilibrium (and perhaps slightly over-loaded) conditions. Control of elution order by selection of the CSP with appropriate configuration (in this instance the (S)-NEU CSP) is an important factor in the design of appropriate methodology.

Absence of any observable (R)-isomer in the analysis of standard (S)-amphetamine (Figure 1f) demonstrated not only the high level of enantiomeric purity of this material, but also that the derivatization step induced essentially no racemization. In addition, the DNB-chromophore introduced by the derivatization provided the requisite uv detectability ($\epsilon \approx 50{,}000$ at 235 nm) for analysis at this trace level.

Since baseline resolution is maintained at all levels, the analytical statistics can be expected to be dependent solely on the instrumental and chromatographic

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limitations: the results should be comparable to any other (i.e., achiral) analytical methodology. Accuracy and precision for the DNB amide/NEU CSP analysis of amphetamine were 97.0 ± 1.7% and 96.4 ± 6.9% at the 1 and 0.1% levels, respectively (five replicates).

Chiral Analysis of Methamphetamine

Methamphetamine (**9**) is the N-methyl derivative of amphetamine. The chiral analysis of this substance is of particular importance to pharmaceutical chemists, since the dextrorotatory (S)-isomer is a controlled substance with a considerable history of abuse, whereas the levorotatory (R)-isomer is marketed as an over-the-counter nasal decongestant. Initial attempts in this laboratory to resolve methamphetamine (as well as many other chiral secondary amines) as amide derivatives on SMI CSPs were invariably unsuccessful; it became apparent that the absence of an -NH- functionality in amide derivatives of secondary amines resulted in loss of a critical solute:CSP interaction, with consequent attenuation of enantiodiscrimination.

Eventually, methamphetamine was successfully resolved on the DNBPG CSP, as the 2-naphthylcarbamate derivative, with an excellent resolution factor of 2.1 (*12*), permitting quantitation and mass spectrometric identification of 1% isomeric contamination (*13*). However, as mentioned previously, difficulty in preparing the required naphthylchloroformate derivatization reagent presented a serious practical drawback to widespread utilization of the method.

Formation of Urea Derivatives. The analytical problem of resolution of secondary amines has been essentially solved by the development of a convenient procedure for the formation of 3,5-DNP urea derivatives, as first described by Pirkle *et al.* (*14*),

$(O_2N)_2C_6H_3\text{-COCl}$ (6) + NaN_3 (7) → $(O_2N)_2C_6H_3\text{-CON}_3$ (8) →(Δ) $(O_2N)_2C_6H_3\text{-NCO}$ (10)

$C_6H_5\text{-CH}_2\text{-CH(CH}_3\text{)-NH-CH}_3$ (9) + $(O_2N)_2C_6H_3\text{-NCO}$ (10) → $C_6H_5\text{-CH}_2\text{-CH(CH}_3\text{)-N(CH}_3\text{)-CO-NH-C}_6H_3(NO_2)_2$ (11)

and by the high enantioselectivities of these chiral derivatives on the NEU CSP. Introduction of the ureide fragment creates a new -NH- group, ensuring that hydrogen-bond interaction between solute and CSP is available for chiral discrimination. In this laboratory, it has been found that ureide derivatives of both primary and secondary amines afford high chiral selectivities on the NEU CSP, often superior to those observed for amide derivatives of primary amines.

In addition, the procedure for urea formation meets the criteria of practicality and procedural convenience: the precursor (**8**) to the reagent DNB-isocyanate (**10**) is

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a stable solid, readily prepared in bulk, the reagent itself is stable in solution for several days (*7*), and the reaction with methamphetamine is essentially instantaneous and quantitative at room temperature. Indeed, this method of ureide formation has been successfully applied to the extractive derivatization of phenylpropanolamine from plasma samples (*7*).

Chromatography. The resolution of methamphetamine DNP-ureide (**11**) on the NEU CSP, as shown in Figure 2a, is representative of an optimized chiral separation for quantitative analytical purposes. The observed separation factor of 1.30 and the resolution factor of 3.9 exceed the system suitability criteria discussed above. In addition, the peaks are highly symmetrical. To obtain this peak symmetry, it was necessary to add a small amount (0.2%) of acetonitrile modifier to the 90:10 hexane:isopropanol mobile phase; this decreased the selectivity from an initially observed value of 1.40, but resulted in a net gain in resolution, since the observed efficiency increased to 5500 plates/25 cm. The results obtained on columns obtained commercially, compared to those obtained on CSPs prepared *in-situ*, were essentially identical with respect to resolution and selectivity.

Trace and Ultra-Trace Analysis. Quantitative trace analysis on such an optimized system is not difficult. The chromatograms of samples of (R)-methamphetamine spiked with the (S)-enantiomer at the 0.1% level exhibit baseline resolution and a precision (RSD, 5 replicates) of 3.8% (Figure 2b). At the sample concentration and detector sensitivity required to monitor 0.1% levels, some extraneous peaks are also observed, arising from the derivatization (no cleanup is performed), which do not interfere with the sample peaks.

The chromatogram of the reference sample of (R)-methamphetamine did exhibit a barely detectible pen deflection coincident with the retention time of the (S)-isomer (Figure 2c). Analytical sensitivity was enhanced by injection of approximately 100 micrograms of sample on the column, both for an 0.01% spike of (S)- in (R)-methamphetamine (Figure 2d), and for the unspiked sample (Figure 2e). Even at this ultra-trace level, baseline resolution was maintained; however, only estimation by comparison of peak heights was possible: a value of 0.004% (R)-isomer content was estimated. More correctly, since the observed peak could represent an analytical artifact, the analysis demonstrated that the reference material contains not more than 0.004% chiral contaminant.

Determination of chiral contamination at such low levels is rarely necessary; however, these results do represent the current state of analytical capability for chiral HPLC. The feasibility of such ultra-trace determinations reinforces the conclusions of this study: that chiral contamination at the higher levels of 0.1 and 1% should now be considered routinely achievable. Therefore, such determinations are reasonable analytical laboratory objectives, and are subject to normal criteria for acceptance of quantitative results.

Chiral Analysis of Tryptophan

The chiral analysis of amino acids is of unquestioned importance, and has played a seminal role in the development of chiral chromatography. No effort will be made to survey the many excellent and varied methods, both in gas and liquid

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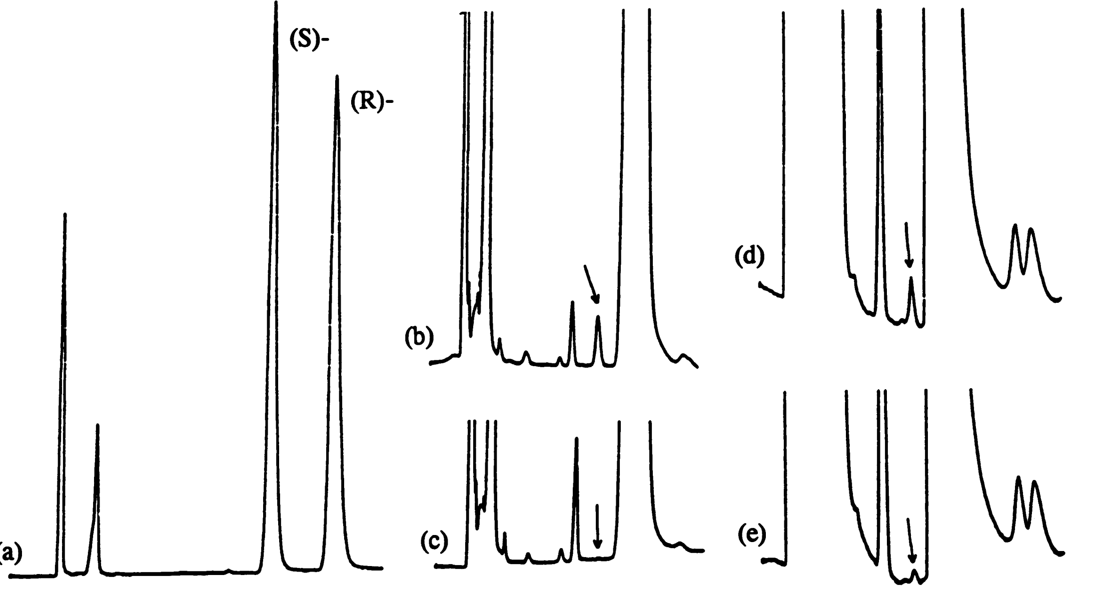

Figure 2. Chiral Resolution of Methamphetamine: (a) racemic 3,5-DNB-ureide derivative on (R)-NEU CSP; (b) and (c) 0.1% spike and unspiked (R)-methamphetamine; (d) and (e) 0.01% and unspiked (R)-methamphetamine at high column load and detector sensitivity.

chromatography, for the resolution of enantiomeric amino acids (the chapter in this volume by Gil-Av discusses this subject in some detail).

Instead, the focus will be on the specific example of determining the chiral purity of (S)-L-tryptophan samples. This recently became an important forensic problem in this laboratory, due to an outbreak of eosinophilia-myalgia syndrome (EMS), which resulted in at least 27 deaths, and which was associated with consumption of L-tryptophan as a dietary supplement. It was necessary to determine if contamination of synthetic (S)-tryptophan with the unnatural (R)-isomer was a factor in the EMS outbreak. (The work described below demonstrated that chiral contamination was not a significant factor.)

Derivatization and Chromatography. Adhering to the analytical criterion that practical methods must be procedurally convenient, yet recognizing that resolution on SMI CSPs is enhanced by introduction of suitable pi-pi interacting groups, as well as by attenuation of strongly polar groups, a method was developed which converted tryptophan (12) to the N-3,5-dinitrobenzoyl methyl ester derivative (14). This

$CH_2-CH(NH_2)-CO_2H$ (12) $\xrightarrow[①]{CH_3OH, H^+}$ $CH_2-CH(NH_2)-CO_2-CH_3$ (13) $\xrightarrow[②]{DNB-Cl}$ $CH_2-CH(NH-CO-C_6H_3(NO_2)_2)-CO_2-CH_3$ (14)

derivatization was accomplished in a two-step, 15-minute, "one-pot" procedure, with no prior or subsequent clean-up. The analytical results (below) also confirmed that essentially no racemization occurred during the derivatization.

Chromatography of the TRP derivative on an (S)-NEU CSP (Figure 3a) showed the typically high selectivity of amino acid derivatives on SMI CSPs; this general approach has been studied extensively by others (*15*). The selectivity (α = 2.28) and resolution (R_s = 6.3) exceed the suggested system suitability criteria; however, the efficiency (N = 1900) was low compared to that observed for other solute types. This is possibly a result of the relatively polar nature of the analyte.

Trace and Ultra-Trace Analysis. Quantitative analysis of samples of (S)-tryptophan spiked at 1 and 0.1% with the (R)-antipode (Figure 3b and 3c, respectively) gave excellent precision (RSD, five replicates) of 0.38 and 1.76%, respectively. The NEU CSP with (S)- configuration was chosen so that the trace component eluted first. This was less to avoid peak overlap (as in the previously discussed examples), since R_s was more than adequate, as it was to compensate for the relatively low efficiency. Analytical sensitivity is enhanced when the first eluted enantiomer is the trace component, since this first peak will typically have a greater height-to-area ratio, and will thus be detectible at lower levels.

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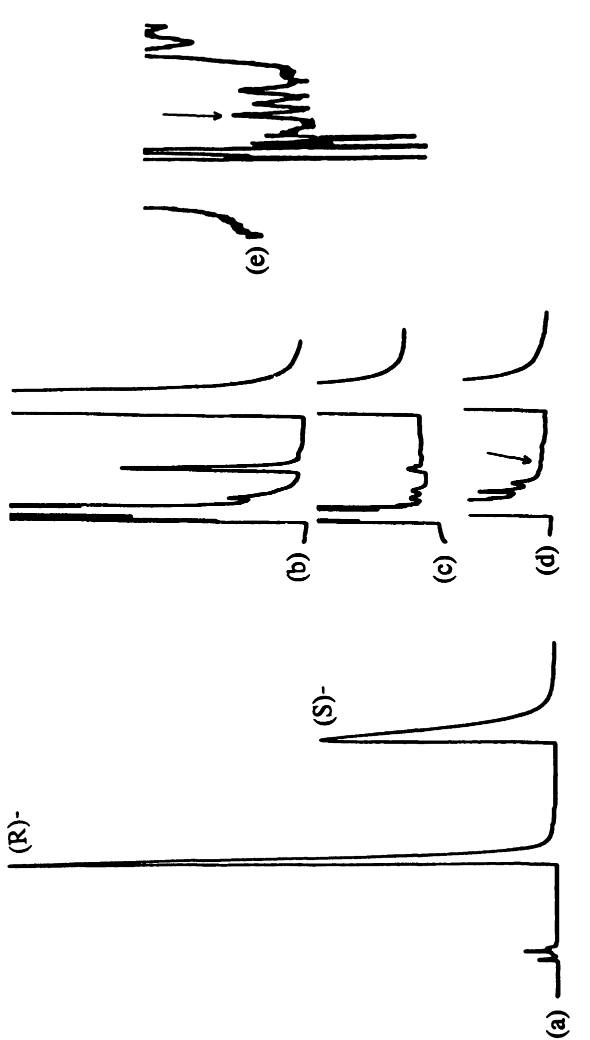

Figure 3. Chiral Resolution of Tryptophan: (a) racemic 3,5-DNB amide, methyl ester derivative on (S)-NEU CSP; (b), (c) and (d) same, 1%, 0.1% spikes and unspiked (S)-tryptophan; (e) unspiked sample at high column load and detector sensitivity.

Since the investigative samples demonstrated that much less than 0.1% (R)-isomer was present in the (S)-tryptophan sample (Figure 3d), efforts to enhance sensitivity, in a manner similar to that described for methamphetamine, were made. The chromtogram (Figure 3e) showed a number of peaks having a uv response representing approximately 0.01% relative to (S)-tryptophan, including one peak coincident with the retention time of the antipode. As with methamphetamine, and in the absence of mass spectrometric confirmation, it was not possible to identify this peak as (R)-tryptophan with certainty, nor to demonstrate that the peak was not an analytical artifact. However, the level of (R)-isomer content was established with confidence to be not more than 0.01%.

Reverse-Phase Analysis. This laboratory also investigated the chiral resolution of tryptophan by reverse-phase elution on a novel NEU stationary phase, in which the chiral moiety is bound to silica via a polyamino support (*16*). The chromatographic results on this CSP also exceeded the analytical criteria for trace analysis; the observed values were α = 1.49, R_s = 2.81. More significantly, esterification was not required; only the N-DNB function was introduced. Because the carboxyl function is free, retention of this (and of other amino acids) is readily controlled by the pH of the water:acetonitrile mobile phases. Trace analyses have not yet been investigated on this reverse-phase system.

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Chiral Analysis of Ibuprofen

Ibuprofen (**15**) is representative of the class of pharmacologically important chiral α-methylarylacetic acids. In humans, members of this class undergo an unusual unidirectional conversion of the (R)-isomer to the therapeutically active (S)-form. Consequently, there has been much attention given to the analytical resolution of α-methylarylacetic acid enantiomers. This laboratory achieved the first enantiomeric HPLC resolution of ibuprofen by chromatography of the 1-naphthylenemethylamide derivative on the DNBPG CSP (*17*). The moderate selectivity observed for this separation (α = 1.12) resulted in a baseline resolution, R_s, of 1.75.

CH-COOH, CH3 (15) → CH-COCl, CH3 (16)

+ O2N, NH2, O2N (17) → CH-CO-NH, CH3, NO2, NO2 (18)

Again, utilization of the NEU CSP and chromatography of the 3,5-dinitroanilide (DNAn) derivative (**18**) proved superior to the original approach. Indeed, as shown in Figure 4a, the resolution is, in a practical sense, "too good" for analytical purposes (α = 3.62; R_s = 18.2; these results were obtained at an efficiency of N = 4400).

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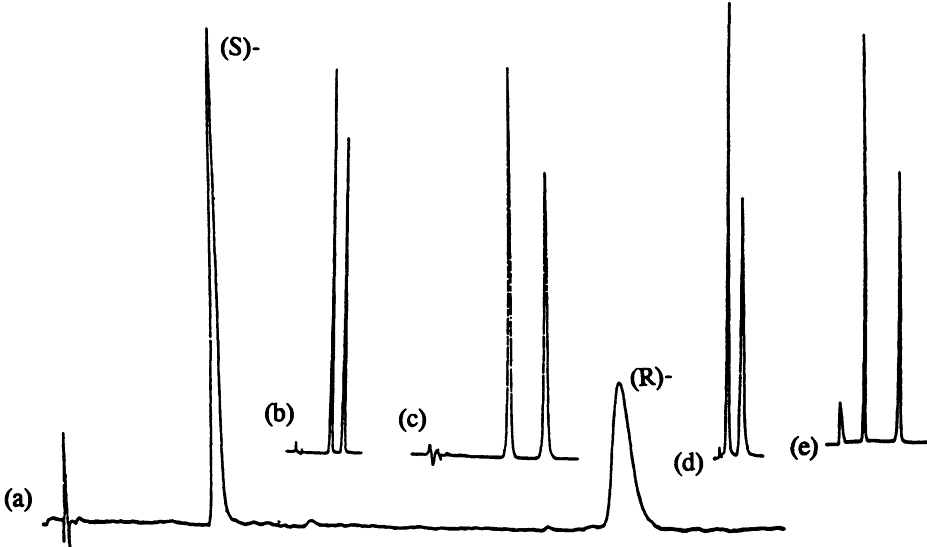

Figure 4. Chiral Resolution of Ibuprofen: (a) racemic 3,5-dinitroanilide derivative on (S)-NEU CSP; (b) m-nitroanilide on same CSP; (c) reverse phase resolution of 3,5-dinitroanilide; (d) 1 inch, five micron column; (e) 10 cm, 3 micron column.

Strategies to Reduce Ibuprofen Resolution. Given the high enantioselectivity of ibuprofen amides on the NEU CSP, the analyst has the rare luxury of devising analytical strategies to reduce resolution, so as to optimize conditions for analytical quantitation. For example, weakening the pi-pi interaction between solute and CSP, by utilization of the (mono-) 3-nitroanilide derivative (instead of 3,5-dinitro-), reduces selectivity to α = 1.34 and resolution to R_s = 4.8 (Figure 4b). Alternatively, conversion to a reverse-phase system had the expected (and in this case desirable) result of attenuating selectivity: the DNAn derivative afforded α = 1.45 and R_s = 6.3 under reverse phase conditions on the NEU CSP (Figure 4c).

Advantages of Reduced Column Length. A trend in analytical laboratories in recent years has been to the use of shorter columns, with the economic advantages of decreased retention times and reduced mobile phase volumes. When selectivity is sufficiently high, as in the case of ibuprofen, chromatographic performance can be impressive on these shorter columns. Figure 4d shows baseline resolution for normal-phase chromatography of the racemic DNAn derivative of ibuprofen on a one inch, five micron particle size NEU stationary phase, wherein the total elution time for the separation at 2 ml/min was less than 60 seconds.

More practically, studies in this laboratory have demonstrated that 10 cm, three micron particle size aminopropyl silica columns, converted *in-situ* to the NEU CSP, consistently provide the same chromatographic performance in every respect, including essentially the same total plate count, as with the more traditional 25 cm, 5 micron columns. This finding parallels the experience of other chromatographers for non-chiral applications. This result was confirmed, not only for ibuprofen, but also for all other chiral pharmaceuticals investigated. Figure 4e shows the resolution of ibuprofen on a 10 cm, 3 micron NEU CSP, while Table I summarizes the chromatographic results on these 10 cm columns, for the four chiral drugs discussed in this work. All of the resolution factors are well in excess of that required for trace and even ultra-trace analysis; furthermore, no analysis requires more than 8 minutes elution time.

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Table I. Chiral HPLC on Short (10 cm) Columns

Solute	α	R_s	N^a	*time*[b]
Amphetamine, DNB	1.21	3.1	4500	8.0
Methamphetamine, DNPU	1.25	6.7	5280	3.9
Tryptophan, DNB, Me Ester	1.89	8.2	3850	6.9
Ibuprofen, DNAn	2.49	11.1	4650	3.3

[a]for 3 micron silica particle size support
[b]for elution of all components at 2 ml/min

For the purpose of acquiring these columns, *in-situ* CSP preparation has been found to be especially convenient, since the precursor aminopropyl columns are widely available in a variety of configurations. In addition, the quantity of chiral

substrate required for binding to the stationary phase is minimal for the shorter columns (typically less than one mmole for a 10 cm column of 4.6 mm. diameter.)

Summary

The current status of analytical criteria for chiral HPLC is summarized below:

(1) Feasibility: Resolution of enantiomeric solutes is now a reasonable objective for the vast majority of chiral solutes. The required chiral stationary phases should be either commercially available, or readily prepared. These CSPs should be rugged and stable to typical analytical conditions. The results should be reproducible from column to column.

(2) Practicality: Although direct resolution of underivatized enantiomers is an ultimate goal, when derivatization is necessary to enhance resolution or improve detectability, this derivatization should be procedurally simple, and should be shown to be non-racemizing.

(3) System Suitability: The primary requirement for quantitative analytical work is an adequate resolution factor. A minimum resolution, R_s, of 1.5 is the normal criterion for baseline resolution, but $R_s \geq 2.0$ is preferred, to provide for ruggedness, or for samples requiring quantitative determination of trace chiral contamination. A secondary requirement is column efficiency: plate counts of approximately 5000/column are currently the norm for SMI CSPs, with a range of 2000-10,000.

(4) Accuracy and Precision: While criteria for quantitation are greatly dependent on the nature of the sample, guidelines which have been accepted in the profession for non-chiral chromatographic procedures are now appropriate and reasonable for chiral chromatography also. Suggested criteria for precision (RSD) are: 1% for racemic standards, 2% for samples such as pharmaceutical dosage forms, and 3% or higher for both standards and other samples where enantiomeric purity of 99% or greater must be established. These criteria are comparable to guidelines routinely employed within the Food and Drug Administration for analysis of pharmaceutical samples (Milda Walters, FDA Detroit District, personal communication, 1991).

Conclusion

Chiral chromatography is now a mature technology. Procedures for the separation and quantitation of enantiomeric solutes have evolved to a point where analytical criteria are the same as for other, achiral, methodologies. Laboratory objectives, acceptance of experimental results, and decisions based on these should all reflect this fact.

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Chapter 3

Commercially Available Brush-Type Chiral Selectors for the Direct Resolution of Enantiomers

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The issue of chirality has become increasingly important to the pharmaceutical, chemical, and agricultural industries in recent years. Chiral compounds can have very different pharmacological activities in the biological system. The determination of these differences in the pharmacological activities will ultimately require a stereoselective analytical assay to evaluate the enantiomeric purity of chiral compounds. The selection of brush-type chiral stationary phases (CSPs) for the liquid chromatographic determination of enantiomeric purity of chiral compounds is reviewed in this chapter.

Chiral, taken from the Greek for hand, means that an object's mirror image is nonidentical with that of the object. Thus, in theory a chiral object may be found in either of two enantiomorphic forms, these "enantiomers" being nonsuperimposable mirror images of one another. Enantiomers lack centers, planes, or improper axes of symmetry. Objects that possess such symmetry elements are superimposable on their mirror images and are termed "achiral". All objects necessarily belong to one of these categories; a hand, a spiral, or a snail shell are chiral, while a cube or a sphere are achiral. Molecules may be chiral or achiral. Since living organisms are themselves chiral, the enantiomers of a chemical substance may elicit rather different responses. For this reason, each enantiomer of a potential pharmaceutical must be obtained in its pure form and tested independently. When prepared synthetically from achiral reagents in an achiral environment, chiral substances are formed as racemates, 1:1 mixtures of the two enantiomers. Hence, the need to separate racemates into the pure enantiomers is common and questions concerning the enantiomeric purity of a substance arise in many fields.

In this chapter, we will review a variety of durable commercially available brush-type chiral stationary phases (CSPs) useful for the direct resolution of enantiomers by liquid chromatography. For enantiomers to be resolved directly over a CSP, they must be interact rapidly and reversibly to form short-lived diastereomeric molecular complexes of nonidentical stability. The greater this stability difference, the easier the

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separation of the enantiomers. The present level of CSP development permits the direct resolution of many enantiomers. While many compounds may be resolved "as is", derivatization with an achiral reagent may be necessary at times to provide the interaction sites essential for chiral recognition or to improve band shape, or to enhance detectability.

In cases where derivatization is necessary, detailed techniques for derivatization with achiral reagents are provided. As the design of CSPs progresses, one may expect an ever diminishing need for derivatization to achieve chiral recognition. However, derivatization to enhance detectability may sometimes still be required.

Determination of Enantiomeric Purity by Chromatographic Methods

Enantiomeric purity is given by the expression 100 x ((a-b)/(a+b)), where a and b are the relative proportions of the two enantiomers. Enantiomeric purity, especially when high, is best determined chromatographically. Such determinations can be fast, accurate, capable of high precision, and carried out on minute amounts of sample which need not be chemically pure. Provided no detected impurities coelute, the relative peak areas from the enantiomers reflect their relative concentrations while the absolute areas can be related to their absolute concentrations.

Separation of enantiomers requires the intervention of some chiral agent. The indirect chromatographic method requires initial derivatization of the mixture of enantiomers with a chiral derivatizing agent to afford a mixture of diastereomers. These may be separable on an achiral chromatography column. Though often satisfactory in practice, the indirect approach has some limitations (in principle) which are absent in the direct approach. The later utilizes either a chiral stationary phase or a chiral mobile phase, the former being more general and in greater use. For many analytes, CSPs can be found which require no prior derivatization of the analytes for enantiomer separation to occur. For others, derivatization with an achiral reagent may be required. As earlier observed, as the design of CSPs evolves, the need for derivatization prior to chromatography will be further reduced.

Derivatization of the analyte, if required, preferably uses an inexpensive, readily available achiral reagent in conjunction with a rapid, simple, and straightforward derivatization technique. Ideally, CSPs of either absolute configuration should be available so that one can control the elution orders of the enantiomers, since it is better to have the trace enantiomer elute before the major enantiomer. The column must be able to tolerate overload conditions and still retain adequate chromatographic efficiency if preparative separations of enantiomers are to be practical.

Although derivatization requires additional effort, the reagents used are typically simple, inexpensive, easily used, and are chosen to facilitate detection, separation, and quantitization. Since achiral derivatization reagents react with the analyte enantiomers in an identical fashion and at identical rates, the ratio of the derivatives is always the same as that of the enantiomeric precursors. Hence, it is not necessary that the reactions proceed to completion. These claims cannot always be made for the chiral derivatizing agents where one is additionally concerned about the enantiomeric purity of the chiral reagent and its stereochemical stability during the reaction. Moreover, detector response is the same for both enantiomers of the derivatives, something not necessarily true for diastereomeric derivatives.

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Chiral Recognition on Brush-Type Phases

If one chiral molecule is to "recognize" the stereochemistry of a second at some instant in time, three or more simultaneous intermolecular interactions are necessary, at least one of these being stereochemically dependent (1). Chromatographically, one observes the weighted time-average result of all possible interactions between the analyte and the stationary phase in the various encounter modes possible. Conformational behavior is quite important. The three interaction sites, if construed as points, lie in a plane. A plane is achiral. That portion of the molecule which lies outside of this hypothetical plane confers chirality to the molecule and impedes "back side" approach by the second molecule.

Major interaction sites are classifiable as π-basic or π-acidic aromatic rings, acidic sites, basic sites, steric interaction sites, or sites for electrostatic interaction. Lipophilic interactions are also possible in reversed mobile phases. Aromatic rings are potential sites for π-π interaction. Acidic sites supply hydrogens for potential intermolecular hydrogen bonds. The hydrogen involved is often an amido proton (NH) from an amide, carbamate, urea, amine or alcohol. Basic sites such as π-electrons, sulfinyl or phosphinyl oxygens, hydroxy or ether oxygens or amino groups may also be involved in hydrogen bond formation. Electrostatic interactions may occur at charged groups or with permanent or induced dipoles. Steric interactions occur between large groups.

As mentioned earlier, enantioselective absorption stems from the formation of transient diastereomeric complexes (between the enantiomers and the CSP) which differ in stability. The enantiomer which is more strongly absorbed is frequently the one that interacts with the greater number of bonding sites or the fewer number of steric repulsion sites. While enthalpic contributions to the binding energy are important, so are the entropic contributions. The latter are more difficult to grasp intuitively and are only now being studied systemically. Typically, the enantiomer adsorbed with the greatest exothermicity also loses the greatest entropy, a situation which reduces the level of enantioselectivity.

For enantiomers to be separated on a brush-type CSPs, the analyte must have the necessary three (or more) interaction sites. While one cannot understand the origins of all chiral separations, it is often possible, within definable limits, to make reasonable estimates as to why a separation occurs. Such chiral recognition models often allow relation of elution order to absolute configuration. Finn elaborates on a proposed labeling scheme for chiral recognition mechanisms, classifying them by how many of the four groups around the stereogenic center participate in the chiral recognition process (2).

Usually, brush-type CSPs utilize a π-π interaction in the recognition process. This type of intermolecular interaction occurs between aromatic ring systems in the enantiomers and those in the CSP and is analogous to the interaction in aromatic charge transfer complexes. Pi-pi interactions can be described as electron donor-acceptor interactions. The extent of interaction is influenced mainly by the electron affinity of the electron acceptor (its tendency to receive an electron) and the first ionization potential of the electron donor (its tendency to donate an electron).

Also common to the enantioselective absorption processes of these CSPs is intermolecular hydrogen bonding between polar groups such as amido and/or carbonyl groups of amides, carbamates, esters, ureas, hydroxyl groups, amino groups, sulfinyl or phosphinyl groups. Even relatively weak hydrogen bonds to the π-electrons in aromatic

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rings or double bonds have been suggested to sometimes be important to chiral recognition.

Figure 1 shows the general chiral recognition model suggested by Pirkle (3) for the more retained enantiomer of an *N*-(3,5-dinitrobenzoyl) (DNB) derivative of an amine upon an *N*-(2-naphthyl)alanine CSP. The CSP's π-donor, acidic, and basic binding sites (naphthyl ring, amido hydrogen, carbonyl oxygen respectively) interact with the complementary sites in the analyte, the more retained enantiomer usually being able to undergo these interactions from a lower energy conformation than can the less retained enantiomer.

Figure 1. Generalized chiral recognition model between a CSP and the more retained enantiomer (A) of a chiral dinitrobenzamide. "B" indicates a basic site on the analyte molecule that is capable of hydrogen bonding to the CSP N-H. Other indicated interactions are π-π interaction between the aryl rings of the CSP and the analyte, and a hydrogen bond between the acid N-H proton of the analyte and the ester (carbonyl) group of the CSP. (Reproduced from ref. 3. Copyright 1986 ACS.)

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Description and Use of Columns Containing Brush-Type Chiral Phases

Phenylglycine: The π-acceptor phenylglycine CSP is based on the 3,5-dinitrobenzoyl derivative of phenylglycine, bound covalently to 3μ or 5μ aminopropyl silica. This CSP is also available in the ionic version. Columns in which the phenylglycine has either the (*R*) or (*S*) absolute configuration are available, enabling one to invert elution order by choice of column. This is sometimes essential for the purification of enantiomers and for the determination of high enantiomeric purity where one wishes the trace enantiomer to elute first. This phase is also available in the racemic form. This π-acceptor CSP resolves both a wide variety of compounds which contain π-basic groups. When such groups are lacking, they can be provided by derivatization. For example, this CSP resolves the 1-naphthamides of amines and amino acids, the anilides of carboxylic acids, and the 1-naphthyl carbamates of alcohols (Figure 2).

Figure 2.

Leucine: The π-acceptor leucine CSP is based on the 3,5-dinitrobenzoyl derivative of leucine, bound covalently to 3μ or 5μ aminopropyl silica. Columns derived from either (*R*) or (*S*) leucine are available. This π-acceptor CSP resolves the same type of compounds as does the phenylglycine CSP. However, this phase often demonstrates

enhanced enantiomeric selectivity for several classes of compounds, including benzodiazopinones (Figure 3).

Figure 3.

Naphthylalanine: The π-donor naphthylalanine CSP is based on the *N*-(2-naphthyl) derivative of alanine, covalently bound to 3μ or 5μ 11-undecanyl silica through an ester linkage. Available are columns of either the (*R*) or (*S*) configurations, in addition to the racemic version. These columns have been used to monitor racemization during peptide coupling reactions. This π-donor CSP resolves 3,5-dinitrophenylcarbamate or 3,5-dinitrophenylurea derivatives of a wide range of amines, alcohols, diols, carboxylic acids, amino acids, thiols, and hydroxy acids. Superior separations of the DNB derivatives of primary amines are noted (Figure 4).

Figure 4.

Naphthylleucine: The π-donor naphthylleucine CSP is based on the *N*-(1-naphthyl) derivative of leucine, which is covalently bound to either 3μ or 5μ 11-undecyl silica through an ester linkage. The (*S*)-*N1N*-naphthylleucine has recently become available. This π-donor CSP resolves DNB derivatives of amino acids as the free acid in a reverse phase mode. In the normal phase mode, this CSP resolves the amides and esters of DNB amino acids with alphas that typically range between 10 and 40. The naphthylleucine columns resolve essentially the same cliental of compounds as does the naphthylalanine column but typically with enhanced enantioselectivities. However, there are scattered instances where the naphthylleucine column shows less enantioselectivity than does the naphthylalanine column (Figure 5).

Figure 5.

α-Burke 1: The π-acceptor α-Burke 1 CSP is named after the graduate student, J. A. Burke III, in Dr. William H. Pirkle's research group who first prepared this phase. The phase is derived from dimethyl *N*-3,5-dinitrobenzoyl-α-amino 2,2-dimethyl-4-pentyl phosphonate covalently bound to 3μ or 5μ mercaptopropyl silica. The α-Burke 1 will be available in both the (*R*) and (*S*) configurations. This π-acceptor CSP resolves the enantiomers of a number of underivatized ß-blockers, as well as many other analytes. This CSP shows unique chromatographic properties. Using an ethanol-dichloromethane-ammonium acetate mobile phase, reduction of column temperature **reduces** the retention of the least retained enantiomer of propranolol and **increases** the retention of the more retained enantiomer without appreciable band broadening (Figure 6).

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Figure 6.

ß-Gem 1: The π-acceptor ß-Gem 1 CSP is named after the graduate student, John (JEM) E. McCune, from Dr. Pirkle's group who first prepared this CSP. The phase is derived from *N*-3,5-dinitrobenzoyl-3-amino-3-phenyl-2-(1,1-dimethylethyl)propanoate covalently bound to 3μ or 5μ silica. This CSP exhibits superior performance to that of the widely used analogue, phenylglycine, for a great many analytes. It shows a general ability to separate the enantiomers of anilide derivatives of a wide variety of chiral carboxylic acids including nonsteroidal anti-inflammatory agents (Figure 7).

Figure 7.

(*tert*)-Buc-(S)-leucine: The (*tert*)-Buc-(*S*)-leucine CSP is thought to derive its enantioselectivity from hydrogen bonding alone. The development of similar types of chiral selectors were reported by Shoji Hara with his (*N*-acyl-1-valylamino)propyl silica based CSPs, and later by Naobumi Oi with the OA-3000. The *t*-Buc-(*S*)-leucine is based on a CSP derived from *N*-(*tert*-butylaminocarbonyl)-(*S*)-leucine, which is covalently bound to 5μ aminopropyl silica. This column affords enantioselectivity for compounds containing both π-donor and π-acceptor sites within the molecule. This column can resolve both 3,5-dinitrophenylcarbamates and 3,5-dinitrophenylurea derivatives as well as 1-naphthylurea derivatives for a wide range of amines, alcohols, diols, carboxylic acids, amino acids, thiols, and hydroxy acids (Figure 8).

Figure 8.

Sumichiral OA-1000: The π-donor OA-1000 CSP is based on the terephthalamide derivative of (*S*)-(1-naphthyl)ethylamine, covalently bound to 5μ aminopropyl silica. This phase contains a chiral amide which has the ability to serve as either a donor or an acceptor in hydrogen bonding. Thus, hydrogen bonding may contribute to the separation of amide derivatives. A π-donor, the OA-1000 affords excellent selectivity for the separation of enantiomers of 3,5-dinitrobenzoyl derivatives of amines and amino acids, the esters and amides of these acids, and other derivatives of carboxylic acids (Figure 9).

Sumichiral OA-2200: The OA-2200 CSP contains three asymmetric carbon atoms and is based on the (1*R*,3*R*)-*trans*-Chrysanthemoyl derivative of (*R*)-phenylglycine. This

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Figure 9.

phase has demonstrated enhanced selectivity for chiral amides. The OA-2200 resolves the 3,5-dinitrobenzoyl derivatives of amines and amino acids, and of the esters and amides of these acids; the 3,5-dinitroanilide derivatives of carboxylic acids; and some underivatized alcohols of interest, such as fungicides (Figure 10).

Figure 10.

Sumichiral OA-2500: The π-donor naphthylglycine CSP is based on the 3,5-dinitrobenzoyl derivative of (*R*)-1-naphthylglycine, which is covalently bound to 5μ aminopropyl silica. This CSP introduces both π-donor and π-acceptor sites as potential chiral selectors. The OA-2500 resolves aromatic chiral carboxylic acids without achiral derivatization. This includes almost the complete series of nonsteroidal anti-inflammatory agents, such as Ibuprofen, Fenoprofen, etc. (Figure 11).

Figure 11.

Sumichiral OA-3000: The OA-3000 is a novel CSP based on the *N*-(*tert*-butylaminocarbonyl)-(*S*)-valylaminopropyl phase, covalently bound to 5μ silica. This CSP, alone among current CSPs, derives its enantioselectivity only from hydrogen bonding. This CSP is very effective in the separation of *N*-acetyl-O-alkyl esters of amino acids. For instance, the methyl ester of *N*-acetyl valine has no ring system and no π-electrons. Nevertheless, the racemate has been clearly separated by the OA-3000. However, the most effective O-alkyl ester for this separation of amino acids is the O-*tert*-butyl ester. The OA-3000 may also be applied to the separation of the 3,5-dinitrophenyl carbamates of hydroxy acids (Figure 12).

Figure 12.

Sumichiral OA-3100: The OA-3100 CSP is based on the *N*-3,5-dinitrophenylaminocarbonyl-(*S*)-valylaminopropyl phase, covalently bound to 5μ silica. This highly efficient π-acceptor CSP separates enantiomers of hydroxy acids, and the dabsyl and dansyl

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derivatives of amino acids, even though the carboxyl group is left underivatized (Figure 13).

Figure 13.

Sumichiral OA-3200: The OA-3200 CSP is based on the *N*-3,5-dinitrophenylaminocarbonyl derivative of (*S*)-(*tert*)-leucine, covalently bound to 5μ aminopropyl silica. Like the OA-2500 and the OA-3100, this π-acceptor CSP resolves aromatic chiral carboxylic acids without derivatization (Figure 14).

Figure 14.

Sumipax OA-4000 and OA-4100: These two diastereomeric π-donor CSPs can reverse the elution orders of the optical isomer pairs they separate. They are derived from the (*R*)- or (*S*)-(1-naphthyl)ethylamine derivatives of (*S*)-valine, each of which is covalently bound to 5μ aminopropyl silica. These phases demonstrate excellent enantioselectivity for the π-acceptor derivatives of amines, carboxylic acids, and amino acids, the esters and amides of these acids, and of underivatized alcohols (Figure 15 and 16).

Figure 15. **Figure 16.**

Sumichiral OA 4400 and OA-4500: The OA-4400 and OA-4500 are CSPs derived from the (*R*)- or (*S*)-(1-naphthyl)ethylamine derivatives of (*S*)-proline, which are covalently bound to 5μ aminopropyl silica. These π-donor CSPs resolve a variety of underivatized ß-blockers, aromatic amines, and pesticides (Figure 17 and 18).

Figure 17. **Figure 18.**

Sumichiral OA-4600 and OA-4700: The OA-4600 and OA-4700 are CSPs derived from the (*R*)- or (*S*)-(1-naphthyl)ethylamine derivatives of (*S*)-(*tert*)-leucine, covalently

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bound to 5μ aminopropyl silica. These π-donor CSPs resolve a variety of underivatized ß-blockers, aromatic amines, cyano alcohols, and pesticides (Figure 19 and 20).

Figure 19.

Figure 20.

Naphthylethylurea: The naphthylethylurea CSP is derived from the reaction between of 1-naphthylethylisocyanate and 5μ aminopropyl silica to form a urea based derivative. Columns of either the (*R*) or (*S*) configuration are available. These π-donor CSPs resolve a wide variety of DNB derivatives of amines and amino acids (Figure 21).

Figure 21.

The Use of Achiral Derivatization Reagents for Chiral Recognition on Brush-Type Phases

Chiral recognition is usually greatest when the three chiral recognition sites are adjacent to the stereogenic center, becoming weaker as these sites are further removed. If a molecule contains all of the aforementioned characteristics, derivatization is usually not required. Examples include the ß-binaphthols, (*S*)-aryl-ß-hydroxyl sulfoxides, C-aryl hydantoins, C-aryl succinimides, and indoline-2-carboxylates.

In some analytes, an adequate number of the necessary interaction sites may not be present. Derivatization with an achiral reagent can provide additional interaction sites and the achiral derivatizing reagents can be chosen to enhance detectability as well. Many enantiomers that otherwise can not be separated by present brush-type CSPs stationary phases can be separated after suitable achiral derivatization. For example, derivatization is typically required for analytes lacking aromatic substituents. The presence of aromaticity in the analytes is one of the usual requirements for chiral recognition by a brush-type CSPs and on most other chiral selectors as well. While many analytes contain π-basic aryl groups and can often be resolved on π-acidic CSPs without derivatization, relatively few analytes originally possess π-acidic groups. Hence, incorporation of such a group by derivatization is a usual first step if a π-donor column is to be used. Reaction with 3,5-dinitrobenzoyl chloride, 3,5-dinitrophenylisocyanate, or 3,5-dinitroaniline is usually simple, easy, and straightforward. In the case of both types of columns, derivatization can reduce analysis time and improve band shapes. For example, amines are often acylated, carboxylic acids esterified or converted to amides or anilides, and alcohols sometimes converted to esters or carbamates.

Derivatization of groups remote from the stereogenic center can improve resolution by reducing the extent to which these groups contribute to achiral retention (*i.e.*, both enantiomers are retained equally).

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If a chiral analyte lacks one or perhaps two of the three sites required for chiral recognition, then derivatization with an achiral reagent may allow separation of the enantiomers of the derivative on the CSP.

In the following derivatization schemes, only analytes that contain interaction sites close to or around the stereogenic center are addressed. Some examples of analytes that contain easily derivatized functional groups are:

CARBOXYLIC GROUPS--Amino acids, hydroxy acids, and carboxylic acids.
AMINE GROUPS--Amines, amino acids, and amino alcohols.
HYDROXYL GROUPS--Alcohols and hydroxy acids.
THIOL GROUPS--Thiols and amino thiols.

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For the selection of the appropriate achiral derivatization reagent and CSP, two selection charts (Tables 1 and 2) review a variety of non-pharmaceuticals and pharmaceuticals that have been resolved over commercially available brush-type chiral selectors. The description and absolute configuration of columns containing brush-type chiral phases used in the two selection charts are found in (Table 3). Techniques in achiral derivatization are found in (Table 4). A list of achiral derivatization reagents with their acronym and chemical names can be found in (Table 5).

In this chapter only the following four cases are provided as examples of possible derivatization schemes. However, one will find several other methods for derivatization in the selection charts.

In the first case, the analyte contains but one derivatizable functional group near the stereogenic center. The task is to select the appropriate achiral derivatization reagent for the application. For example, the analyte is 1-phenylbutanoic acid. The choice of achiral derivatization reagents are 3,5-dinitroaniline (DNA) and 3,5-dimethylaniline (DMA). From the selection chart (Table 1), one finds that the ß-Gem 1 resolves both derivatives. The 3,5-dimethylanilide derivative provides the needed enantioselectivity for your reverse-phase application. Although, the other derivative would be adequate in most cases. This procedure applies to other analytes, such as amines, alcohols, and thiols that contain only one derivatizable functional group.

In the second case, the analyte contains two similar or identical functional groups near the stereogenic center. The same achiral reagent is used to derivatize either one or both functional groups. In this example the analyte is 1-phenyl-1,2-ethanediol. Originally, 3,5-dinitrophenylisocyanate was used as the derivatization reagent. However, this reagent is not commercially available. A new acylation reagent, *N*-imidazole-*N*'-carbonic acid-3,5-dinitroanilide (ICDNA), is now available as an alternative achiral derivatization reagent. From the selection chart (Table 1), 1-phenyl-1,2-ethanediol is resolved as its *bis*-derivative using the naphthylalanine column.

In the third case, the analyte contains two dissimilar functional groups which may be treated with the same derivatization reagent. The analyte to be derivatized in this case is propranolol. From the selection chart (Table 2), one finds that propranolol can be resolved as either its oxazolidone or several other acylated derivatives. In one achiral derivatization scheme for amino alcohols, phosgene (PHOS) is used to acylate both the hydroxy and amino to afford the cyclic oxazolidone derivatives. In contrast, ß-naphthylisocyanate (NIC-1) derivatizes only the amino group in most cases. This reagent is safe, easy to use, and the derivatization scheme is straightforward. In the selection chart

TABLE 1: The Selection Chart for the Resolution of Non-Pharmaceuticals Over Brush-Type Phases

[a] Analytes: Analytes are classified according to their structural feature and functional group(s). An alcohol and its functional group--hydroxyl. [b] Reagent: Reagents are listed in Table 5 by their acronym and chemical name. If not applicable (N/A), indicates that no derivatization is required. [c] Derivative: Derivatives are classified according to their functional group(s). An alcohol derivative and its functional group as a derivative--carbamate. [d] Procedures: If derivatization is required, each procedure is listed in Table 4. [e] CSP: CSPs are listed in Table 3 by their description and absolute configuration. Literature cited: References for analytes resolved on CSP [f].

Analytes[a]	Reagent[b]	Derivative[c]	Procedure[d]	CSP[e]	Literature Cited[f]
ALCOHOLS:HYDROXYL MOIETY					
Aromatic/aliphatic	ICDNA	Carbamate	1	7,8,10,20,21	5,6,25,50
Aromatic/aliphatic	NIC-1	Carbamate	2	1,2,12	46
2-Hexanol	ICDNA	Carbamate	1	14	22
2-Octanol	ICDNA	Carbamate	1	14,20,21	22,25
2-Decanol	ICDNA	Carbamate	1	14	22
1-Methoxy-2-propanol	ICDNA	Carbamate	1	14	22
4-Methyl-2-pentanol	ICDNA	Carbamate	1	14	22
1-Phenylethanol	ICDNA	Carbamte	1	7,8,14,20,21	5,6,22,25
Menthol	ICDNA	Carbamate	1	14	22
Benzyl	N/A			1-3	26,27
Cyclic	N/A			1-3	26,27
ALCOHOLS:HYDROXYL MOIETY					
Bi-β-naphthols	N/A			1-3	26,27
2,2,2-Trifluoro-1-(9-anthryl)-ethanol	N/A			1-3,12	44,45
AMINES:AMINO MOIETY					
Aromatic/aliphatic	ICDNA	Urea	1	7,8	5,6
Aromatic/aliphatic	NC-2	Amide	3	1,2	11
Aromatic/aliphatic	DNBC	Amide	3	14,20,21	21,25
Heterocyclic	NC-1	Amide	3	1,2,12	35,44
N-Acyl 1-(2-fluorenyl)-1-amino alkanes	N/A			1,2,5,6	9
1-Aryl-amino alkanes	NC-1	Amide	3	1,2	14,15
Phenethyl	DNBC	Amide	3	7,8,15,20,21 26,27	5,6,23,25,52
sec-Butyl	DNBC	Amide	3	7,8,15	5,6,23
Octyl	DNBC	Amide	3	7,8,15,20,21	5,6,23,25

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TABLE 1 (Continued)

Analytes[a]	Reagent[b]	Derivative[c]	Procedure[d]	CSP[e]	Literature Cited[f]
α- and β- AMINO ACIDS: AMINO/ACID MOIETY					
Aromatic/aliphatic	DNBC/ROH	Amide/ester	4	7,8,10,14, 15,20,21	5,6,21,23,25,50
Aromatic/aliphatic	AC/ROH	Amide/ester	4	17	24
Aromatic/aliphatic	DNBC	Amide	5	7,8	49
N-Aryl-α-amino esters	N/A			1,2,5,6,14	12,21
2-Carboalkoxyindolines	N/A			1,2,5,6	12
Aromatic/aliphatic	AC/ROH	Amide/ester	4	17	24
α- and β- AMINO ALCOHOLS: AMINO/HYDROXYL MOIETY					
Aromatic/aliphatic	ICDNA	Urea	1	7,8	5,6
CARBOXYLIC ACIDS: ACID MOIETY					
Aromatic/aliphatic	DNA	Amide	6	7,8,10,14, 20,21	6,21,25,50
Aromatic/aliphatic	ANL	Amide	6	1,2	11
1-Methylphenylacetic	DNA	Amide	6	15	23
1-Bromo-2,2-dimethylbutyric	DNA	Amide	6	15,20,21	23,25
trans-Chrysanthemic	DNA	Amide	6	15	23
2-Phenylpropionic	DNA	Amide	6	20,21	25
α-Methoxyphenylacetic	NMA-1	Amide	7	1,2	43
α-Phenylbutanoic	DNA	Amide	6	12	56
α-Phenylbutanoic	DMA	Amide	6	12	56
DIOLS: HYDROXYL MOIETY					
Aromatic/aliphatic	ICDNA	Carbamate	1	7,8	6,7
Dihydro	N/A			1-3,5,6	28,29,39-42
Tetrahydro	N/A			1-3,5,6	28,29,39-42
α-Phenyl-1,2-ethanediol	ICDNA	Carbamate	1	7,8	6,7
HYDROXY ACIDS: HYDROXY/ACID MOIETY					
Aromatic/aliphatic	ICDNA	Carbamate	1	7,8	6
PEPTIDES					
Dipeptides	DNBC	Amide	3	1,2,4,7-9	20
PIPERDINES					
2-Methyl	NC-1	Amide	3	1,2,12	44

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TABLE 1 (Continued)

Analytes[a]	Reagent[b]	Derivative[c]	Procedure[d]	CSP[e]	Literature Cited[f]
POLYCYCLIC AROMATIC HYDROCARBONS					
Benzo[a]pyrene	N/A			1-3,5,6	28,29,39-42
Benz[a]anthracene	N/A			1-3,5,6	28,29,39-42
THIOLS:SULFUR MOIETY					
Aromatic/aliphatic	ICDNA	Carbamate	1	7,8	5,6
INSECTICIDES, PESTICIDES & FUNGICIDES: HYDROXY/ESTER/ ACID MOIETIES					
Fenvalerate	N/A			1-3	19
Permethrin	N/A			1-3	19
Cypermethrin	N/A			1-3	19
Cyfluthrin (Bay FCR 1272)	N/A			1-3	19
Fenpropanate	N/A			1-3,20,21	19,25
Deltamethrin	N/A			1-3	19
Flucythrinate	N/A			1-3	19
S-3308	N/A			15,20,21	23,25
S-3307	N/A			20,21	25
Allethrolone	N/A			20,21	25
Propargyllone	N/A			20,21	25

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TABLE 2: The Selection Chart for the Resolution of Pharmaceuticals Over Brush-Type Phases

[a] Analyte: Analytes are classified according to their pharmacological activity. The drug propranolol is an ß-adrenergic drug. [b] Reagent: Reagents are listed in Table 5 by their acronym and chemical name. If not applicable (N/A), indicates that no derivatization is required. [c] Derivative: Derivatives are classified according to their functional group(s). An alcohol derivative and its functional group as a derivative--carbamate. [d] Procedures: If derivatization is required, each procedure is listed in Table 4. [e] CSP: CSPs are listed in Table 3 by their description and absolute configuration. Literature cited: References sited for analytes resolved on CSP [f].

Analytes[a]	Reagent[b]	Derivative[c]	Procedure[d]	CSP[e]	Literature Cited[f]
α- and β-ADRENERGIC DRUGS					
Propranolol	N/A			11	51
Propranolol	PHOS	Oxazolidone	8	1,2	17
Propranolol	NIC-1	Urea	2	1,2	47
Alprenolol	NIC-1	Urea	2	1,2	47
Oxprenolol	N/A			11	51
Oxprenolol	NIC-1	Urea	2	1,2	47
Pindolol	N/A			11	51
Metoprolol	N/A			11	51
Metoprolol	NIC-1	Urea	2	1,2	47
Proenthalol	N/A			11	47
Bufuralol	N/A			11	51
Timolol	NIC-1	Urea	2	1,2	47
α- and β-ADRENERGIC DRUGS					
Ephedrine	NA-2	Oxazolidine	9	1,2	33,53
Ephedrine	PHOS	Oxazolidone	8	1,2	54
Ephedrine	NCF-2	Carbamate	10	1,2	10
Ephedrine	NIC-1	Urea	2	1,2	47
Norephedrine	NCF-2	Carbamate	10	1,2	10
Norpseudoephedrine	NCF-2	Carbamate	10	1,2	10
Pseudoephedrine	NCF-2	Carbamate	10	1,2	10
p-Hydroxyephedrine	NCF-2	bis-Carbamate	10	1,2	10
ANTIANXIETY AGENTS					
Benzodiazepinones and analogues:					
Lorazepam	N/A			1,2,5,6	30
Oxazepam	N/A			1,2,5,6	30
Diazepam	N/A			1,2,5,6	30
ANTIBIOTICS					
β-Lactams	N/A			5,6	8

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TABLE 2 (Continued)

Analytes[a]	Reagent[b]	Derivative[c]	Procedure[d]	CSP[e]	Literature Cited[f]
ANTICONVULSANTS					
Mephenytoin	N/A			1-3,5,6	16
Glutethimide	N/A			1-3,5,6	16
Ethosuximide	N/A			1-3,5,6	16
Methsuximide	N/A			1-3,5,6	16
Phensuximide	N/A			1-3,5,6	16
ANTIHYPERTENSIVES					
MK-286	N/A			1,2	32
Debrisoquine and 4-hydroxydebrisoquine	N/A			1,2	48
ANTIINFLAMMATORY AGENTS					
1-Methylarylacetic Acids:					
Benoxaprofen	NMA-1	Amide	7	1,2	13,34
Fenoprofen	NMA-1	Amide	7	1,2	13,34
Fenoprofen	DMA	Amide	6	12	56
Ibuprofen	NMA-1	Amide	7	1,2	13,43
Ibuprofen	DMA	Amide	6	12	56
Naproxen	NMA-1	Amide	7	1,2	13
Naproxen	DNA	Amide	6	7,8,10	50
Etodolac	MeOH	Ester	11	1,2	31
Flurbiprofen	NMA-1	Amide	7	1,2	43
ANTIMALARIAL DRUGS					
Primaquine	N/A			1,2	36
ANTICHOLINERGIC DRUGS					
Tropicamide	N/A			1,2	37
HYPERTENSIVE DRUGS					
Methoxamine	NIC-1	Urea	2	1,2	47
HYPONOTICS AND SEDATIVES:					
Barbiturates:					
Butabarbital	N/A			1-3,5,6	16
Hexobarbital	N/A			1-3,5,6	16
Mephobarbital	N/A			1-3,5,6	16
Secobarbital	N/A			1-3,5,6	16

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TABLE 2 (Continued)

Analytes[a]	Reagent[b]	Derivative[c]	Procedure[d]	CSP[e]	Literature Cited[f]
SYMPATHOMIMETIC AMINES					
Amphetamines:					
Amphetamine	NCF-2	Carbamate	10	1,2	10
Amphetamine	DNBC	Amide	3	1-3	18
Amphetamine	NC-1	Amide	3	1-3	18,34,55
Amphetamine	NC-2	Amide	3	1-3	18,38,55
p-Methoxy-amphetamine	AC	Amide	3	1-3	18
Benzphetamine	NCF-2	Carbamate	10	1,2	10
p-Hydroxy-amphetamine	NCF-2	Carbamate	10	1,2	10
Methamphetamine	NCF-2	Carbamate	10	1,2	10
Methoxyphenamine	NCF-2	Carbamate	10	1,2	10

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Table 3: A List of Commercially Available Brush-Type Chiral Stationary Phases

	Chiral Stationary Phase	Description/Absolute configuration
CSP 1	(D)-Phenylglycine	(*R*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
CSP 2	(L)-Phenylglycine	(*S*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
CSP 3	(D)-Phenylglycine (Ionic)	(*R*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
CSP 4	(D,L)-Phenylglycine	(*R*,*S*)-*N*-(3,5-Dinitrobenzoyl)phenylglycine
CSP 5	(D)-Leucine	(*R*)-*N*-(3,5-Dinitrobenzoyl)leucine
CSP 6	(L)-Leucine	(*S*)-*N*-(3,5-Dinitrobenzoyl)leucine
CSP 7	(D)-*N2N*-Naphthylalanine	(*R*)-*N*-(2-Naphthyl)alanine
CSP 8	(L)-*N2N*-Naphthylalanine	(*S*)-*N*-(2-Naphthyl)alanine
CSP 9	(D,L)-*N2N*-Naphthylalanine	(*R*,*S*)-*N*-(2-Naphthyl)alanine
CSP 10	(*S*)-*N1N*-Naphthylleucine	(*S*)-*N*-(1-Naphthyl)leucine
CSP 11	α-Burke 1	(*R*)-Dimethyl *N*-3,5-dinitrobenzoyl-α-amino-2,2-dimethyl-4-pentyl phosphonate
CSP 12	ß-Gem 1	(*S*,*S*)-*N*-3,5-dinitrobenzoyl-3-amino-3-phenyl-2-(1,1-dimethylethyl)propanoate
CSP 13	(*tert*)-Buc-(*S*)-leucine	*N*-(*tert*-Butylaminocarbonyl)-(*S*)-leucine
CSP 14	OA-1000	(*S*)-(1-Naphthyl)ethylaminoterephthalic acid
CSP 15	OA-2200	(1*R*,3*R*)-*trans*-Chrysanthemoyl-(*R*)-phenylglycine
CSP 16	OA-2500	(*R*)-*N*-(3,5-dinitrobenzoyl)-1-naphthylglycine
CSP 17	OA-3000	*N*-(*tert*-Butylaminocarbonyl)-(*S*)-valine
CSP 18	OA-3100	*N*-3,5-Dinitrophenylaminocarbonyl-(*S*)-valine
CSP 19	OA-3200	*N*-3,5-Dinitrophenylaminocarbonyl-(*S*)-(*tert*)-leucine
CSP 20	OA-4000	(*R*)-*N*-(1-Naphthyl)ethylaminocarbonyl-(*S*)-valine
CSP 21	OA-4100	(*S*)-*N*-(1-Naphthyl)ethylaminocarbonyl-(*S*)-valine
CSP 22	OA-4400	(*S*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-proline
CSP 23	OA-4500	(*R*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-proline
CSP 24	OA-4600	(*R*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-(*tert*)leucine
CSP 25	OA-4700	(*S*)-(1-Naphthyl)ethylaminocarbonyl-(*S*)-(*tert*)leucine
CSP 26	(*R*)-Naphthylethylurea	(*R*)-1-(Naphthyl)ethylurea
CSP 27	(*S*)-Naphthylethylurea	(*S*)-1-(Naphthyl)ethylurea

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Table 4: Techniques in Achiral Derivatization

1. [Acylation] Reactions of chiral compounds with Imidazoles (*N*-Imidazole-*N*'-carbonic acid-3,5-dinitroanilide (ICDNA)).

   Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound, 15.0 mg (0.05 mmol) of ICDNA, and 2 mL of DMF. Stir and heat at 60°-70°C for 1-2 hours. Cool to room temperature; evaporate solvent to dryness under a stream of N_2. Take up the residue in 3 mL of CH_2Cl_2. Transfer mixture to a separatory funnel and wash organic layer with 2 x 1 mL of a 0.1 M $NaHCO_3$ solution, 2 x 1 mL of a 0.1 M HCl solution, and 2 x 1 mL of H_2O. Filter organic layer through anhydrous Na_2SO_4 and evaporate solvent to dryness under a stream of N_2. Take up the residue in 1-2 mL of mobile phase. From the solution, inject 10 µL.

2. [Acylation] Reactions of chiral compounds with isocyanates (1-naphthylisocyanate (NIC)).

   Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound, 0.007 mL (0.05 mmol) of NIC-1, and 2 mL of toluene. Stir and heat at 60°-70°C for 30 minutes. After cooling, evaporate solvent to dryness under a stream of N_2. Take up the residue in 3 mL of CH_2Cl_2. Transfer mixture to a separatory funnel and wash organic layer with 2 x 1 mL of a 0.1 M $NaHCO_3$ solution, 2 x 1 mL of a 0.1 M HCl solution, and 2 x 1 mL of H_2O. Filter organic layer through anhydrous Na_2SO_4 and evaporate solvent to dryness under a stream of N_2. Take up the residue in 1-2 mL of mobile phase. From the solution, inject 10 µL.

3. [Acylation] Reactions of chiral compounds with acid chlorides (1-naphthoyl chloride (NC-1), 2-naphthoyl chloride (NC-2), and 3,5-dinitrobenzoyl chloride (DNBC)).

   Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound, 2 mL of methylene chloride, and add 6 mg (0.03 mmol) of the acid chloride. Stir or shake for 2 minutes. Then add 2 mL of a 0.1 N NaOH solution. Shake for 1 minute and discard upper aqueous layer. Add an additional 2 mL of 0.1 N NaOH solution and shake for 2 minutes. Discard upper aqueous layer and wash organic layer with 2 x 1 mL of H_2O and discard upper layer. Filter organic layer through Na_2SO_4 and evaporate solvent to dryness under a stream of N_2. Take up the residue in 1-2 mL of mobile phase. From the solution, inject 10 µL.

4. [Acylation/alkylation] Reactions of amino acids with acid chlorides and alcohols.

   Follow procedure 11 for alkylation with any alcohol. Then follow procedure 5 for acylation with any acid chloride.

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Table 4. Continued

5. [Acylation] Reaction of chiral compounds with acid chlorides (3,5-dinitrobenzoyl chloride (DNBC)).

   Into a 5 mL reaction vial, place 5.0 mg (0.05 mmol) of the chiral compound with 3 mL of dry THF. Add 12.0 mg (0.05 mmol) of DNBC and 0.1 mL (1.5 mmol) of propylene oxide. Stir reaction mixture for 15 minutes; filter to remove traces of residual amino acid. Evaporate solvent under a stream of N_2. Take up residue in 1-2 mL of mobile phase. From this solution, inject 10 μL.

6. [Acylation] Reaction of chiral compounds with amines (3,5-dinitroaniline (DNA)/oxalyl chloride (OXY-Cl)).

   Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound and 0.01 mL (0.12 mmol) of oxalyl chloride. Reflux reaction mixture for 15 minutes. Evaporate solvent to dryness under a stream of N_2. To the reaction vial, add slowly 1.0 mL of $CHCl_3$ and 0.006 mL (0.04 mmol) of NMA-1. Stir mixture for 1 hour. Transfer reaction mixture to a separatory funnel and wash organic layer with 3 x 1 mL of a 0.1 M $NaHCO_3$ solution, 3 x 1 mL of a 0.1 M HCl solution, and 3 x 1 mL of H_2O. Filter organic layer through anhydrous Na_2SO_4 and evaporate solvent to dryness under a stream of N_2. Take up residue in 1-2 mL of mobile phase. From this solution, inject 10 μL.

7. [Acylation] Reaction of chiral compounds with amines (1-naphthalenemethylamine (NMA-1)/EDC)

   Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound, 2.0 mL of CH_2CL_2, 0.003 mL (0.02 mmol) of NMA-1, 4.0 mg (0.02 mmol) of EDC. Cool the reaction mixture to 4°C for 1-2 hours. Let stand at room temperature for either 2-3 hours or until a white precipitate (urea) forms. Transfer mixture to a separatory funnel and wash organic layer with 2 x 1 mL of a 0.1 M $NaHCO_3$ solution, 2 x 1 mL of a 0.1 M HCl solution, and 2 x 1 mL of H_2O. Filter organic layer through anhydrous Na_2SO_4 and evaporate solvent to dryness under a stream of N_2. Take up the residue in 1-2 mL of mobile phase. From the solution, inject 10 μL.

8. [Acylation] Reactions of chiral compounds with acid chlorides (phosgene (PHOS)).

   Into a round bottom flask, place 5.0 mg (0.03 mmol) of the chiral compound, 0.09 mL of a 10% NaOH solution, and 0.15 mL of ether. Cool the round bottom flask to 0°C with an ice water bath and add dropwise 0.07 mL of phosgene. Stir mixture for 30 minutes. Transfer reaction mixture to a separatory funnel and remove aqueous layer. Filter organic layer through anhydrous Na_2SO_4 and evaporate solvent to dryness under a stream of N_2. Take up residue in 1-2 mL of mobile phase. From this solution, inject 10 μL.

Continued on next page

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Table 4. Continued

9. [Acylation] Reaction of chiral compounds with aldehydes (2-naphthaldehyde (NA-2)).

   Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound, 31.0 mg (0.02 mmol) NA-2, and 2 mL of benzene. Reflux mixture for 2 hours. Remove the calculated amount of water using a Dean-Stark trap. Cool to room temperature; evaporate solvent to dryness under a stream of N_2. Take up residue in 1-2 mL of mobile phase. From this solution, inject 10 μL.

10. [Acylation] Reactions of chiral compounds with chloroformates (2-naphthychloroformate (NCF-2)).

    Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound, 2 mL of CH_2Cl_2, and 2-3 drops of triethylamine. While stirring vigorously, slowly add 5.0 mg (0.03 mmol) of the chloroformate. Continue stirring for 10 minutes at ambient temperature. Transfer mixture to a separatory funnel and wash organic layer with 2 x 1 mL of a 0.1 M $NaHCO_3$ solution, 2 x 1 mL of a 0.1 M HCl solution, and 2 x 1 mL of H_2O. Filter organic layer through anhydrous Na_2SO_4 and evaporate solvent to dryness under a stream of N_2. Take up the residue in 1-2 mL of mobile phase. From the solution, inject 10 μL.

11. [Alkylation] Reactions of chiral compounds with alcohols (any alcohol/HCl).

    Into a 5 mL reaction vial, place 5.0 mg (0.02 mmol) of the chiral compound and add 4 mL of 1.25 N HCl in ROH. Stir at room temperature for 1-2 hours, then evaporate to dryness under reduced pressure at 60°C. **Note**: The alcohol is usually methyl alcohol, however a variety of alcohols have been used for improved chiral recognition. Refer to Table 1 and Table 2 for specific examples of separations in which different lengths of alkyl chains of alcohols were used for improved chiral recognition.

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Table 5: A List of Achiral Derivatization Reagents

Reagent Acronym	Reagent Chemical Name
AA	Acetic anhydride
AC	Acetyl chloride
ANL	Aniline
CDI	1,1'-Carbonyldiimidazole
DABSYL-Cl	4-(Dimethylamino)-azobenzene-4'-sulfonyl chloride
DANSYL-Cl	5-Dimethylamino-1-naphthalenesulfonyl chloride
DMA	3,5-dimethylaniline
DMF	*N,N*-dimethylformamide
DNA	3,5-Dinitroaniline
DNBC	3,5-Dinitrobenzoyl chloride
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EF	Ethyl formate
HOBT	1-Hydroxybenzotriazole hydrate
IDCNA	*N*-Imidazole-*N'*-carbonic acid-3,5-dinitroanilide
NAL-2	2-Naphthaldehyde
NA-1	1-naphthylamine
NA-2	2-naphthylamine
NC-1	1-Naphthoyl chloride
NC-2	2-Naphthoyl chloride
NCF-2	2-Naphthylchloroformate
NIC-1	1-Naphthylisocyanate
NMA-1	1-Naphthalenemethylamine
OXY-Cl	Oxalyl chloride
PHOS	Phosgene
PY	Pyridine
ROH	Any alcohol

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(Table 2), propranolol can be resolved directly without derivatization using the α-Burke 1.

In the fourth and final case described in this chapter, the analyte contains two dissimilar functional groups that may be treated with two different reagents. The analyte in this example is any amino acid. The amino group is acylated with 3,5-dinitrobenzoyl chloride (DNBC) and the free acid of leucine resolved under reversed-phase conditions with an ion pair reagent in the mobile phase using the naphthylalanine column (4). Other similar cases could be made for the separation of hydroxy acids and amino thiols.

Conclusion

Brush-type CSPs have several advantages relative to CSPs derived from proteins, enzymes, cellulosics, or cyclodextrins. They are usually less expensive and much more durable than the bipolymer CSPs. They have a higher density of binding sites than the bipolymer CSPs and are consequently better suited for preparative separations. While it is certainly true that bipolymer CSPs are capable of separating the enantiomers of some compounds that cannot be resolved on present-day brush-type CSPs, one can, as the understanding of chiral recognition principles advances, look forward to brush-type CSPs of increasing scope and enantioselectivity. For example, CSPs capable of separating the enantiomers of underivatized ß-blockers and underivatized nonsteroidal anti-inflammatory drugs (NSAIDs) have been designed using mechanistic approaches and found to perform as expected. As the design of brush-type CSPs continues to advance, they will largely supplant the protein, enzyme, and cellulosic CSPs owing to their superior characteristics as stationary phases for chromatography.

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Chapter 4

Chiral Separations Using Native and Functionalized Cyclodextrin-Bonded Stationary Phases in High-Pressure Liquid Chromatography

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Cyclodextrin bonded stationary phases for HPLC have had a pronounced effect in the area of chiral separations. They are hydrolytically stable and have demonstrated unusual selectivity in the reversed and normal phase modes. Their selectivity is in part due to the inclusion complexation properties of cyclodextrins. Native and derivatized cyclodextrin bonded stationary phases have shown versatility for chiral separations. They are discussed in terms of their retention and chiral recognition mechanisms. Structural-selectivity relationships were observed for analytes using these columns. Optimization parameters are reviewed.

Many recent advances in the field of chiral chromatography are making the separation of enantiomers much more routine. Cyclodextrins have played a significant role in this area. Cyclodextrin was discovered by Villiers in 1891. Because of its apparent similarity to cellulose, he called it cellulosine (1). Schardinger was the first to characterize cyclodextrin as an oligosaccharide and to give a detailed description of its preparation and isolation (2,3). Schardinger isolated a bacillus, B. macerans, which was responsible for the formation of cyclodextrin from starch. B. macerans (along with other bacteria) are common sources of the enzyme, Cyclodextrin-Trans-Glycosylase (CTG), which is used in the production of cyclodextrins today. Cyclodextrins may be isolated by selective precipitation from the digest in which they are formed. Adsorption chromatography and cellulose column chromatography also have been used to isolate and identify cyclodextrins (4).

Freudenberg et. al., recognized the ability of cyclodextrin to form inclusion complexes (5). In the last few years there has been a tremendous increase in the research involving cyclodextrins and their inclusion complexation properties. Cyclodextrin bonded stationary phases for HPLC and derivatized cyclodextrins, some of which are suitable as coated stationary phases in capillary GC, are becoming very valuable tools in the area of enantioselective separations. The subject of the present report is the expanding application of cyclodextrins in high performance liquid chromatography (HPLC).

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Structure and Physical Properties

Cyclodextrins are chiral, toroidal shaped molecules composed of six or more D(+)-glucose residues bonded through α-(1,4) glycosidic linkages in which all of the glucose units are held in a C-1(D) chair conformation. Cyclodextrins are referred to by the number of glucose residues which they contain; α-CD (cyclohexaamylose) contains 6, β-CD (cycloheptaamylose) contains 7, and γ-CD (cyclooctaamylose) contains 8. Cyclodextrins with fewer than 6 glucose residues have not been found; this is probably due to excessive ring strain of a hypothetical 5 glucose residue torus. Cyclodextrins with more than 8 glucose residues have been identified, and some with branched structures have been reported (4). The structure of β-CD (7 glucose units) is shown in Figure 1 along with a 2 glucose segment depicting the numbering scheme employed.

The mouth of the cyclodextrin molecule has a larger circumference than its base and is lined with secondary hydroxyl groups of the C-2 and C-3 atoms of each glucose unit. The primary C-6 hydroxyl groups are located at the base of the cyclodextrin torus. While the primary hydroxyl groups (C-6) are free to rotate, partially blocking the opening at the base, the secondary hydroxyl groups (C-2 and C-3) are fixed in space with all of the C-2 hydroxyl groups pointed in a clockwise direction and all of the C-3 hydroxyl groups pointed in a counter-clockwise direction (when viewing down into the mouth of the CD cavity). Each glucose residue contains 5 stereogenic centers, hence there are 30, 35, and 40 stereogenic centers in α-CD, β-CD, and γ-CD respectively. The interior of the cyclodextrin cavity has two rings of C-H (C-5 and C-3) and one ring of glycosidic oxygens. Although the polarity of the cavity of β-CD has been compared to that of n-octanol, on a Pyrene solvent polarity scale, further experimentation is needed (6). Therefore, a cyclodextrin molecule can be described as a truncated cone with a partially blocked base, a hydrophobic cavity, hydrophilic edges, and multiple stereogenic centers. Important physical properties common to cyclodextrins are: i) they are non-reducing, ii) glucose is the only product of acid hydrolysis, iii) their molecular weights are always integral units of 162.1 (glucose), and iv) they do not appreciably absorb ultraviolet or visible light in the 200 to 800 nm wavelength range (4). Important physical properties of α-CD, β-CD, and γ-CD are listed in Table I.

Early Uses of Cyclodextrins in Chromatography

The low solubility of cyclodextrins in water and various other organic solvents hindered their use in chromatography. This problem was first overcome by incorporating cyclodextrins into polymeric gels. One of the first reports of such a CD-gel which retained the inclusion complexation properties of cyclodextrin was by Solms and Egli (7). Cyclodextrin was reacted with epichlorohydrin to produce CD-gels that could be made into particles of appropriate dimensions for standard column chromatography. Other procedures also were used to prepare CD-gels that were suitable as packing materials for liquid chromatography (8-12). In general, these CD-gel stationary phases showed that solute retention was a function of CD-solute binding constants, with the exception of solutes which did not form inclusion complexes. Anomalies to this retention behavior were observed and could be attributed to different interactions of the solute with the various crosslinkages (7-12).

The use of cyclodextrins as mobile phase modifiers is an alternative approach whereby their unique complexation properties can be used to advantage in

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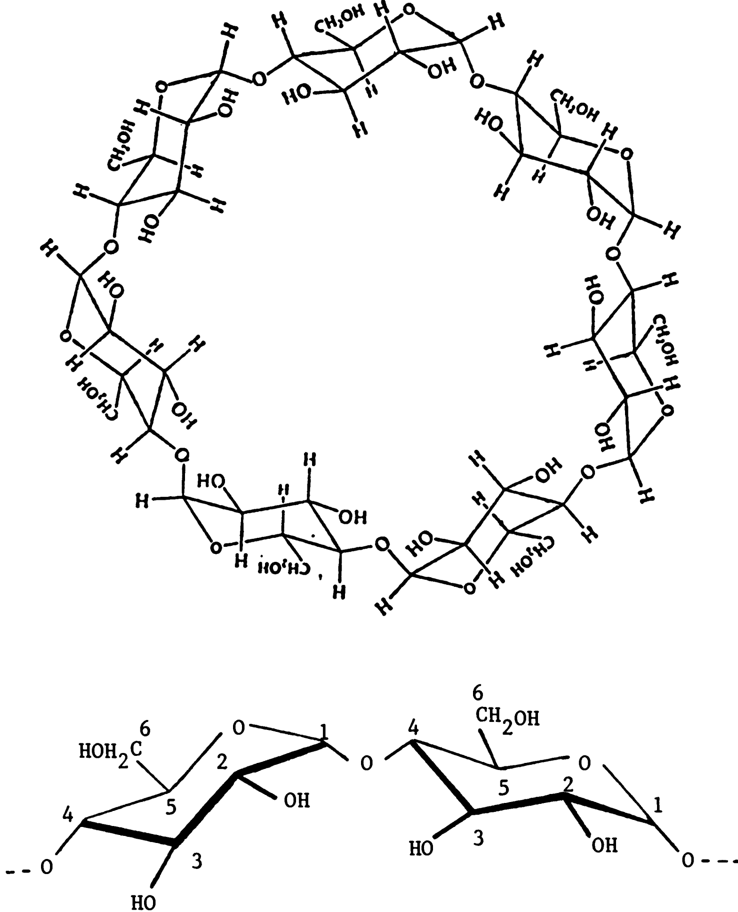

Figure 1. Structure of β-cyclodextrin along with a two glucose segment showing the numbering scheme employed.

TABLE I. Physical Properties of Cyclodextrin

CD	Glucose Units	Molecular Weight	Cavity Dimensions, Å Diameter External	Internal	Depth	Water Solubility, M
α-CD	6	973	13.7	5.7	7.8	0.114
β-CD	7	1135	15.3	7.8	7.8	0.016
γ-CD	8	1297	16.9	9.5	7.8	0.179

chromatography. The earliest report of such use was by Uekama et al., with the separation of A, B, and E prostaglandins by ion exchange chromatography (13). Cyclodextrins have been used since as mobile phase modifiers to affect separations in TLC (14-16), reversed phase (17- 19), and microbore HPLC (20-21). The role of the organic modifier, pH, ionic strength, and temperature in complexation has been studied. Equations relating capacity factors (k') to binding constants as functions of CD concentration, mobile phase composition, pH, and greater than (1:1) complexation have been derived (22-25). Three disadvantages of cyclodextrin as a mobile phase modifier are poor efficiency, low solubility of cyclodextrin in aqueous/organic systems, and prohibitive cost. Increasing the pH or adding Urea can increase the solubility of CD (26). Derivatization is an alternative method of increasing the solubility of CD (14). An advantage of using CD as a mobile phase additive is the possibility of enhancing the luminescence of some analytes such as dansyl amino acids and polyaromatic hydrocarbons (6,27).

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Bonded Cyclodextrin Stationary Phases in HPLC

With increased interest in using cyclodextrins in separations, CD-bonded stationary phases with the mechanical strength necessary for HPLC were needed. One of the earliest attempts to develop such a stationary phase was to bond CD to silica gel via nitrogen containing linkage arms (28). This linking was made by bonding [3(2-aminoethyl)amino] propyl trimethylsilane to silica gel and then reacting CD, which had previously been tosylated at the primary (C-6) hydroxyl, to this derivatized silica gel. Other nitrogen containing linkages also were developed (29-31). All of the CD-based stationary phases containing nitrogen in the linkage arms suffered from low coverage and instability under typical reversed phase chromatographic conditions.

In 1983 we developed a hydrolytically stable linkage (32). The spacer arm is attached to the silica gel through a silane linkage. Either an epoxy-silane, an organohalosilane or a vinyl-alkyl silane may be used. It is necessary that this reaction be done in the absence of water. The CD is then attached through its hydroxyls to the epoxide or the organohalide functionality. This is done by the reaction of a strong base (NaH) with the CD. A solvent such as dimethylformamide or pyridine is used. The use of the vinyl alkylsilane requires the conversion of the vinyl-alkyl ligand to either the epoxide or halide derivative (33). Several studies have been done to measure the reactivity and relative pKa's of the cyclodextrin hydroxyls (4, 34). Although linkage of the CD may occur through either the primary or secondary hydroxyl, the C-6 and C-2 hydroxyls are known to be the most reactive. These non-nitrogen containing bonded CD silica gel stationary phases have proven themselves to be relatively stable under standard HPLC conditions (32, 35-36). A schematic of this linkage is shown in Figure 2. Today these stable stationary phases are marketed as Cyclobond. Cyclobond I, II and III are the β-, γ- and α-CD forms, respectively.

Cyclobond has seen immediate and sustained success as only few liquid chromatographic stationary phases have experienced. Its impact on the field of chiral separations has been pronounced. Its versatility is due, in part, to the fact that they have shown unusual selectivity and stability. When used in the reversed phase mode, inclusion complex formation (in which the hydrophobic portion of the analyte resides in the cyclodextrin cavity) is largely responsible for retention and selectivity (32). In the normal phase mode, the hydrophobic mobile phase occupies the cavity of the cyclodextrin. Analyte interactions are primarily with the hydroxyl groups at the mouth and base of the cyclodextrin. Hence, in the normal phase mode, selectivity is similar to that of a diol type column (36-38). Chiral selectivity has been achieved in the reversed phase mode with all of the original Cyclobond I, II, and III columns, and

separations of geometric and position isomers have been achieved in both the reversed (34) and normal phase modes using Cyclobond I, II and III (37-43). Recently, there have been a few reports of enantiomeric separations in the normal phase mode using Cyclobond I.

Currently, the useful range of the Cyclobond series is being extended by derivatizing the CD bonded silica gel. Five different functional pendant groups have been placed on the Cyclobond I: i) acetyl, ii) 2-hydroxypropyl, iii) naphthylethyl carbamate, iv) dimethylphenyl carbamate, and v) *para*-toluoyl ester. These stationary phases are listed in Table II. Imparting additional chirality are ii and iii of which both the (R), (S), and racemic derivatives can be made (44-47). These derivatized Cyclobond I columns have been used in both the reversed and normal phase modes making them the first successful "multimodal" chiral stationary phases (48).

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General Mechanism of Retention on Cyclobond

In the reversed phase mode, the feature of the native CD bonded stationary phase which imparts their unique selectivity and influences retention is inclusion complexation (32, 35-36, 49-51). This complexation involves interaction of a nonpolar portion of the analyte molecule with the relatively nonpolar cyclodextrin cavity. This type of interaction is depicted in Figure 3. The inclusion complex gains stability from Van der Waals'-London dispersion forces and hydrogen bonding (4, 52). The size, shape, and polarity of the analyte are the most critical factors influencing the stability of the inclusion complex. If an analyte is too large to fit into the hydrophobic cavity, then inclusion is impossible. Hydrogen bonding of polar molecules to the C-2 and C-3 hydroxyl groups generally occurs preferentially to inclusion complexation (52). The depth which benzene is able to penetrate the cavity of α-CD is 50% that of β-CD, and β-CD shows 50% less affinity for pyrene than does γ-CD (52- 53). Aromatic groups have the ability to share electrons with the glycosidic oxygens of the cyclodextrin cavity, given the proper orientation (49, 52). The separation of polyaromatic hydrocarbons (PAH) shows that their retention is more a function of their shape and size than molecular weight (51). Saturated rings also can be included, but a more random fit and hence less selectivity is observed (51, 54). Amines and carboxylate groups will interact strongly with the hydroxyl groups of cyclodextrin through hydrogen bonding if the proper geometric and pH conditions are met. These can be the important second and third interactions necessary for chiral selectivity. Changing a single atom in a molecule may dramatically change retention as in the series: carbazole, fluorene, dibenzothiophene, biphenyl, and dibenzofuran shown in Figure 4 (51). This is due to interactions with the secondary hydroxyls of the β-CD.

The relative polarity of the CD bonded stationary phase contributes to retention. Many other interactions are possible and may also contribute to retention. For example, interactions with residual silanols on the surface of the silica gel and with the linkage arm are two effects that have been discussed (55).

Retention has been observed in both the reversed and normal phase modes on native CD bonded stationary phases. To date, the greatest success for enantioselective separations has been in the reversed phase mode with aqueous/organic mobile phases. Normal phase mode separations have been shown for structural isomers (37- 43) and carotenes (56). Hexane or cyclohexane with alcoholic modifiers are typical normal phase solvents used with these columns.

Silica Gel–O–(O–)Si(OH)–CH_2–CH_2–CH_2–O–CH_2–CH(OH)–CH_2–O–

Figure 2. Diagram of the non-nitrogen containing bonded stationary phase Cyclobond.

TABLE II. Derivatized Cyclobond I

R group [a]	Substituent	Name
$-C(=O)-CH_3$	Acetyl	Cyclobond I Ac
$-CH_2CH(OH)-CH_3$	(S) or racemic 2-hydroxypropyl	Cyclobond I SP or RSP
$-C(=O)-NH-CH(CH_3)-C_{10}H_7$	(R), (S) or racemic Naphthylethyl Carbamate	Cyclobond I RN, SN or RSN
$-C(=O)-NH-C_6H_3(CH_3)_2$	Dimethylphenyl Carbamate	Cyclobond I DMP
$-C(=O)-C_6H_4-CH_3$	*para*-toluoyl ester	Cyclobond I PT

[a] R groups are attached through the C-2, C-3, and/or C-6 atoms of β-CD.

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Figure 3. Schematic showing the reversible inclusion complex formation typical of cyclodextrin bonded stationary phase.

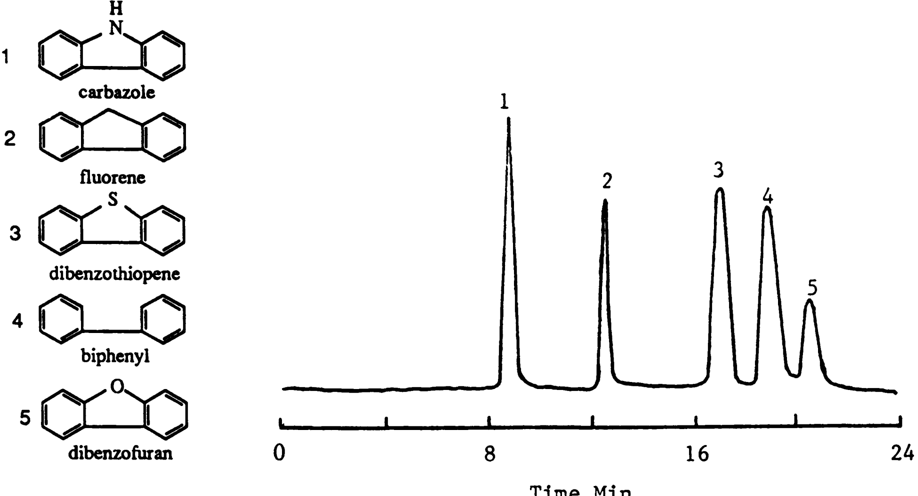

Figure 4. Chromatogram showing effect on retention of changing a single atom in a homologous series.

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Chiral Selectivity

In the reversed phase mode using native cyclodextrin bonded stationary phases, at least two things must be present in order to achieve enantioselectivity. First, a relatively tight fit must occur between the analyte and the cavity of the cyclodextrin. Second, there must be some interaction between the analyte and the C-2 and C-3 hydroxyls at the mouth of the cyclodextrin cavity (36). Both of these things must contribute to the three simultaneous but energetically different points of interaction of the analyte with the chiral stationary phase which are required for chiral recognition (57- 58).

A good rule of thumb is that the better the molecular fit, the greater the potential for chiral recognition (49). Generally, it has been observed that at least one or more rings must be present in an analyte to achieve this snug fit, and in most cases, an aromatic ring is necessary (36, 59- 63). Of the three commercially available native cyclodextrin bonded stationary phases, Cyclobond I (β-CD) has been the most adept at chiral separations. β-CD bonded stationary phases generally require a structure with at least two aromatic rings such as dansyl amino acids, but some success has been shown with single ring structures (59- 63). This illustrates that β-CD will include structures the size of naphthyl or biphenyl moieties tightly. The larger γ-CD bonded stationary phase (Cyclobond II) has shown success separating enantiomers containing fused rings such as naphthlene or multiple fused ring moieties in the reversed phase mode (64).

Chromatographic studies, as well as computer modeling (65- 66), relating analyte structures to enantioselectivity have shown that definite structure-selectivity relationships exist and that they may be used to predict what types of compounds can be separated by CD bonded stationary phases (59- 63). The solutes in these studies may be grouped by the number of rings in their structures. The α-CD bonded stationary phase was used in conjunction with compounds that contained 1 or 2 rings (63). The β-CD bonded stationary phase was used to evaluate compounds containing 1 or more rings (59- 62). Structures containing two or more rings can be further divided into groups that contain either a single or double ring aromatic substituent off the stereogenic center, two aromatic ring substituents off the stereogenic center, and structures that have the stereogenic center as part of a ring.

The enantiomers of aromatic amino acids; phenylalanine, trytophan, tyrosine, and 19 analogues were separated on an α-CD bonded stationary phase (63). Chromatographic data for these separations are listed in Table III. All structures had a single aromatic ring or the aromatic indole substituent β to the stereogenic center. Opposite the stereogenic center to the aromatic ring were the amine and acid functionalities, both of which effectively hydrogen bond. Substitutions on the aromatic ring tended to decrease selectivity with the exception of *para* - NO_2, -OH, or -halogens which may enter the CD cavity and enhance complexation. Nonpolar substituents such as 6-methyl- or 6-fluorotryptophan that force the analyte to tilt inside the cavity (steric interactions) increased selectivity. Derivatization of the amine increased retention but did not increase selectivity (63).

Chiral compounds which contain a single aromatic ring and whose enantiomers could be separated on a β-CD bonded stationary phase showed similarities to those separated on α-CD bonded stationary phases. Chromatographic data for these separations are shown in Table IV (62). All racemates had an aromatic ring α to the stereogenic center except for the five which were amino acids. They were

TABLE III. Chromatographic Data of Resolution of Racemates of Aromatic Amino Acid and Analogues on a-Cyclodextrin Bonded Stationary Phase (Cyclobond III) [a]

Compound	Structure	k'[b]	α	Rs	Mobile Phase[c]
D,L-Phenylalanine	C_6H_5–CH_2–C(H)(NH_2)–COOH	1.1	1.09	0.85	100:0
α-methyl-*m*-methoxy-		0.7	1.20	1.10	100:0
N-acetyl-*m*-fluoro-		5.2	1.03	0.55	100:0
α-methyl-		0.9	1.09	1.00	100:0
p-fluoro-		1.5	1.08	1.00	100:0
m-fluoro-		1.0	1.12	1.10	100:0
p-chloro-		5.0	1.05	0.80	90:10
N-benzoyl-		14.2	1.03	0.45	90:10
N-carbamyl-		3.0	1.03	0.70	90:10
N-carbobenzoxy-		5.8	1.02	0.55	74:36
D,L-Tryptophan	indol-3-yl–CH_2–C(H)(NH_2)–COOH (ring positions 1(N–H), 2, 3, 4, 5, 6, 7)	2.7	1.20	1.90	100:0
α-methyl-		2.6	1.26	1.90	100:0
1-methyl-		5.7	1.12	1.10	100:0
5-methyl-		4.9	1.07	1.00	100:0
6-methyl-		6.4	1.48	4.57	100:0
N-formyl-		8.0	1.04	0.70	100:0
4-fluoro-		2.4	1.07	0.85	100:0
5-fluoro-		2.7	1.08	1.00	100:0
6-fluoro-		3.7	1.64	6.18	100:0
D,L-Tyrosine	HO–C_6H_4–CH_2–C(H)(NH_2)–COOH	0.1	1.40	0.90	100:0
O-methyl-D,L-tyrosine		4.4	1.21	2.70	100:0
(Dinitrobenzoyl)leucine N-butyl thioester		1.3	1.16	0.95	60:40

[a] Adapted from reference 58. [b] k' is of first eluting enantiomer. In these cases the L enantiomer always eluted first. [c] Mobile phase reported as percentage (v/v) buffer:organic modifier. The buffer was 1.0% triethylammonium acetate (TEAA) at pH 5.1. The organic modifier was methanol.

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TABLE IV. Enantiomeric Separation of Structures Containing one Aromatic Ring on β-CD Bonded Stationary Phase (Cyclobond I) [a,b]

Compound	Structure	k' [c]	α	R	Mobile Phase[d]
(±)-1-Phenylethyl propionate	$CH_3CH_2C(=O)OC(H)(CH_3)C_6H_5$	3.71	1.01	0.5	60:40 [e]
(±)-α-Methylbenzyl acetate	$CH_3C(=O)OC(H)(CH_3)C_6H_5$	2.54	1.03	0.5	60:40 [e]
(±)-2-Chloro-2-phenylacetic acid	$C_6H_5CH(Cl)C(=O)OH$	3.49	1.03	0.5	60:40 [e]
(±)-Ethyl-3-phenyl-glycidate	$C_6H_5CH—CHC(=O)OCH_2CH_3$ (epoxide, O)	0.58	3.32	5.8	50:50 [f]
D,L-3,4-dihydroxy-phenyl-α-propyl-acetamide	$CH(CH_2CH_2CH_3)C(=O)NH_2$ on ring with OH, OH	0.16	1.31	0.6	70:30 [e]
(±)-2-Methoxy-α-methylbenzyl alcohol	$CH(CH_3)OH$ on ring with OCH_3	2.73	1.04	0.8	80:20 [e]
D,L-2-phenyl-propionaldehyde	$CH_3C(H)(C_6H_5)—CHO$	6.00	1.22	3.0	90:10 [e]
D,L-α-amino-3-thio pheneacetic acid	thiophene–$CH(NH_2)C(=O)OH$	2.14	1.05	0.7	70:30 [e]
(±)-Mandelic acid	$C_6H_5CH(OH)C(=O)OH$	2.04	1.07	1.4	96:4 [e]
(±)-Mandelic acid methyl ester	$C_6H_5CH(OH)C(=O)OCH_3$	1.92	1.07	1.5	96:4 [e]
(±)-O-acetyl-mandelic acid	$CH_3C(=O)O—CH(C_6H_5)C(=O)OH$	1.87	1.05	0.9	98:2 [f]

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TABLE IV. cont.

(±)-Ephedrine	OH—CHCH(NHCH$_3$)CH$_3$ (phenyl)	0.4	1.10	0.8	98:2 [e]
D,L-phenylalanine-amide	CH$_2$CH(NH$_2$)CNH$_2$ (C=O) (phenyl)	1.34	1.04	0.6	98:2 [f]
D,L-tyrosine	CH$_2$CH(NH$_2$)COH (C=O) (phenyl, OH)	1.02	1.06	0.5	90:10 [f]
D,L-tyrosine methyl ester	CH$_2$CH(NH$_2$)COCH$_3$ (C=O) (phenyl, OH)	2.04	1.03	0.7	98:2 [f]
3-Hydroxy-D,L-kynurenine	OH, CCH$_2$CNH$_2$COH (C=O, H, C=O), NH$_2$ (phenyl)	1.01	1.08	1.0	98:2 [f]
N-(3,5-dinitrobenzoyl)-D,L-leucine	CNHCH[CH$_2$CH(CH$_3$)$_2$]COH (C=O), O$_2$N, NO$_2$ (phenyl)	2.03	1.03	0.5	98:2 [f]

[a] Adapted from reference (57). [b] These data were generated by using two 25 cm Cyclobond I columns in series. [c] k' is of first eluting enantiomer. [d] Mobile phase reported as percentage (v/v) buffer:organic modifier. The buffer was 1.0% TEAA. The organic modifier was methanol. [e] pH 7.1 [f] pH 4.1

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either β or γ substituted. All compounds except one also had a carbonyl substituent off the stereogenic center. All of the racemates are believed to have at least one hydrogen bonding group in close proximity to the hydroxyls at the mouth of the cyclodextrin when the inclusion complex is formed. The stereogenic center of all of the racemates appears to be the main point of rotation between two π systems. This "sandwich" of two π systems would impart a degree of rigidity to the structure, and this rigidity is believed to be, in part, responsible for enhanced enantioselectivity (60,62).

Table V lists racemic compounds with two or more rings which can be resolved using a β-CD bonded stationary phase. For these solutes, all of the previous requirements for single ring compounds apply as well. Aromatic substituents α to the stereogenic center showed better selectivity than β and γ substituted aromatic compounds. Compounds with two or more aromatic ring systems generally showed greater retention than single aromatic ring structures. If the stereogenic center was between two aromatic rings or an aromatic ring system and another π system, then enantioselectivity was greater (60).

The enantiomeric separation of a series of nicotine analogues was analyzed to show the effects of different steric, electronic, and hydrogen bonding capabilities on enantioselectivity (61). Table VI contains structural and chromatographic data for these enantiomeric separations. Variations in the substitution of the N'- pyrrolidine ring showed that the presence of a tightly complexed substituent, such as the phenyl group on N'-benzylnornicotine, facilitated chiral separation. Other substituents off the N'- pyrrolidine ring which offered a tightly complexing substituent but had unfavorable steric interferences such as N'-α-naphthylnornicotine showed no enantioselectivity. It should be noted that N'-α-naphthylnornicotine along with other α substituted naphthyls can be resolved using the larger γ-CD bonded stationary phase (64). This data also indicates that fluorine substituents can be included and act as hydrogen bond acceptors to enhance chiral recognition. The presence of the pyrrolidine ring (which imparts some structural rigidity) was not necessary for enantioselectivity as shown by the separation of the enantiomers of N'-benzyl-N,α-dimethyl-3-pyridinemethanamine. Addition of alkyl groups to the pyridine ring did not enhance enantioselectivity, but the addition of a cyclic alkyl substituent allowed enantiomeric separation (61).

The separations of racemates with the stereogenic center as part of a ring system have also been studied, and the chromatographic data are shown in Table VII (60). Separation of the enantiomers of 5-phenylhydantion analogues show that when two aromatic ring systems were attached to the stereogenic center, enantioselectivity was greater than if only one aromatic ring was attached. If the stereogenic center is α to two aromatic rings and contained in a fused ring system such as in (+/-)-1-[5-chloro-2-(methylamino)phenyl]-1,2,3,4-tetrahydroisoquinoline, the chiral selectivity is tremendous (60). This can be attributed to the rigidity and the optimum spatial arrangement of the analyte.

Chiral Selectivity of the Functionalized Cyclodextrins

Acetyl and hydroxypropyl derivatized β- CD bonded stationary phases have extended the selectivity of Cyclobond in the reversed phase mode (44- 45). The secondary hydroxyl groups are relatively fixed in space on the native β-CD.

TABLE V. Chromatographic Data of Racemates that Contain Two Rings as Part of Their Structures on β-CD Bonded Stationary Phase (Cyclobond I)[a,b]

Compound	Structures	k'[c]	α	R	Mobile Phase[d]
α-Methyl-tryptamine	(indole)–$CH_2CH(NH_2)CH_3$	1.60	1.06	1.0	95:5
4-Chloro-2-(α-methylbenzyl)-phenol	C_6H_5—CH(CH_3)—(HO-phenyl)—Cl	9.44	1.06	1.3	60:40
Bromopheniramine	(pyridyl)—CH($CH_2CH_2N(CH_3)_2$)—(phenyl)—Br	2.70	1.10	0.8	70:30
Mandelic acid benzyl ester	C_6H_5—CH(OH)—C(=O)—$OCH_2C_6H_5$	3.05	1.02	0.6	70:30
Benzoin	C_6H_5—CH(OH)—C(=O)—C_6H_5	3.17	1.08	1.0	70:30
(±)-2-Phenylbutyro-phenone	C_6H_5—C(=O)—CH(C_2H_5)—C_6H_5	2.38	1.16	1.2	50:50
N,N'-dibenzyl-D,L-tartramide	$[-CH(OH)C(=O)NHCH_2C_6H_5]_2$	1.95	1.04	0.6	70:30

[a] Adapted from reference (55). [b] These data were generated by using two 25cm Cyclobond I columns in series. [c] k' is of the first eluting enantiomer. [d] Mobile phase reported as percentage (v/v) buffer:organic modifier. Buffer is 1% TEAA, pH 4.1 and the organic modifier is methanol.

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TABLE VI. Chromatographic Data for Enantiomeric Separation of Nicotine and Nicotine Analogous on β-CD Column [a]

Compound	Structure	k'[b]	α	Rs	Mobile Phase[c]	
Nicotine				d		
1-Methyl-2-(2-pyridyl)-pyrrolidine				d		
1-Methyl-2-(4-pyridyl)-pyrrolidine				d		
1-Methyl-2-phenyl pyrrolidine				d		
N-ethyl-N,α-dimethylphenyl methanamine				d		
N'-ethylnornicotine				d		
N'-(2,2-difluoroethyl) nornicotine		3.86	1.03	1.14	90:10 [g]	II [f]
		0.60	1.00	d	0:100 [g]	II [f]
N'-(2,2,2-trifluoro-ethyl nornicotine		1.03	1.09	1.51	75:25 [g]	II [f]
		0.64	1.19	1.29	0:100 [g]	II [f]
N'-benzylnornicotine		2.82	1.18	2.36	70:30 [g]	II [f]
		2.23	1.14	1.47	0:100 [g]	II [f]
N'-(2-methylbenzyl)-nornicotine		8.13	1.07	1.54	90:10 [h]	I [e]
		1.22	1.15	1.17	0:100 [g]	I [e]
N'-(1-naphthylmethyl)-nornicotine				d		

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TABLE VI. cont.

Compound	Structure					
N'-(2-naphthylmethyl)-nornicotine		5.35 3.37	1.07 1.02	2.02 1.12	70:30[h] 0:100[h]	I[e] I[e]
N-benzyl-N,α-dimethyl-3-pyridinemethanamine		2.05 2.53	1.03 1.00	0.80 d	80:20[i] 0:100[i]	I[e] I[e]
N,α-dimethyl-N-(2-naphthylmethyl)-3-pyridinemethanamine		1.64 2.83	1.09 1.00	1.21 d	80:20[i] 0:100[h]	I[e] I[e]
N,α-dimethyl-N-(1-naphthylmethyl)-3-pyridinemethanamide				d		
N'-benzoylnornicotine		4.75 4.06	1.05 1.00	1.32 d	95:5[i] 0:100[g]	I[e] I[e]
6-ethylnicotine				d		
6-butylnicotine				d		
5,6-cyclohexeno-nicotine		2.97 4.16	1.04 1.07	1.50 1.57	75:25[g] 0:100[g]	II[f] II[f]

[a] Adapted from reference (56). [b] k' of the first eluting enantiomer. [c] Mobile phase reported as percentage (v/v) buffer:organic modifier. The buffer is 1% TEAA pH 7.1. The organic modifier is acetonitrile. [d] The enantiomers were not resolved. [e] One 25cm cyclobond I column was used. [f] Two 25cm cyclobond I columns were used in series. [g] The flow rate 1.0 ml/min. [h] The flow rate 0.8 ml/min. [i] The flow rate 0.5 ml/min.

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TABLE VII. Chromatographic Data for Enantiomeric Separation of Compounds Which have the Stereogenic Center as Part of a Ring [a,b]

Compound	Structure	k'[c]	α	Rs	Mobile Phase[d]
5-(4-Methylphenyl)-5-phenylhydantoin		10.17	1.12	2.0	70:30[e]
5-(4-Hydroxyphenyl)-5-phenylhydantoin		2.96	1.35	2.0	80:20[f]
5-Methyl-5-phenylhydantoin		1.20	1.07	1.1	85:15[e]
α-Methyl-α-phenylsuccinimide		6.0	1.07	2.0	70:30[e]
(±)-4-Benzyl-2-oxazolidinone		2.08	1.07	2.0	96:4[e]
5-Ethyl-5-(p-tolyl)-2-thiobarbituric acid		4.66	1.03	0.5	50:50[f]
(±)-1-[5-Chloro-2-(methylamino)phenyl-1,2,3,4-tetrahydroisoquinoline		0.32	2.34	3.0	60:40[e]
9-Methyl-$\Delta^{5(10)}$-octaline-1,6-dione		4.51	1.04	1.0	80:20[f]
N-Ethyl-3-phenyl-2-norbornanamine		0.91	1.06	0.7	70:30[e]

[a] Adapted from reference (55). [b] Two Cyclobond I columns were used in series. [c] k' is for the first eluting enantiomer. [d] Mobile phase reported as percentage (v/v) buffer: organic modifier. The buffer was 1% TEAA. [e] pH= 4.1 [f] pH= 7.1

Acetylated and hydroxylpropyl derivatives allow the rim of the cyclodextrin to be "extended" and somewhat flexible. The 2-hydroxypropyl β-CD bonded stationary phase has been useful in the separation of enantiomers with stereogenic centers incorporated into ring systems, external or β to an aromatic ring, and for atropic compounds such as (+/-) 1,1' di-2-naphthol (45). Table VIII lists chromatographic data for enantiomeric separations that have been reported using the 2-hydroxypropyl β-CD bonded stationary phases. The acetylated β-CD bonded stationary phase has been useful for the separation of the enantiomers of norphenylephrine and multi-ring structures such as (+/-) norgestrel (44) and scopalamine (88).

Although many normal phase achiral separations have been achieved with native cyclodextrin bonded stationary phases (37- 43), the normal phase separation of enantiomers was not reported until recently (46). As mentioned before, in the normal phase mode, nonpolar organic mobile phase molecules occupy the cyclodextrin cavity. Without inclusion, enantiomeric selectivity often was not seen. To overcome this problem, a variety of different substituents were attached to the hydroxyl groups of native cyclodextrin. To date, four of the five new derivatized β-CD stationary phases have been successful in performing chiral separations in the normal phase mode (46). Table IX lists chromatographic data for chiral separations using derivatized β-CD bonded stationary phases in the normal phase mode. Acetylated, dimethylphenyl carbamate, napthylethyl carbamate, and *para*-tolouyl ester β-CD bonded stationary phases have been used successfully in the normal phase mode for the separation of enantiomers. With the addition of aromatic and carbonyl functionalities, opportunities for π-π interactions arise. Also, interactions with the residual hydroxyl groups of β-CD exist. Each derivatized β-CD stationary phase was shown to have distinct enantioselectivity, but in some respects they were similar to derivatized cellulosic phases (67). The isocyanate derivatized β-CD phases seemed to have the best enantioselectivity. This may be attributed to the additional hydrogen bonding and stronger dipole-dipole interactions available through the carbamate linkage relative to the ester linkage. Although in some cases the derivatized β-CD stationary phases exhibits selectivity analogous to derivatized cellulosic phases, there are examples of separations unique to each type of chiral phase (46). In addition to similarities to the derivatized cellulosic phases, the napthylethyl isocyanate derivatized β-CD shows enantioselectivity similar to the napthylvaline reciprocal chiral stationary phase developed by Pirkle and Pochapsky (68).

Some of the racemates that were separated on derivatized β-CD bonded stationary phases in the normal phase mode were reported previously to be resolved on native cyclodextrin in the reversed phase mode. However, these normal phase separations were achieved with shorter retention times and increased resolution (46). Mechanistic studies concerning requirements for the enantioselectivity of the derivatized cyclodextrin chiral stationary phases are currently underway; however, general retention may be related to the degree of substitution of each type of pendant on the β-CD. The acetylated β-CD stationary phase appears to be ca. 90% derivatized, while the aromatic pendant groups show an average degree of substitution to be ca. 6 for napthylethyl isocyanate, 10 for dimethylphenyl isocyanate, and 13 for *para*-toluoyl ester. That is 33, 50, and 66% respectively. The decreased percentage of coverage for the larger pendant groups is probably due to increased steric hindrance (46).

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TABLE VIII. Chromatographic Data for Separation of Racemates using Cyclobond I SP or RSP [a]

Compound	Structure	k'[b]	α	Rs	Column[c]	Mobile Phase[d]
(±)-1-Benzocyclo-butene carboxylic acid	COOH	2.98	1.14	1.0	A	100:0
		1.60	1.10	0.6	B	95:5
		3.30	1.09	0.6	C	90:10
1-Indanol	OH	1.27	1.07	0.6	A	100:0
		1.00	1.11	0.6	B	100:0
		3.00	1.04	0.5	C	95:5
(±)-1,2,3,4-Tetrahydro 1-naphthol	OH	2.36	1.08	0.7	A	100:0
		1.92	1.08	0.5	B	100:0
		1.71	1.03	0.6	C	80:20
3-Phenylphthalide	O, O	5.92	1.04	0.5	B	90:10
Chlorthalidone	O, NH, HO, Cl, O-S-O, NH_2	1.33	1.38	2.2	A	95:5
		1.50	1.31	2.8	B	95:5
		2.80	1.21	1.6	C	90:10
Idazoxan	N, O, N H, O	0.89	1.07	0.55	A	100:0
		0.63	1.06	0.50	B	100:0
Methylidazoxan	N, O, N H, O	0.75	1.19	1.09	A	100:0
		0.62	1.11	0.60	B	100:0
		1.17	1.08	0.55	C	95:5
BAY COOH	CF_3, O_2N, COOH, Me, N H, Me	1.16	1.28	1.56	A	98:2
		0.65	1.12	0.8	B	95:5
		3.74	1.11	0.7	C	95:5
SQ 28 873	CF_3, O_2N, CO_2Me, Me, N H, Me	2.66	1.06	0.6	A	95:5

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TABLE VIII. cont.

Compound						
SQ 30 840	4.32	1.22	2.00	A	85:15
	1.33	1.38	1.50	B	80:20
	5.46	1.14	1.07	C	80:20
BAY CNET	3.27	1.10	0.7	A	98:2
	1.54	1.12	0.5	B	95:5
Nisoldipine	9.81	1.13	1.04	A	90:10
	3.17	1.07	0.6	C	80:20
SQ 31 236	2.00	1.21	2.00	A	85:15
	10:86	1.28	1.25	B	80:20
	2.54	1.13	0.83	C	95:5
SQ 31 579	5.08	1.14	0.55	A	95:5
3,3',5-Triiodo-D,L-thyronine	7.18	1.05	0.65	A	85:15
3,5-Diiodo-D,L-thyronine	8.42	1.07	1.12	A	90:10
	4.16	1.05	0.5	C	80:20
α-(1-Naphthyl)ethylamine	2.10	1.05	0.3	A	100:0[e]
	2.58	1.06	0.6	B	90:10[e,f]
	5.40	1.06	0.4	C	80:20[e,f]
D,L-3-(α-acetonyl-4-chlorobenzyl)-4-hydroxycoumarin	4.41	1.06	0.6	A	95:5[e]
	5.38	1.04	0.6	B	95:5[e]
(±)-1,1'-Bi-2-naphthol	4.40	1.08	0.6	A	20:80[f]

Continued on next page

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TABLE VIII. cont.

[structure: binaphthyl with $O-(CH_2)_8-O$ bridge]	0.64	1.08	0.5	A	70:30[g]
	4.16	1.15	1.1	A	70:30[g]

[a] Adapted from reference (40). [b] k' is of the first eluting enantiomer. [c] Column A is a 25 x 0.46 cm. S-2-hydroxypropyl derivatized β-CD of 300 Å pore silica and 3.1% carbon loading. Column B is 15 x 0.56 cm racemic 2-hydroxypropyl derivatized β-CD on 300 Å pore silica gel with 4.3% carbon loading. Column C is 25 x 0.46 cm. S-2-hydroxypropyl β-CD derivatized on 120 Å pore silica gel with 5.0% carbon loading. [d] Mobile phase reported as percentage (v/v) buffer:acetonitrile. Buffer is 1% TEAA at pH 4.1. [e] Buffer is 1% TEAA pH 7.1. [f] Methanol was used as organic modifier. [g] Aqueous is not buffered.

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TABLE IX. Chromatographic Data of Enantiomeric Separations in the Normal Phase Mode using Derivatized β-CD Bonded Stationary Phases [a]

Compound	Structure	k'[b]	α[c]	Mobile Phase[d]	Column
(±)-γ-Phenyl-γ-butyrolactone		14.5	1.10	98:2 Hex:ipa	DMP
(R,S)-ciprofibrate		2.36	1.20	80:20:02 ACN:ETOH:HOAC	SN
(±)-Phensuximide		11.20	1.30	90:10 Hex:ipa	DMP
(R,R)-(S,S)-(±)-N, N'-bis(α–methylbenzyl)sulfamide		4.33	1.21	90:10 Hex:ipa	Acetyl
(R,S)-N-trichloroacetyl-1,2,3,4-tetrahydro-1-naphthylamine		7.70 1.28	1.3 1.06	95:5 Hex:ipa 90:10 Hex:ipa	DMP RN
D,L-3-(α-acetonyl-4-chlorobenzyl)-4-hydroxycourarin		23.6 21.5	1.12 1.13	70:30 ACN:ipa 60:40 Hex:ipa	RN Acetyl
(R,S)-α-methoxyphenylacetic acid		2.55	1.27	99.5:0.5 ETOH:HOAc	PT
(R,S)-2-methoxy-2-phenylethanol		4.5	1.10	95:5 Hex:ipa	DMP
(±)-glutethimide		14.3	1.10	90:10 Hex:ipa	DMP

Continued on next page

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TABLE IX. cont.

N-CBZ-DL-proline	3.1 13/3	1.08 1/16	90:10 Hex:ipa 99.9:0.1 ETOH:HOAc	PT RN
(±)-5-(α-Phenethyl)-semioxamazide	3.31	1.03	90:10 Hex:ipa	Acetyl
(R,S)-N-(3,5-di-nitrobenzoyl-α-methylbenzylamine	6.2	1.61	70:30 Hex:ipa	DMP
N'-benzylnornicotine	2.61	1.18	98:2 Hex:ipa	RN
N'-(2-naphthyl-methyl)nornicotine	4.4	1.13	98:2 Hex:ipa	RN
(R,S)-2,2'-bi-2-naphthol	23.4	1.10	98.2 Hex:ipa	DMP
(R,S)-2,2'-bi-naphthyldiyl-crown-4	2.6	1.23	95:5 Hex:ipa	RN
(R,S)-2,2'-bi-naphthyldiyl-17 thiacrown-5	5.2	1.08	95:5 Hex:ipa	PT
	2.9	1.06	98:2 Hex:ipa	RN
	4.2	1.07	98:2 Hex:ipa	RN

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TABLE IX. cont.

[structure: binaphthyl-O-O-$(CH_2)_4$]	3.14	1.07	98:2 Hex:ipa	RN

[a] Adapted from reference (41). [b] k' is of first eluting enantiomer. [c] The resolution Rs of all separations are greater than 1.5 except compound last 3 which were 1.0-1.3. [d] Mobile phases reported are (v/v) percentages. Hex is the abbreviation for Hexane, ipa for Isopropal alcohol, ACN is acetonitrile, HOAc is acetic acid.

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Mechanistic studies have been done using the napthylethyl carbamate β-CD stationary phase. Napthylethyl carbamate, dimethylphenyl carbamate, and *para*-toluoyl β-CD are π-basic phases. The napthylethyl carbamate β-CD stationary phases showed definite structure-selectivity relationships for enantiomers in at least one group of compounds. Solutes containing an aromatic π-acidic group and an amide, urethane, or urea functionality were resolved (46- 47). A large number of chiral amines, alcohols, and carboxylic acids were derivatized to contain the π-acidic 3,5 dinitrobenzoyl (3,5 DNB) group and were subsequently resolved (47).

For naphthylethyl carbamate β-CD bonded stationary phases, the best enantioselectivity of 3,5 DNB derivatized amines was seen if the carbon α to the stereogenic center was 2° or 3°. Generally, within a homologous series of compounds, retention decreased as hydrocarbon chain length increased; however, aromatic groups increased retention. Benzyl and naphthyl containing amines showed a reversal of elution order on the (R) and the (S)- naphthylethyl carbamate β-CD bonded stationary phase columns, but non-aromatic amines did not show this reversal. This implies different retention and chiral recognition mechanisms for aromatic and non-aromatic amines. Aromatic amines can have strong interactions with the naphthylethyl carbamate substituent. Aliphatic amines have a reduced potential for π–π, steric, and repulsive interactions. Enantioselectivity was observed for 3,5 DNB derivatized aliphatic amino acids on the naphthylethyl carbamate phases only with a mobile phase mixture of acetic acid/ acetonitrile/ ethanol. For derivatized aromatic amino acids, a mobile phase of acetic acid/ methanol gave good separations. This is due, in part, to the relatively reduced competition for the hydrogen bonding sites of the chiral stationary phase shown by the acetic acid/ acetonitrile/ ethanol mobile phase (47). The enantioselectivity shown for 3,5 DNB derivatized aromatic and aliphatic amino esters by the naphthylethyl carbamate β-CD bonded stationary phase further demonstrates that the naphthylethyl isocyanate group interacts in an enantioselective manner to a greater extent with aromatic than aliphatic compounds (47).

Chromatographic data showed that the enantioselectivity of 3,5 DNB derivatized alcohols decreases as the similarity of the substituents of the stereogenic center increases. Increasing the chain length of one of the substituents increases enantioselectivity. It should be noted that the derivatization of alcohols introduces a new atom between the stereogenic center and the 3,5 DNB group. This lengthening and therefore further removal of the stereogenic center and its substituents from interactions with the derivatized cyclodextrin reduces enantioselectivity (47).

From a mechanistic point of view, the addition of a new stereogenic center as in (R) or (S)-napthylethyl carbamate β-CD, and (R) or (S)- 2-hydroxypropyl allows one to ascertain what portion of the chiral selector (or which specific interactions) are primarily responsible for enantioselectivity. For example: i) the cyclodextrin moiety could totally dominate enantioselectivity, ii) the chiral substituent could totally dominate enantioselectivity, and iii) both the CD and its substituent could contribute (in varying degrees) to the separation (47). In cases where the cyclodextrin dominates enantioselectivity, the same retention order would be observed with the racemic, the (R) or the (S) derivatized β-CD bonded stationary phases. When enantioselectivity is totally dominated by the chiral pendant group, there would be no separation on the racemic derivatized β-CD, but a reversal of enantioselectivity and elution order for the (R) and the (S) derivatized β-CD. Approximately one third of the racemates separated with the naphthylethyl carbamate β-CD column showed this second type of behavior

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(47). This enables one to choose the elution order for a pair of enantiomers. If one enantiomer is present in only trace amounts in the presence of the other, then it is desirable to have trace enantiomer elute first in order to avoid overlap with the tailing peak of the more concentrated enantiomer. In cases where both the CD and substituents contribute to enantioselectivity, the chiral pendants and the β-CD may act in a synergistic or an antagonistic manner. A reversal of elution may occur between the (R) and (S) derivatized columns. Generally it was shown that the best enantioselectivity was with either the (R) or the (S) derivative, and was somewhat less for the racemic derivatized β-CD column. The dominant enantioselective effects are difficult to predict (47).

Acetyl and naphthylethyl isocyanate derivatized β-CD bonded phases have shown enantioselectivity in both the reversed and normal phase modes (44, 48). With the increasing number of chiral stationary phases commercially available (>50), the need for consolidation and the elimination of different chiral stationary phases is apparent (48). The derivatized cyclodextrin stationary phases have been effective in separations analogous to derivatized cellulosic and Pirkle type chiral stationary phases (46). Derivatized cellulosic phases consist of broad range MW polymers coated onto large pore silica gel. Aqueous and some organic(CH_2Cl_2, $CHCl_3$, THF, DMF, etc.) mobile phases would destroy this coating. Even less polar alcoholic mobile phases can destroy the secondary structure often thought to be responsible for some of their chiral selectivity (67). Polar mobile phases also are not recommended for "Pirkle" type chiral stationary phases (68). Bonded CD stationary phases have shown the ability to operate in both the normal and reversed phase modes; however, for a chiral stationary phase to be considered "multimodal", it needs to be able to separate different classes of compounds in both modes (48).

The napthylethyl carbamate β-CD bonded stationary phase has become the first useful "multimodal" column. In the reversed phase mode, a totally different set of racemates were separated than was separated in the normal phase mode (46, 48). Enantiomers of the pesticides Dyfonate, Ruelene, Ancymidol, and Coumachlor, as well as a variety of pharmaceuticals such as Tropicamide, Indapamide, Althiazide, Tolperisone, and a sulphonamid were reported resolved (48). Other racemates that had been previously reported separated on other columns also were resolved. Further investigations of the derivatized β-CD bonded stationary phases' "multimodal" chiral capabilities are needed.

Optimization

Methanol and acetonitrile have been the most frequently used organic modifiers for chiral separations with cyclodextrin bonded phases in the reversed phase mode. This is not to say that other organic modifiers such as ethanol, propanol, dimethylformamide, and dioxane could not be used. Organic modifiers compete with the solute molecule for residence in the cyclodextrin cavity. The nature of the cyclodextrin inclusion complex with organic molecules is such that it is usually strongest in water and decreases upon addition of organic modifiers (36). Methanol is the weakest displacer (*i.e.* the poorest competitor for residence in the cyclodextrin cavity) of the alcohols, and acetonitrile is a stronger displacer than methanol or ethanol (36, 49). Enantiomeric selectivity is generally independent of the organic modifier used, but there are cases where enantioselectivity is observed using acetonitrile but not methanol. The best efficiencies were generally achieved with acetonitrile as the mobile phase modifier.

Figure 5 shows the three basic profiles of retention vs. percentage organic modifier that have been observed using Cyclobond (36). For type A, retention

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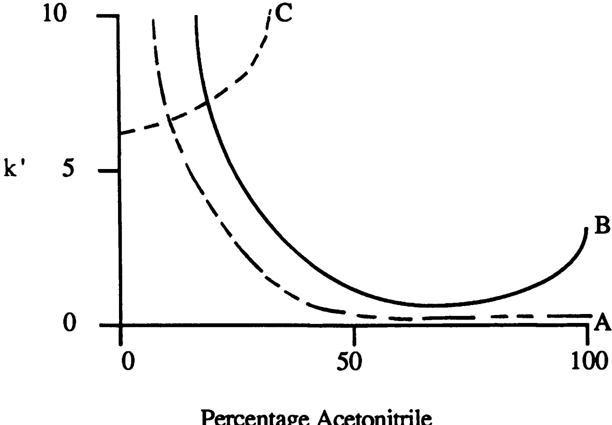

Figure 5. Typical curves showing the effect of mobile-phase composition on the retention of different solutes.

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decreases with increased organic modifier. This is usually accompanied by a decrease in enantioselectivity to a point where the enantioselectivity is zero. This is the most common type of retention behavior.

Type B behavior is similar to type A except that at higher percentages of organic modifier, retention increases, sometimes with an increase in enantioselectivity (36, 49). At first, this was thought to be due to normal phase adsorption to residual silanol groups. However with an increase in enantioselectivity, it had to be attributed to interaction with the cyclodextrin which is the only chiral part of the stationary phase. This behavior was observed for some dansyl amino acids (36, 49) and nicotine analogues (see Table VI).

For type C behavior, the retention increases with higher percentages of organic modifier. This results from the insolubility of the solute in the organic modifier and is not unique to chromatography with cyclodextrin bonded stationary phases. Changing the organic modifier may shift the curves in Figure 5, but these general types of behavior are still observed (36, 49).

Normal phase eluants for CD bonded stationary phases are generally, but not limited to, hexane/ isopropanol mixtures. The fact that the CD are covalently bonded to silica gel in Cyclobond allows a wider range of solvents to be used in comparison to other normal phase chiral stationary phases (46). Chiral selectivity for CD bonded stationary phases in the normal phase mode is still new, and more is being learned about the effects of various mobile phase solvents (46- 47).

The greatest effects on selectivity and efficiency in the reversed phase mode are usually obtained with variations in the pH and ionic strength of the mobile phase. The addition of salts to the mobile phase, in the form of buffers, generally has a more significant effect on retention and efficiency than it does on selectivity. Buffers have been known to compete with the analyte for the cyclodextrin cavity (36, 49). With increasing buffer concentration, retention is decreased and efficiency is increased; however, these effects can be pH and concentration dependent (36). There can be a tremendous change in retention with changes in pH. Ionizable solutes tend to show the greatest sensitivity to changes in the pH (36). Triethylammonium acetate (TEAA) is one of the more useful buffers because it is noncorrosive and the pH can be easily adjusted. In the normal phase mode, because the primary mechanism is π-π interaction and hydrogen bonding, pH control generally is not used (46- 47).

The binding constant of a solute to cyclodextrin is very temperature dependent (4, 36, 49, 52). There is an increase in the binding constant at lower temperatures. Chromatographic studies using CD bonded stationary phases have shown that decreasing the temperature almost always increases selectivity, but the same cannot be said for resolution. Increasing the binding constant of the solute in the cyclodextrin cavity by lowering the column temperature may lead to reduced resolution. This is due to a loss of efficiency which is a consequence of poorer mass transfer. The increasing selectivity and band broadening effects may work antagonistically and are hard to predict. Increasing the column temperature may even improve the separation of strongly retained solutes (36). In the normal phase mode, since inclusion is not the only mechanism responsible for retention and enantioselectivity, band broadening at reduced temperatures may not be a problem, but this effect has not been studied specifically.

Typical flow rates for a 25 cm analytical Cyclobond column range from 0.5 to 2.0 ml/ min. At high flow rates (i.e. 1.0 to 2.0 ml/ min.) band broadening from mass transfer effects will tend to decrease efficiency and lead to poorer resolution in the reversed phase mode (36). Lowering the flow rate can improve efficiencies by a factor of 1.5 to 4 (36, 49). The greatest changes are seen between 1.0 and 0.4 ml/ min, after which further reduction only allows small gains in efficiency (49).

Applications

Chiral L-amino acids and D-sugars are the basic building blocks of all living systems. Phospholipids, steriods, nucleotides, nucleosides, and bile salts make up some of the other chiral molecules present in living things. Approximately 57% of the drugs prescribed in the United States are chiral (69- 70, 72). It also has been reported that the enantiomers of a racemic drug may show significantly different biological activities. A tragic example is that of the drug Thalidomide (N-(2,6-dioxo-3-piperidinyl) phthalimide). The d-form is a soporific drug, but the l-form is a powerful teratogen. The administration of the racemic d,l-Thalidomide to pregnant women resulted in a large number of birth defects and the subsequent rapid withdrawal of this drug from the market (71). Another case of different biological activities for each enantiomer is that of (R) and (S) propranolol, a β-adrenergic blocker used as an antianginal. The (S)-form of propranolol is as much as 100 times more potent than the (R)-form (69). Great differences in the pharmacological activities such as these between enantiomers of drugs are typical.

Between 1983 and 1985, 88% of the new chiral drugs were marketed as the racemic form (70, 72). With the advancement of technology in the field of chiral separations and tragic cases such as Thalidomide in the past, new and stricter regulations for the testing and marketing of chiral drugs are being set forth (73). Predictions have been made that racemic compounds will soon be considered as drugs with a 50% impurity (70, 72- 73). The separation of these chiral compounds is a very important analytical problem.

Cyclobond columns have been useful in the separation of many drug stereoisomers (65- 66, 72, 74- 75). Table X lists chromatographic data of some chiral drugs whose enantiomers could be separated using CD bonded stationary phases. Also listed are chromatographic data of some drugs found as diastereomers. Many clinical applications have been reported for cyclodextrin bonded stationary phases. These analyses include separations of chiral drugs and chiral metabolites. The analysis and quantitation of the enantiomers of ibuprofen and its metabolites (76) and the structural isomers of suprofen (77) in biological fluids were reported. The determination of the enantiomeric composition of urinary phenolic metabolites of phenytoin (78), hydantion and analogues (79) are shown. The separation of the enantiomers of d,l-hexabarbitol and chiral anti-inflammatory agents and metabolites in rat blood was also done using a β-CD bonded stationary phase (80- 81). The clinical applications of β-CD columns in HPLC are not limited to analyzing drugs, but Cyclobond columns also have been used to separate and quantitate the metabolites of industrial solvents such as toluene and xylene (82). The level of *o*-, *m*-, and *p*-methylhippuric acid in the urine is a valuable clue in the investigation of the exposure of workers to toluene and xylene. Other biological applications include separations of mycotoxins (50), cyclopropanes (83), and the enantiomers of rotenoids (84) which are used as pesticides.

The separation of carbohydrates by liquid chromatography has received increased attention in the past decade (85- 87). CD bonded stationary phases have shown a high degree of selectivity for sugars and other carbohydrates (85). Many mono-, di-, tri-, tetra-, and deoxysaccharides and sugar alcohols have been separated using either α-CD or β-CD bonded stationary phases. Retention of saccharides on CD bonded stationary phases could be related to size with the smaller saccharides usually eluting before the larger saccharides. There was a close relationship between the number of hydroxyl groups on the sugar available for hydrogen bonding and retention, with the deoxy sugars eluting first. α-CD and β-CD bonded stationary phases are very adept at separating cyclodextrins from each other as well (86).

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Table X. Chromatographic Data for the Separation of Enantiomeric and Diastereomeric Drugs

Therapeutic Catagory Drug Name	k'[a]	α	Rs	Column[b]	Mobile Phase[c]	Ref.[d]
Anti- Inflammatory						
Ibuprofen	8.04	1.10	0.7	CB I	70:30	68
Ketoprofen	7.67	1.06	1.2	2 CB I	73:27	68
Sedative- anticonvulsants						
Hexabarbital	9.39	1.14	1.5	CB I	85:15	68
Mephobarbital	14.8	1.14	1.6	CB I	80:20	68
Mephenytoin	0.48	1.33	1.8	CB I	60:40	68
Phensuximide	1.97	1.15	1.1	CB I	90:10[e]	68
Etomidate	1.80	1.70	1.1	CB I	95:5[e,f]	68
Triazoline	5.00	1.15	1.5	CB I	90:10	60
β-Adrenergic Blocker						
Propanolol	2.78	1.04	1.4	2 CB I	75:25	68
Metaprolol	3.51	1.03	0.9	2 CB I	68:32	68
Antihistimine						
Chloropheniramine	5.86	1.07	1.5	CB I	85:15[e]	68
Anticorticosteroid						
Aminoglutethimide	7.49	1.03	0.9	CB I	Gradient[e]	68
Vasodilator						
Verapamil	2.94	1.03	0.7	CB II	85:15	68
Progestrin						
Norgestrel	0.48	1.24	1.1	2CBII	70:30[e,g]	68
Diuretic						
Chlorthaldone	0.50	1.44	1.9	CB I	70:30	68
Central Nervous System Stimulant						
Methylphenidate	1.17	1.14	1.6	CB II	90:10[e]	68
Huperzine A	1.83	1.05	0.8	CB I	90:10[e,h]	68
Nomifensine	3.20	2.50	4.2	CB I	80:20	71

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Table X. cont.

Narcotics, Analgesics						
Methadone	2.38	1.04	0.8	CB I	Gradient[e]	60
Scopolamine	1.67	1.10	1.8	CB I	96:4[e]	70,88
Atropine	6.83	1.04	0.6	CB I	96:4[e]	70
Homotropine	1.98	1.07	1.4	CB I	96:4[e]	70
Cocaine	6.28	1.04	0.9	CB I	96:4[e]	70
Diastereomeric Drugs						
Cinchona Alkaloids						
Quinidine	2.16					
Quinine	1.78	1.21	1.76	CB I	90:10	60
Cinchonidine	1.62					
Cinchonine	2.12	1.31	1.86	CB I	90:10	60
Antiestrogen						
Tamoxifen	0.41		2.6	CB I	75:25	60
Clomiphene	3.60		2.00	CB I	35:65	60

[a] k' is of the first eluting enantiomer or isomer. [b] Column is 25 X 0.46 cm Cyclobond I (CBI), Cyclobond II (CBII), or 2 Cyclobond columns in series. [c] Mobile phase reported as percentage (v/v) aqueous buffer: organic modifier. Unless otherwise noted the buffer was 1% TEAA at pH 4.1 and the organic modifier was methanol. [d] Adapted from this reference. [e] Acetonitrile was used as the organic modifier. [f] The buffer was 5% TEAA pH 7.0. [g] The mobile phase was unbuffered.[h]The buffer was 1% TEAA pH 7.0.

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The separation of anomers of sugars has long posed a difficult problem to the chromatographer. The rapid mutarotation of anomers leads to peak doubling and band broadening. The separation of 36 different pairs of anomers was reported using either an α-CD or a β-CD bonded stationary phase (87). A high percentage of organic modifier was used in the majority of sugar separations using these phases. Because of this, hydrogen bonding to the surface of cyclodextrin is believed to be the dominate retention mechanism and not inclusion complexation. This high percentage of organic modifier also slowed mutarotation (87).

Conclusions

From this review it can be concluded that CD bonded stationary phases have shown tremendous versatility in HPLC. A wide range of structural classes of compounds were separated including many classes of enantiomers. The enantioselectivity shown by both the native and the derivatized phases can be explained at least partially with structural-selectivity relationships. These phases are being used more and more in clinical and pharmaceutical applications. With the growing need for chiral separations, versatile chiral stationary phases such as these are needed. It also should be noted that a reemergence of interest in chiral separations by GC is presently underway. Derivatized CDs which are capable of being used as coated capillary GC stationary phases are the main reason for this increased interest (89-93).

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Chapter 5

Chromatographic Optical Resolution on Polysaccharide Carbamate Phases

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Phenyl-, aralkyl-, and alkylcarbamates of polysaccharides, cellulose and amylose, were prepared by the reaction of the polysaccharides with corresponding isocyanates. The carbamates were adsorbed on silica gel to use as chiral stationary phases for HPLC. Optical resolving abilities of the carbamates were evaluated by using a hexane-2-propanol mixture as eluent. Alkylcarbamates of both cellulose and amylose showed low chiral recognition. Optical resolving abilities of phenylcarbamate derivatives having a substituent on phenyl group depended on the kind and position of the substituent, and 3,5-dimethylphenylcarbamates of both polysaccharides gave practically useful chiral stationary phases. 1-Phenylethylcarbamates also exhibited interesting chiral recognition. Optical resolving abilities of 3,5-dimethylphenylcarbamates of oligosaccharides were also evaluated.

In the past ten years, many chiral stationary phases (CSP) for high-performance liquid chromatography (HPLC) have been developed (1-3). CSP can be prepared from both optically active small molecules and polymers. These are usually chemically bonded or adsorbed on silica gel. In the chromatographic system with a CSP of a small molecule, the mechanism of chiral recognition can be estimated through spectroscopic studies on the interaction between the chiral compound used for CSP and the compound to be resolved. On the other hand, in the system with a CSP of a polymer, the understanding of chiral recognition mechanism in a molecular level is

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Cellulose tris(phenylcarbamate)

Amylose tris(phenylcarbamate)

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usually difficult because in many cases the exact steric structures of a polymer is not available. Nevertheless, polymeric CSPs are interesting because their chiral recognition depends on the higher-order structure of the polymer and unexpected high chiral recognition ability may appear due to the higher-order structure of the polymer. Polysaccharides such as cellulose and amylose are the most accessible optically active polymers. These polysaccharides themselves show rather low chiral recognition, but their derivatives, particularly carbamate derivatives exhibit high chiral recognition and can separate broad racemic compounds into optical isomers (2,4). In this article, optical resolution by tris(p-substituted phenylcarbamate)s of cellulose is briefly described, and then chiral recognition by 3,5-disubstituted phenylcarbamates of cellulose, and amylose, and their oligomers is discussed.

Optical resolving powers of aralkylcarbamates such as benzylcarbamate and 1-phenylethylcarbamate of the polysaccharides are also evaluated.

Experimental

Polysaccharide carbamates were prepared by the reaction of polysaccharides with corresponding isocyanate derivatives. ^{1}H-NMR and elemental analyses indicated that hydroxy group were almost quantitatively converted to urethane moieties. The derivatives were adsorbed on macroporous silica gel (particle size 7 or 10 μm, pore size 400 nm) which had been treated with 3-aminopropyltriethoxysilane; the weight ratio of the carbamate to silica gel was 25:100. Each of the packing materials obtained was packed in a stainless-steel tube (25 x 0.46 (id) cm) by a slurry method. Chromatographic analysis was performed on a JASCO TRIROTAR-II chromatographic equipped UV and polarimetric (JASCO DIP-181C) detectors using a hexane-2-propanol (90:10) mixture as an eluent at a flow rate of 0.5 ml/min at 25°C. Dead time (t_0) was determined with 1,3,5-tri-tert-butylbenzene. Most columns exhibited theoretical plate numbers between 3000-6000 for benzene.

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Resolution on 4-Substituted Phenylcarbamates of Cellulose

Optical resolving abilities of various 4-substituted phenylcarbamates (1) of cellulose have been evaluated (4-6). Table I shows the separation factors (α) for 9 racemic compounds (3-11) on eleven 4-substituted phenylcarbamates (**1a-k**). The substituents are arranged in the increasing order of electron-withdrawing power from left to right in the table. The optical resolving ability is influenced very much by the substituent introduced on the phenyl group. Phenylcarbamates **1a** and **1k** containing a hetero atom show very low chiral recognition. The best chiral discrimination is attained either alkyl-substituted or halogen-substituted phenylcarbamates. This results indicates that inductive effect of substituents influences the chiral recognition of CSP. t-Butylphenylcarbamate **1e** exhibits particularly high optical resolving power. In this case, sterical effect of t-butyl group may also play an important role.

A schematic hydrogen bond interaction between a phenylcarbamate group and the carbonyl or hydroxy group of a solute is depicted in Figure 1. Both NH and CO of the carbamate moiety can interact with a solute through hydrogen bond. The introduction of an electron-donating group like t-butyl probably increases the electron density of the carbonyl oxygen, which may facilitate the hydrogen bond on this oxygen. On the other hand, an electron-withdrawing substituent like halogen increases the acidity of NH proton. This has been confirmed by the down-field shift of NH resonance in ^{1}H NMR spectra of the cellulose phenylcarbamates (5). The increase of acidity of NH proton should enhance the capability of the hydrogen bond formation of this proton with an electron-donating group like carbonyl. Thus, the introduction of alkyl or halogen group is expected to raise the chiral recognition ability of cellulose tris(phenylcarbamate)s. Polar substituents like CH_3O and NO_2 themselves can interact

CH-OH, CF_3 (4); O, Ph, Ph (5); CONHPh, CONHPh (6); OH HO, CH_3 CH_3 (7)

3 4 5 6 7

$(C_6H_5)_3C$-CHOH, C_6H_5 (8); O, O, C_6H_5 (9); $Co(acac)_3$ (10); O, C_6H_5 (11)

8 9 10 11

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Table I. Separation factors (α) of racemates (3-11) on cellulose 4-substituted tris(phenylcarbamate)s (1a-k)[a)]

	a	b	c	d	e	f	g	h	i	j	k
	CH_3O	CH_3	CH_3CH_2	$(CH_3)_2CH$	$(CH_3)_3C$	H	F	Cl	Br	CF_3	NO_2
3	~1	1.48	1.11	1.17	1.74	1.37	1.14	1.16	1.19	1.23	~1
4	1.35	1.52	1.57	1.59	1.75	1.45	1.26	1.29	1.29	1.30	~1
5	1.34	1.55	1.55	1.43	1.27	1.46	1.38	1.68	1.70	1.61	1.33
6	1.00	1.35	2.12	2.14	2.24	1.45	1	1.44	1.17	1.22	~1
7	1.15	1.30	1.33	1.39	1.50	1.65	1.17	1.20	1.21	2.04	~1
8	1.00	1.37	1.59	1.47	1.36	1.22	1.64	1.95	1.95	1.48	1.00
9	~1	1.16	1.22	1.23	1.45	1.10	1.13	1.12	1.13	1.14	1.00
10	~1	1.75	1.76	2.46	2.50	1.24	1.53	1.46	1.79	2.06	~1
11	1.13	1.20	1.19	1.15	1.22	1.17	1.12	1.16	1.17	1.18	~1

a) Eluent: hexane-2-propanol (90:10).

α=(retention time of second-eluted isomer-t_0)/(retention time of first-eluted isomer-t_0)

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Figure 1. Interaction between carbamate moiety and carbonyl or hydroxy group of a solute.

1

X =

a: 4-CH_3O
b: 4-CH_3
c: 4-CH_3CH_2
d: 4-$(CH_3)_2CH$
e: 4-$(CH_3)_3C$
f: H
g: 4-F
h: 4-Cl
i: 4-Br
j: 4-CF_3
k: 4-NO_2
l: 3,5-(CH_3)
m: 3,5-Cl_2

2

X =

a: 3,5-$(CH_3)_2$
b: 3,5-Cl_2

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with a polar solute. Since the substituents exist at remote position from the chiral glucose unit, the interaction on these substituents can not efficiently discriminate enantiomers. Therefore, the introduction of a polar substituent is not suitable for the enhancement of chiral recognition ability of CSP.

When methyl or chloro group was introduced at 2- or 6-position of the phenyl group of cellulose tris(phenylcarbamate), the derivatives showed very low chiral recognition. These substituents may hinder the interaction between the urethane moiety of CSP and a solute. Most of the phenylcarbamates which have a high chiral recognition ability form a liquid crystalline phases. However, the phenylcarbamates with a substituent at 2- or 6-position do not form such a phase. This means that the phenylcarbamates without a substituents at 2-position probably take an ordered structure on silica gel, whereas those with a substituents at 2-position do not. Regular higher-order structure of cellulose tris(phenylcarbamate)s may also be important to attain efficient optical resolution.

Resolution on 3,5-Disubstituted Phenylcarbamates of Cellulose and Amylose

To increase the inductive effect, 3,5-dimethylphenylcarbamates and 3,5-dichlorophenylcarbamates of the polysaccharides were prepared and their optical resolving abilities were evaluated (Table II) (5,7). Most compounds are better resolved on one of the four CSPs than on the 4-substituted phenylcarbamates shown in Table I. The compounds carrying a hydroxy group, **4**, **7**, and **8**, are more retained on 3,5-dimethylphenylcarbamates **1*l*** and **2a** and

the compounds carrying a carbonyl group, **10** and **11** on dichloro derivatives **1m** and **2b**. These compounds may interact with the CSPs as depicted in Figure 1. Although the dichloro derivatives exhibit high optical resolving power for several compounds, the life time of the packed columns is short because the derivatives are soluble or swell in hexane containing more than 10 % of 2-propanol. The dimethyl derivatives are much less soluble in solvents and the packed columns are not damaged by use of a more polar eluting system such as hexane containing more than 30 % of 2-propanol. Enantioselectivity of **1*l*** and **2a** for a few racemic compounds is opposite. For instance, on **1*l*** (-)-isomer of **5** elutes first while on **2a** (+)-isomer elutes first. This means that both CSPs are useful and a variety of compounds can be resolved with these two.

Table II. Optical resolution on tris(3,5-dimethylphenylcarbamate) (**1*l*** and **2a**) and tris(3,5-dichlorophenylcarbmate)s (**1m** and **2b**) cellulose (**1**) and amylose (**2**)[a]

	1*l* (Me)		**1m** (Cl)		**2a** (Me)		**2b** (Cl)	
	k_1'	α	k_1'	α	k_1'	α	k_1'	α
3	0.97(+)	1.32	0.87(+)	1.65	0.53(+)	1.58	0.84(+)	1.34
4	2.13(-)	2.59	0.28(-)	1.38	1.30(+)	1.15	0.37	1.00
5	0.74(-)	1.68	0.56(+)	1.84	0.42(+)	3.05	0.50(+)	1.32
6	0.83(+)	3.17	0.59(+)	1.41	3.25(+)	2.01	0.59(-)	1.11
7	2.36(-)	1.83	1.62(+)	1.11	2.46(-)	2.11	1.10(+)	~1
8	1.37(+)	1.34	0.40(+)	1.29	2.65(+)	1.98	0.88(+)	2.25
10	0.42(+)	~1	0.76(+)	1.82	0.25(-)	~1	0.63(+)	~1
11	1.17(-)	1.15	2.65(-)	1.26	0.61(-)	~1	1.26(-)	~1

a) Eluent: hexane-2-propanol (90:10).
The sign in parenthesis represents optical rotation of the first-eluted isomer.

So far, the optical resolution of 483 racemic compounds on cellulose tris(3,5-dimethylphenylcarbamate) (**1*l***) has been examined in our group and 221 of them were completely resolved and 86 of them were partly resolved by showing two peaks with an overlap. Therefore, 64 % of 483 compounds are separable on **1*l***. We have also examined the optical resolution of 363 racemic compounds on amylose derivative **2a**, and 105 of them were completely resolved and 97 of them were partly separated. In this case, 56 % of 363 racemic compounds are separable. With two 3,5-dimethylphenylcarbamates, 182 compounds are separable only on **1*l***, 77 only on **2a** and 125 on the both. Totally, 343 (80 %) of 483 racemic compounds can be resolved at least on one of the CSPs.

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Chiral Recognition by 3,5-Dimethylphenylcarbamates of Oligosaccharides

Optical resolving abilities of 3,5-dimethylphenylcarbamates of cellooligosaccharide (12), maltooligoaccharide (13), and cyclodextrin (14) have been evaluated and compared with these of corresponding polysaccharide carbamates (8). The carbamates of cellobiose (12, n=2) and cellotetraose (12, n=4) shows chiral recognition quite different from that of cellulose derivative (Table III). For instance, elution order of enantiomers of 3 and 5 are reversed on the oligomer and cellulose derivatives. This suggests that the conformation of glucose units of the oligomers may be different from that of cellulose derivative. On the other hand, the carbamates of maltooligosaccharides except for dimer show rather similar chiral recognition (Table IV). The conformation of the carbamates of the oligomers (n=4 and 7) may be similar to that of the amylose derivative.

Cellooligosaccharide derivative (n = 2, 4) 12

Maltooligosaccharide derivative (n = 2, 4, 7) 13

Cyclodextrine derivative (n = 6, 7, 8) 14

R = 3,5-$(CH_3)_2C_6H_3$-NHCO-

Table III. Optical resolution on 3,5-dimethylphenylcarbamates of cellobiose, cellotetraose, and cellulose[a]

	Cellobiose		Cellotetraose[b]		Cellulose	
	k_1'	α	k_1'	α	k_1'	α
3	0.26(-)	1.37	1.54(+)	1.21	1.23(-)	2.61
4	0.25(-)	~1	2.13(-)	1.13	2.21(-)	1.15
5	1.38(+)	1.37	9.38	1.00	2.84(-)	1.39
9	0.29(+)	1.38	3.70(+)	~1	2.10(+)	1.20
11	0.25(-)	~1	not eluted		2.13(-)	2.59[c]

a) Eluent: hexane-2-propanol (98:2), 0.5 ml/min.
b) Eluent: hexane. c) Eluent: hexane-2-propanol (90:10).
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Table IV. Optical resolution on 3,5-dimethylphenylcarbamates of maltooligosaccharides and amylose[a)]

	n=2		n=4		n=7		amylose	
	k_1'	α	k_1'	α	k_1'	α	k_1'	α
3	0.59(-)	~1	2.26(+)	1.34	1.58(+)	1.07	5.63(+)	1.42
4	3.26(+)	~1	18.5(-)	1.54			1.30(+)	1.15[b)]
5	0.20(+)	~1	1.04(+)	1.11	0.74(+)	1.07	1.39(+)	4.29
9	0.94	1.00	5.05(+)	1.60	2.65(+)	1.28	0.93(+)	1.12[b)]
11	0.49	1.00	1.98(-)	1.06	1.19(-)	1.12	2.67(-)	1.10

Capacity factor: k_1=(retantion time of the first eluted isomer-dead time)/(dead time)
Separation factor: α=(capacity factor of the second-eluted isomer)/k_1
Sign of optical rotation of the first eluted isomer is shown in parenthesis.
a) Eluent: hexane-2-propanol (99:1), 0.5 ml/min.
b) Eluent: hexane-2-propanol (90:10).

Table V. Optical resolution on 3,5-dimethylphenylcarbamates of cyclodextrins and amylose[a)]

	α-CD[b)]		β-CD		γ-CD		Amylose	
	k_1'	α	k_1'	α	k_1'	α	k_1'	α
3	2.42(-)	~1	1.46(-)	1.15	0.95(-)	1.52	5.63(+)	1.42
4	2.49(-)	1.30	1.76(-)	1.22	1.36(+)	1.05	1.30(+)	1.15[c)]
5	1.00(+)	1.36	0.87(+)	1.37	0.66(-)	1.16	1.39(+)	4.29
10	not eluted		2.50(-)	1.15	1.83(-)	1.11	2.98(-)	1.11
11	not eluted		7.75	1.00	7.34(-)	1.10	2.67(-)	1.10

a) Eluent: hexane-2-propanol (99:1), 0.5 ml/min.
b) Eluent: hexane. c) Eluent: hexane-2-propanol (90:10).
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Chiral recognition by the 3,5-dimethylphenylcarbamates (14) of α-, β-, and γ-cyclodextrines (n=6,7, and 8) are quite different from that of the amylose derivative (Table V). The higher-order structure of the carbamates of cyclic oligosaccharides must be different from that of a linear polysaccharide amylose.

The existence of these structural differences and similarity between the carbamates of oligomers and polymers is also expected from the CD spectra of the carbamates (8).

Optical Resolution on Aralkylcarbamates of Cellulose and Amylose

Tris(alkylcarbamate)s such as methylcarbamate and cyclohexylcarbamate of cellulose show rather low chiral recognition. Optical resolving abilities of aralkylcarbamates

such as benzyl- (**15**), (±)-1-phenylethyl- (**16**), (±)-1-(1-naphtylethyl)- (**17**), and diphenylmethylcarbamtes (**18**) have been tested (Table VI) (9). Three carbamates **15**, **17**, and **18** did not efficiently separate 5 racemic compounds into optical isomers. Although four columns had similar theoretical plate numbers for benzene, **15**, **17**, and **18** exhibited broad peaks for the racemic compounds in Table VI. However, **16** show rather high optical resolving power for 4 compounds, and can separate also completely into enantiomers. Among four aralkylcarbamates, only **16** forms lyotropic liquid crystalline phase in tetrahydrofrane. Regularly-ordered structure of the cellulose carbamates seems to be essential to obtain an efficient CSP. Analogous results were also obtained on the four aralkylcarbamates of amylose. Only (RS)-1-phenylethylcarbamate (**20**) exhibited high optical resolving power (Table VII). Optical resolving ability of **20** is higher than that of **16** in most cases.

1-Phenylethyl group of **16** and **20** is chiral. The influence of chirality of this group on optical resolving ability was investigated. In case of **16**, (R) or (RS) derivative showed higher resolving ability than (S) derivative, and on **20**, (S) or (RS) derivative shows higher resolving ability (Table VIII). The compounds which can be better resolved on (S)-**20** than other polysaccharide carbamate including phenylcarbamates are shown in Figure 2.

OCONH-R OCONH-R OCONH-R
Cellulose derivative

OCONH-R OCONH-R OCONH-R
Amylose derivative

R = $-CH_2-C_6H_5$ $-CH(CH_3)-C_6H_5$ $-CH(CH_3)-C_{10}H_7$ $-CH(C_6H_5)-C_6H_5$

Cellulose	15	16	17	18
Amylose	19	20	21	22

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Figure. 2. Racemic compounds resolved on amylose tris((S)-1-phenylethylcarbamate).

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Table VI. Optical resolution on cellulose aralkylcarbamates (15-18)

	15		16		17		18	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
3	0.39(+)	~1	0.62(+)	~1	0.51	1.00	0.97(+)	~1
5	0.27	1.00	0.52(-)	1.12	0.62(-)	~1	0.50(-)	~1
9	2.57(-)	~1	3.18(-)	1.20	1.80	1.00	1.54(-)	1.08
10	0.57(+)	~1	0.61(+)	1.37	0.25(+)	~1	1.25(+)	~1
Benzoin	2.03	1.00	3.67(+)	1.18	4.22	1.00	3.58	1.00

The sign of optical rotation of the first-eluted isomer is shown in parentheses.

Table VII. Optical resolution on amylose aralkylcarbamates (19-22)

	19		20		21		22	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
3	0.66(+)	~1	0.72(+)	~1	0.79(+)	~1	1.01(+)	~1
5	0.43(+)	~1	1.68(+)	1.15	0.66(-)	~1	0.67(+)	1.09
7	1.00(+)	~1	1.69(-)	1.24	1.16(+)	~1	1.60(-)	~1
10	0.41(+)	~1	0.75(-)	~1	2.50(+)	~1	1.92(+)	~1
Benzoin	2.41(+)	~1	3.51(+)	1.41	3.33(+)	~1	4.11(+)	1.21

The sign of optical rotation of the first-eluted isomer is shown in parentheses.
Eluent: hexane-2-propanol (90:10).

Table VIII. Optical resolution of racemates on (R)-, (S)-, and (RS)-20

	(R)-20			(S)-20			(RS)-20		
	k'_1	α	Rs	k'_1	α	Rs	k'_1	α	Rs
3	0.74(+)	1.86	2.41	0.90(+)	2.38	4.43	0.72(+)	2.60	4.20
4	1.97(+)	1.05		1.95(-)	1.88	5.67	1.80(-)	1.14	0.86
5	0.61(+)	1.19	0.83	0.61(+)	1.28	1.52	1.68(+)	1.15	0.83
6	4.46(+)	1.18	1.01	4.79(+)	1.19	1.67	4.30(+)	1.24	1.91
7	1.93(-)	1.18	1.10	1.75(-)	1.31	1.69	1.69(-)	1.24	1.37
9	2.07(+)	1.07		3.02(-)	~1		2.13(+)	1.11	0.83
11	1.10(-)	~1		1.50(+)	1.21	1.68	1.18(+)	~1	

The sign of optical rotation of the first-eluted isomer is shown in parentheses.
Eluent: hexane-2-propanol (90:10).

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Chapter 6

Protein Column-Based Chiral Reversed-Phase Liquid Chromatography

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Great progress has been made in the field of direct optical resolution by liquid chromatography on protein-containing columns. A main contributing factor is due to improvements in immobilization technology and the production of crosslinked silica-bonded phases which are highly compatible with organic solvents, meaning that the columns can now be used with the same range of mobile phase systems as the common alkylsilica columns. A wide variety of pharmaceuticals and other biologically active compounds have now been resolved on such columns and certain guidelines for mobile phase selection have emerged. Precolumn derivatization of amino acids with various fluorogenic reagents has led to new methods for chiral amino acid analysis using BSA-based columns in the final step.

One of the fastest expanding areas of liquid chromatography concerns separation of enantiomers, which is also manifested by the growing literature in the field (*1-5*). The main interest has been focused on *direct* methods, *i.e.* methods by which the enantiomers are separated via the use of a chiral phase system. In liquid chromatography this means that either of the phases can be chiral. Thus, a number of systems have been described working with columns containing chiral stationary phases, as well as others which operate with conventional columns (containing an achiral stationary phase) and chiral mobile phases. The latter are usually produced by dissolving a chiral additive in the solvent used as the mobile phase. A majority of the research, however, has been directed towards new chiral stationary phases. These now range from the so-called "brush" type phases consisting of small homochiral molecules, immobilized onto the surface of the support, to the "biopolymer" phases, which are much more complex due to their supermolecular structures. To the latter type belong the protein phases, which have exhibited a broad enantioselectivity and proven to be useful for separation of the enantiomers of a variety of racemates,

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particularly of polar or charged compounds, such as many of the common pharmaceuticals. It is the purpose of this paper to highlight some of the most recent advances in the continuing research on enantiomer separation by liquid chromatography on protein phases.

Background

Already in 1958, as a result of studies of the binding of small ligands to proteins in solution, enantioselectivity in binding to bovine serum albumin (BSA) was observed (*6*). By the use of isotopically labeled compounds, it was then found that the affinity of L-tryptophan for BSA was roughly 100 times higher than that of the D-enantiomer. These results were verified in 1973 by the first affinity-type chromatographic separation of enantiomers described (*7*). In this single experiment, a column containing BSA immobilized to agarose was used for the separation of the tryptophan enantiomers in a discontinuous elution procedure. In 1982, it was demonstrated that such columns could be used in low-pressure LC-systems for separation of the enantiomers of a series of other compounds (other aromatic amino acids, a chiral sulfoxide and sulfimide, a N-benzoylamino acid) under isocratic conditions (*8,9*). These studies also revealed the strong influence of the mobile phase composition on retention and resolution. The technique seemed very promising and an improvement was made by the introduction of a silica-based BSA column for HPLC (*10*). In the same year another protein column, containing α_1-acid glycoprotein (orosomucoid), appeared (*11*). A third protein column (*12,13*), based on ovomucoid (another acid glycoprotein, isolted from hen's egg white) was reported in 1987. Quite recently, a column based on a cellulase obtained in a microbial process (cultivation of a mould, *Trichoderma reesei*) has been described (*14,15*). Three of the protein columns described are now commercially available from different manufacturers. A summary is given in Table I.

Table I. Analytical Columns for HPLC Based on Proteins

Protein	*Source*	*Trade name (manufacturer)*
BSA	Bovine plasma	Resolvosil (Macherey-Nagel GmbH, Düren, FRG)
AGP	Human plasma	Enantiopac (LKB, Bromma, Sweden) Chiral AGP (ChromTech, Norsborg, Sweden)
Ovomucoid (OVM)	Egg white (hen)	Ultron ES-OVM (Shinwa Kako, Kyoto, Japan)
Cellobiohydrolase (CBH-I)	*T. reesei*	------

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Immobilization Techniques

Due to the size of a protein and the large number of functional groups present, a variety of possibilities for immobilization exist. Since the loading capacity of protein columns in general is very low, it is essential to obtain a high degree of surface coverage with protein on the silica. Another important factor to consider concerns the contribution of the immobilization technique used to non-stereoselective binding. Since hydrophobic interaction makes a major contribution to retention on protein columns, any additional contributions from achiral structure elements of the stationary phase should be avoided. Therefore, the immobilization reagents should preferably not lead to an increased hydrophobicity of the chiral sorbent since this would lead to increased retention of the analyte with decreased enantioselectivity (separation factor) as a result. This seems to be a complex problem, however, and is not yet fully understood.

The simplest approach to immobilization is to couple the native protein to a suitably activated silica. By such a process a number of the free amino groups in the protein (probably those of lysine side chains) are engaged. It has been found, however, that the stability of the sorbent is increased if the protein is crosslinked during the immobilization procedure. In the case of the glycoproteins (AGP, OVM, CBH-I) this can be carried out without the use of any external crosslinker since oxidation with periodate yields aldehyde groups in the carbohydrate moiety which then react with primary amino groups of the protein giving easily reducible imino functions. If the silica matrix has been activated to contain either amino- or aldehyde groups, the process will simultaneously anchor the crosslinked protein to the silica.

Albumins, on the other hand, require a crosslinker, *i.e.* a bifunctional reagent. We have recently studied immobilization of BSA to silica under various conditions using glutaraldehyde (GA) and N,N'-disuccinimidyl carbonate (DSC), respectively. The chromatographic properties of the resulting sorbents were then compared under identical conditions. First, immobilization to silica of varying pore size gave the result that a 100 A material was optimal with respect to the amount (215 mg/g silica) of protein bound (*16*). Secondly, large differences in sorbent properties, depending on the immobilization method used, were found (*17*). Some of the main results are summarized in Table II.

It is likely that the introduction of further positive charges in sorbent II causes a large increase in retention of acidic compounds while uncharged analytes are less influenced. The effect on the separation factors is, however, more difficult to rationalize. One might conclude, though, that different immobilization techniques can create different microenvironments at the chiral binding sites of the protein, resulting in an overall change in enantioselectivity. Such an assumption is not unreasonable since it has been shown previously that physically immobilized BSA can act quite differently from covalently bound. For example, while columns of the latter type have been used to separate the enantiomers of the drug Omeprazole (trade mark of AB Hässle, Sweden) under various conditions, the resolving property is lost in columns containing adsorbed BSA (*18*). The most plausible interpretation of this phenomenon is that in the adsorbed protein, the stereoselective binding site for this

particular analyte is blocked or sterically unaccessible due to the conformation attained by the protein upon adsorption.

Table II. Retention Data from BSA-Sorbents Showing the Influence of the Procedure Used for Immobilization

A. BSA crosslinked with GA. Immobilization to 100A silica;
I: non-functionalized, II: 3-aminopropyl-functionalized. Mobile phase: Phosphate buffer (50 mM, pH 7.6) with 4% of 1-propanol

Analyte	*Sorbent I*			*Sorbent II*		
	k_1'	k_2'	α	k_1'	k_2'	α
D,L-Tryptophan	0.76	1.88	2.47	1.41	7.53	5.34
DNP-D,L-Aspartic acid	5.85	19.0	3.25	21.1	47.1	2.23
Oxazepam	9.00	28.4	3.16	9.68	41.1	4.25

B. BSA immobilized to 100A 3-aminopropyl silica;
II: With GA, III: With DSC. Mobile phase: Borate buffer (50 mM, pH 8.3) with 2% of 1-propanol

Analyte	*Sorbent II*			*Sorbent III*		
	k_1'	k_2'	α	k_1'	k_2'	α
Bendroflumethiazide	10.9	17.3	1.59	4.5	5.85	1.30
Benzoin	3.75	6.75	1.80	1.6	2.2	1.38
D,L-Kynurenine	0.77	2.67	3.47	0.83	5.1	6.14

Chromatographic Properties and Use

Since the protein phases are charged as well as hydrophobic, there are numerous ways to regulate retention and resolution of the enantiomers of an analyte. The crosslinked protein phases are highly resistant to protein-denaturing organic solvents (*16,19*) and can therefore be used under conditions similar to those applied in reversed-phase LC on alkylsilica columns. Retention and resolution are regulated via the mobile phase composition, where the pH of the buffer used, the ionic strength and the nature and concentration of the organic modifier are the most important factors. Charged, amphiphilic organic modifiers are often very effective at low concentrations for charged analytes (*20-22*). Recent applications of computer-based optimization techniques using multivariate analysis strategies, particularly a modified sequential simplex optimization approach, have facilitated certain separation problems to a large extent (*5,23*).

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A wide variety of racemates, including numerous pharmaceuticals, have so far been successfully resolved on protein columns, mainly with the use of the BSA- and AGP-based types which have been available since 1983. A fairly exhaustive review is given in ref. 4. Both of these column types have been used for uncharged as well as charged analytes, in the latter case also with the use of ion-pairing mobile phase additives (*24*) which can drastically change the retention behaviour. Empirical results, however, seem to indicate a slight difference in the applicability of these two columns; the BSA-type being most useful for anionic analytes (carboxylic acids), whereas the AGP-type should be more favourable for cationic compounds (amines). Whether this is correlated to the lower isoelectric point of the AGP ($\Delta pI = 2$), caused by the sialic acid content, is not yet clear. It is interesting, however, that the two newer phases, based on OVM and CBH-I, both contain acid glycoproteins. These phases are both very useful within the field of cationic analytes (α-values obtained on CBH-I for three common β-blockers: 4.4 (metoprolol), 6.9 (propranolol), 8.3 (alprenolol). The relation between protein structure and resolving ability is, however, an extremely complex subject which will require much more study. Quite recently, experiments with OVM-phases have indicated that the sugar part is essential for the chiral recognition of many analytes, whereas the sialic acid moiety only affects retention and not the chiral resolution (*25*). An example on recent resolutions of an anionic (Etodolac) and a cationic (Mefloquin; one of the two diastereomers) drug on a BSA-based column (S. Andersson, unpublished data) is shown in Figure 1.

The BSA-columns show a remarkable ability to resolve N-substituted amino acids, particularly various N-acyl derivatives. In a search for highly fluorescent derivatives, which should permit detection of very small amounts of an enantiomeric contaminant, we have recently investigated a series of amino acid derivatives obtained via a reaction with a new reagent, N-(chloroformyl)-carbazole (*26*). Derivatives prepared with the use of fluorescein isothiocyanate (FITC, isomer I) and FMOC-Cl were also studied (Scheme 1). Due to the extremely high detection sensitivity obtained with FITC-derivatization, this technique of chiral amino acid analysis shows great promise. Some typical chromatograms are shown in Figure 2. The very mild conditions used in reaction c) (Scheme 1), however, make this an even more attractive route. Further, these derivatives (CC) often showed large α-values. The pronounced effect from the side chain structure on retention is evident from the data given in Table III. Many of the separation factors (*e.g.* of methionine and phenylalanine) can be increased considerably by increasing the methanol content of the mobile phase which also leads to faster elution. Further work in this field is still in progress.

The role of an aromatic group in the structure of the compound to be resolved is interesting since no resolution of aliphatic racemates on protein columns can be found in the literature. With the use of an aliphatic amino acid having different radioisotopes in its two enantiomers, we recently investigated its optical resolution, after acylation with an aliphatic acid chloride, using on-line liquid scintillation counting in a flow cell. The instrumentation permitted simultaneous monitoring of both radioisotopes (^{3}H and ^{14}C). The results show that an aromatic group is not necessary for enantiomer discrimination by the protein, since a number of simple N-acyl derivatives of the amino acid used (aspartic acid) were resolved with quite

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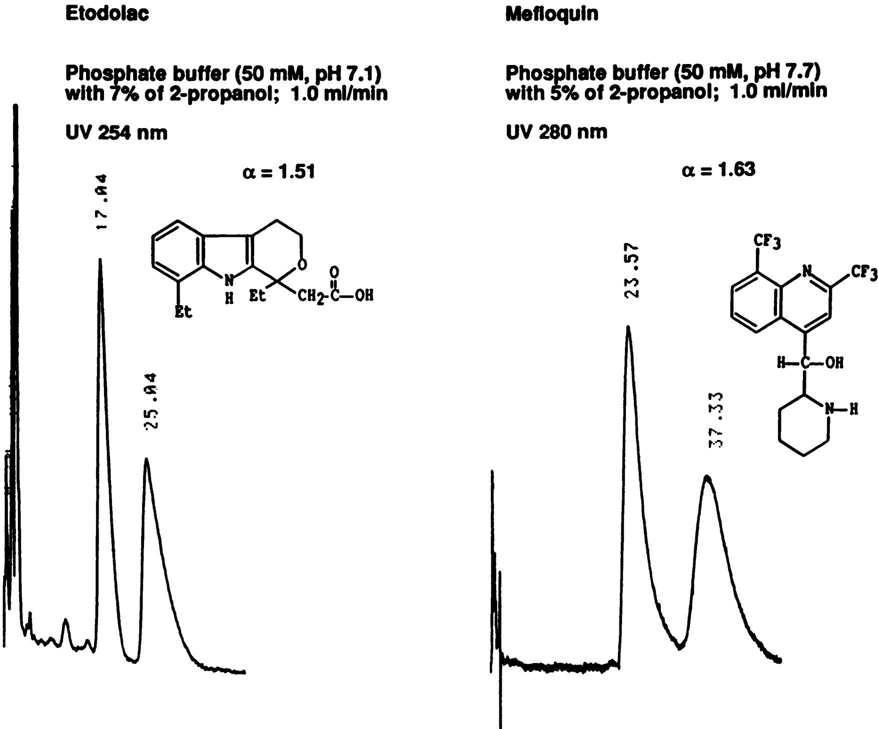

Figure 1. Separation of the enantiomers of Etodolac (left) and Mefloquin (right) on a BSA-column (4.0x125 mm).

a)

b)

c)

Scheme 1. Reactions used to obtain fluorescent N-derivatives of amino acids.

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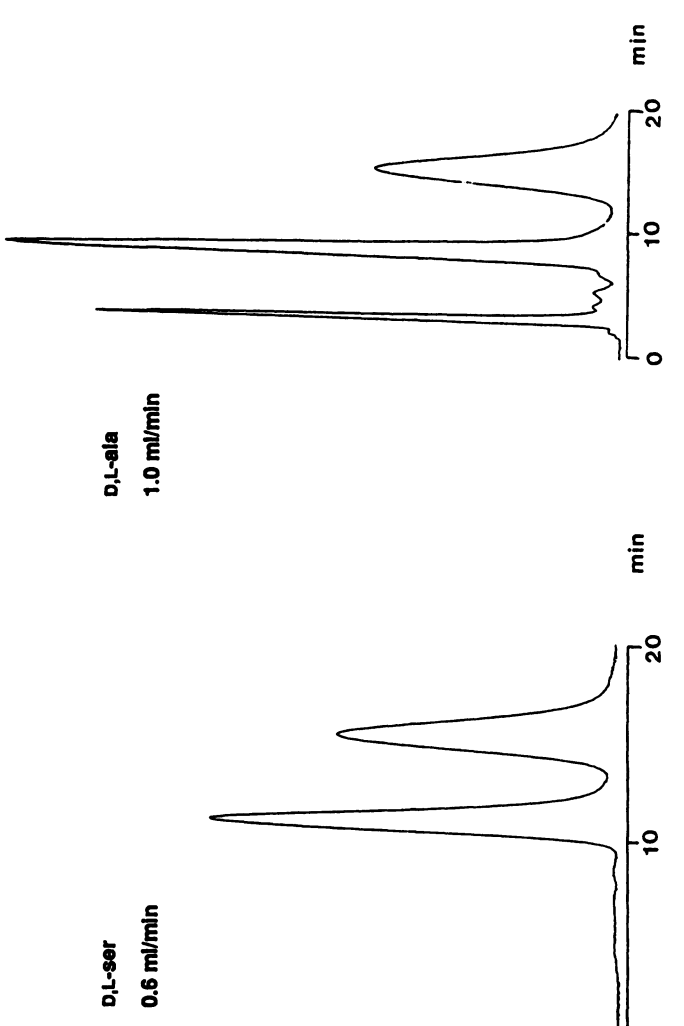

Figure 2. Chromatograms showing the chiral separation of D,L-serine and D,L-alanine as FITC-derivatives. BSA-column. Mobile phase: Phosphate buffer (50 mM, pH 8.2) with 10% of methanol. Fluorescence detection (488/520 nm).

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significant α-values (Table IV). No resolution could be achieved, however, of free aspartic acid, which eluted close to the solvent front, under any of the conditions used. Although isotopic substitution can influence rentention to some extent (*e.g.* deuterium isotope effects sometimes shown in gas chromatography) the separation factors given in Table IV are much too large to be caused by isotope differences. A typical chromatogram is shown in Figure 3. The apparent peak broadening is caused by the large detector cell (500 µl) used.

Table III. Substituent Effects on Retention and Resolution of CC-Amino Acids
BSA-column; phosphate buffer (50 mM, pH 8.0), 30% of methanol

Amino acid side chain	k_1'	k_2'	α
CH_2OH	5.84	8.92	1.53
CH_3	5.12	15.0	2.93
$CH(CH_3)_2$	8.93	13.2	1.48
$CH(CH_3)OH$ (threo)	3.59	11.6	3.23
$CH_2CH_2SCH_3$	21.8	25.3	1.16
$CH_2CO_2^-$	7.94	10.0	1.26
$CH_2CH_2CO_2^-$	3.07	4.20	1.37
$CH_2C_6H_5$	28.8	107.4	3.73

Table IV. Separation Factors Obtained for a Series of Aliphatic N-Acylaspartic Acids

Acyl group	Mobile phase (Phosphate buffer)	α
CH_3CO	10 mM, pH 5.7	1.29
CH_3CH_2CO	25 mM, pH 5.4, 10% CH_3OH	1.17
$(CH_3)_2CHCO$	-"-	1.13
$ClCH_2CO$	25 mM, pH 5.4, 5% CH_3OH	1.22
$BrCH_2CO$	-"-	1.19

Since protein columns are operated in the reversed-phase mode, retention and optical resolution possible to optimize via the mobile phase composition, and a wide spectrum of racemates of varying structure and polarity can be baseline resolved, they should be ideal tools for bioanalytical work such as studies of pharmacokinetics of racemic drugs. To a certain extent, however, such applications have thus far been hampered by the rather moderate column efficiency. This leads to a reduced sensitivity and sometimes lack of resolution from interfering peaks in the chromatograms. A certain remedy to such problems has recently been found through the application of column enrichment techniques (27). Via a system of coupled

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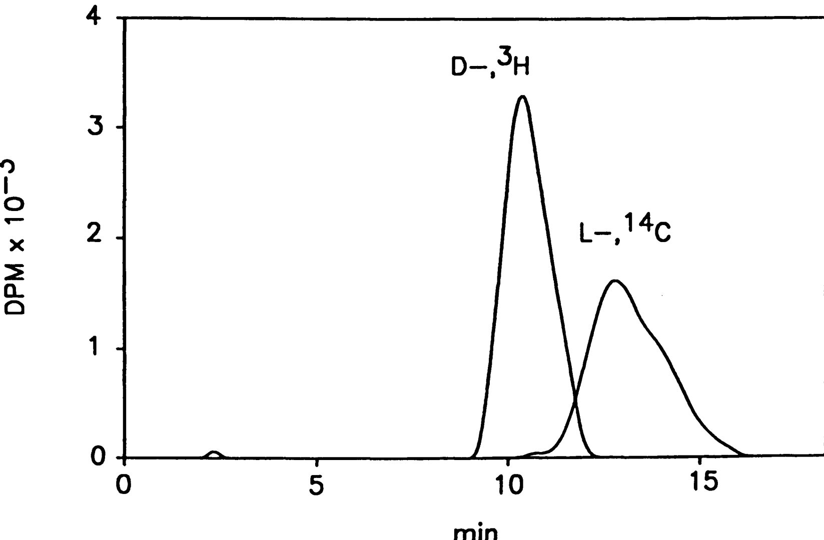

Figure 3. Separation of the radiolabeled enantiomers of N-acetyl-D,L-aspartic acid. BSA-column. Mobile phase: Potassium phosphate buffer (10 mM, pH 5.7). 1.0 ml/min.

columns, the two separated enantiomers from a protein column can each be introduced on separate alkylsilica (C-18) columns. This yields a peak compression upon backflushing and reelution from an achiral analytical column. The sensitivity enhancement can be substantial.

Conclusions

Proteins are highly versatile chiral stationary phases for analytical columns operating in the reversed-phase mode. Immobilization techniques based on crosslinking of the protein yield stable chiral sorbents which are compatible with high concentrations of mobile phase cosolvents, such as methanol or acetonitrile. In many cases, enantiomeric separation factors have been found to increase with increasing concentration of organic modifier due to a larger reduction of the capacity ratio of the first eluted enantiomer. Although enantioselectivity is preserved in most cases, different methods for immobilization of the protein will generally cause large differencies in the retention properties of the chiral sorbent. A broad enantioselectivity is not a unique property of proteins from animal sources, since recently a microbially produced acid glycoprotein with cellulase activity was found to optically resolve a number of important racemic pharmaceuticals with quite inpressive separation factors.

A new method for chiral amino acid analysis, based on fluorogenic labeling with N-(chloroformyl)-carbazole followed by separation of the individual amino acid derivatives on a C-18 column and reanalysis of the collected fractions on a BSA-column, is under development. There are no requirements of aromatic structures in an analyte for enantioselectivity, since a series of aliphatic N-acylamino acids have been resolved on a BSA-column with α-values ranging between 1.1 and 1.3.

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Chapter 7

Optimization of Chiral Separations on Silica-Bonded α_1-Acid Glycoprotein by Mobile Phase Additives

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Chiral separations on silica–bonded α_1–acid glycoprotein can be controlled by pH as well as charged and uncharged addivites in the mobile phase. Optimization of resolution and sensitivity can be based on a simple multivariate design. Examples show the use of three variables at most for the separation of cationic and anionic enantiomers. Further improvements of the chiral selectivity can be achieved by chiral anionic and cationic additives. The effects indicate interactions with several sites, binding by cation–exchange and ion–pairing mechanisms. Peak symmetry and resolution can be improved by increased flow rate and considerable improvements have also been achieved by solvent and pH gradients.

The rapid development of the chiral separations during the last decade has been achieved by many different chromatographic techniques. The theoretical basis for the separation process is incompletely known in many cases and the methodology is then mainly based on practical experiences. The empirical approach is particularly justified when the selector has several binding sites of different character and can be used for enantiomers of highly different structures as is the case with α_1–acid glycoprotein (AGP).

Control of Retention. Silica–bonded AGP (CHIRAL–AGP) can be applied for separation of charged as well as uncharged enantiomers. It is an acidic protein, isoelectric point 2.7, containing anionic as well as

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uncharged, hydrogen-bonding sites *(1)*. The retention and the stereoselectivity can be varied within a wide range by such properties of the mobile phase as pH and content of charged and uncharged organic components *(2)*. The effect of these changes is highly dependent on the structure of the analyte. Studies of the effects of additives on the enantiomers of a solute have indicated binding to several sites that are affected differently by the additive *(3)* and even a reversal of the retention order can be obtained *(4)*.

Chiral separations for analytical purposes should give not only a complete resolution but also such a low retention and small peak width that a sufficient detection sensitivity can be achieved. An optimization with so many variables and such a goal can be very time-consuming if the "one variable at a time" method is applied. A more efficient approach is the use of factorial design which gives a maximum of information from a limited number of experiments. This method was used in the studies of the effects of the mobile phase components.

The aim of the study was to develop methods based on a simple multivariate design for optimizing chiral separations on silica-bonded AGP. Special techniques for improving the stereoselectivity and the peak width are also presented.

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Experimental.

Apparatus. The chromatographic system consisted of an LKB-pump, Model 2150 (Bromma, Sweden), a Rheodyne 7010 injector with a 20µl loop and a Spectroflow 783 variable-wavelength detector (Kratos, Ramsey, NJ. USA). The separation column, injector and connecting tubes were thermostated using a RM6 (Messgeräte-Werk Lauda, Lauda-Köningshofen F.R.G.). The chromatograms were recorded on a Perkin Elmer 56 instrument or an SP 4270 integrator (Spectra-Physics, San José, CA, U.S.A.).

Chromatographic Conditions and Chemicals. The separation column was a CHIRAL-AGP (100 x 4.0 mm ID., 5µm) from ChromTech (Stockholm, Sweden). The flow-rate was 0.4 ml/min and the system was thermostated at 22°C. The mobile phases were phosphate buffers, if not otherwise stated (ionic strength, $\mu = 0.05$) to which modifiers were added. The wavelength of detection was the UV-adsorption maximum. Atropine sulphate, homatropine bromide and sodium octanoate were purchased from Merck (Darmstadt, F.R.G.). Metoprolol succinate was synthesized at AB Hässle, Mölndal, Sweden. 1-(2-hydroxyphenyl)-2-(*tert.*-butylamino)ethanol (2HPE), 1-(4-hydroxyphenyl)-2-(*tert.*-butylamino)ethanol (4HPE) and ipratropine were kindly supplied by AB Draco (Lund, Sweden). 4-Phenylbutyric acid, R-and S-forms of 3-phenylbutyric acid and the racemate of 2-

phenylbutyric acid were purchased from Fluka (Buchs, Switzerland). The S– and R–forms of 2–phenylbutyric acid were purchased from Sigma (St. Louis, U.S.A.).

Factorial Design. Each of the variables is used at two levels which are assumed to have different effects on the chiral selectivity. A complete factorial design with n variables will then need 2^n experiments.

The effects of the mobile phase components can be estimated by a statistical treatment of the results obtained *(5)*. A model describing the response (retention or stereoselectivity) with three variables can be expressed as

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 + \varepsilon$$

β_n describes the effect of variable x_n and β_{nm} describes the combined effects of variables x_n and x_m. The ß–coefficients are calculated by multiplying the level of each variable with its response, adding the products and dividing the sum by the number of experiments. The variables are given two levels: –1 and +1.

In a system with three variables, x_1, x_2 and x_3, the composition of the mobile phases, expressed as levels, are given in Table I.

Table I. Composition of Mobile Phases Expressed as Levels

Expt. No	x_1	x_2	x_3
1	–1	–1	–1
2	–1	–1	+1
3	+1	–1	+1
4	+1	–1	–1
5	–1	+1	–1
6	–1	+1	+1
7	+1	+1	+1
8	+1	+1	–1

Results and Discussion.

Binding Processes. Previous retention studies with charged additives have indicated that AGP can bind solutes by charged as well as uncharged sites *(3)*. When the binding site is charged the solid phase can act as an ion–exchanger. The number of charged groups changes with pH and the binding ability is also affected by mobile

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phase ions with the same charge as the solute. Counter ions will not have a direct effect on the binding to the charged sites.

An uncharged site can bind a charged solute as an ion pair with mobile phase ions and the retention is affected by the concentration and the nature of counter ions as well as ions with the same charge as the solute *(6)*. Weak protolytes might also be distributed to the solid phase in uncharged form and the actual pH range depends on the pK_a–values of the solute as well as on its hydrophobicity.

The effect of charged mobile phase additives is highly dependent on the structure of the solute and even small differences in structure can give rise to considerable changes in retention and stereoselectivity. Hence, it is important to use such experimental methods that the effects of the mobile phase additives can be evaluated.

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Effect of Mobile Phases Expressed as Separation Factors. A study was performed with three variables in the mobile phase, pH, 2–propanol and sodium octanoate. The mobile phases were designed as shown in Table I:

x_1 = pH in the mobile phase: 6.0 (–1) and 7.5 (+1)
x_2 = concentration of 2–propanol: 0% (–1) and 3% (+1)
x_3 = concentration of octanoate: 0 mM (–1) and 4 mM (+1)

The results obtained with two esteralcohols, atropine and homatropine, are shown in Figure 1. The only structural difference between the two solutes is a CH_2 group in the substituent at the chiral center but despite the similarity the chiral separations are affected quite differently. Atropine can only be separated into enantiomers in the presence of octanoate whereas the enantiomers of homatropine are easily separated without additives and both octanoate and 2–propanol have negative effects on the stereoselectivity.

Chiral separations of two arylsubstituted alkanolamines are shown in Figure 2. The only structural difference between them is the position of a phenolic substituent. 2HPE is easily separated in most of the mobile phases. 4HPE on the other hand requires pH 7.5 and octanoate in the mobile phase.

Effects of the Variables Expressed as ß–Coefficients. If the aim of the study is selecting the best of the eight systems tested, the problem is solved by combining the results in Figures 1 and 2 with the corresponding k'–values. However, it is of greater interest to find a way to use the chromatographic data for optimizing resolution and retention even though such conditions are not covered by the phases included in the study.

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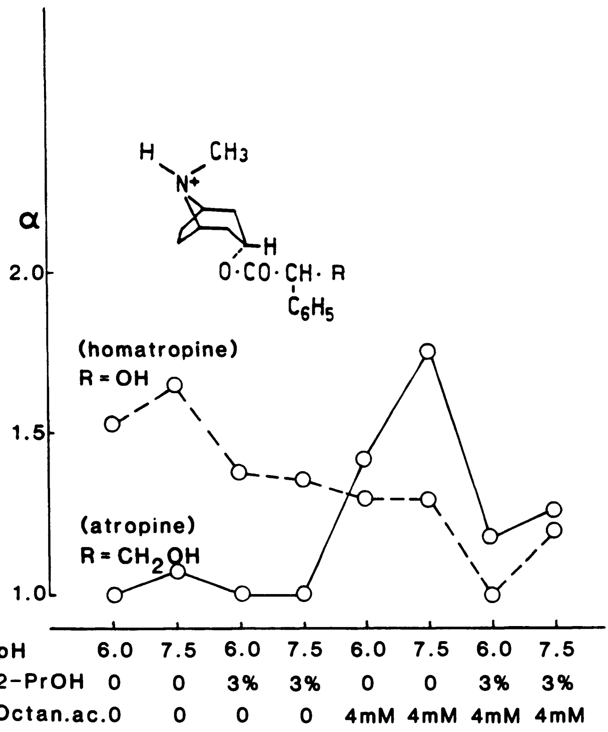

Fig. 1. Separation factors: dependence on mobile phase composition

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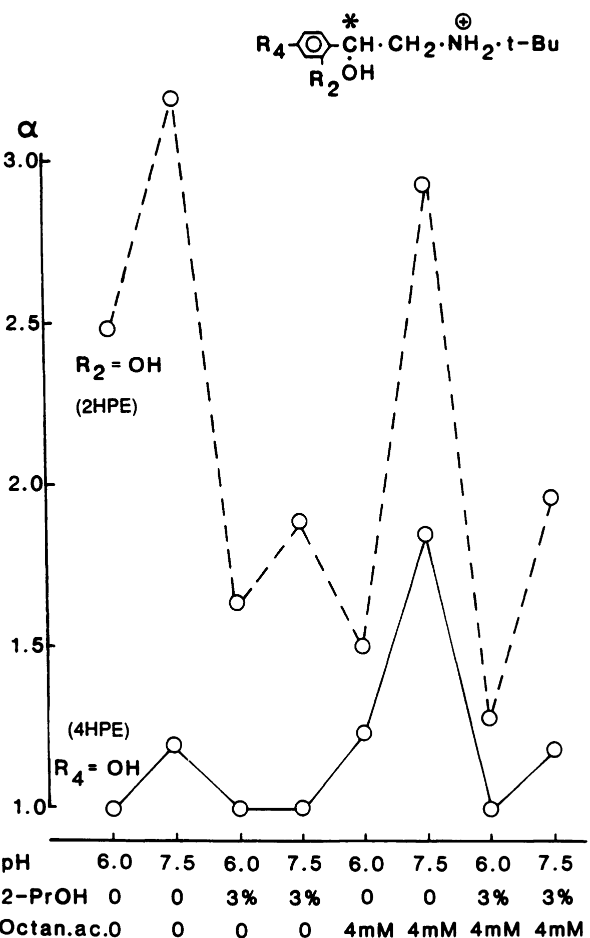

Fig. 2. **Separation factors: dependence on mobile phase composition**

For this task it is necessary to have such expressions for the effect of each of the variables as the ß-coefficients. The values calculated for the solutes in Figures 1 and 2 are summarized in Table II. It must be emphasized that the magnitude of a ß-coefficient depends on the range of the variable as well as on the magnitude of the response. Hence, the ß-coefficient can not be used in a quantitative comparison of the effect of a variable on different solutes.

The variance of the coefficients has not been calculated. However, the experimental variation has been estimated by normal probability plots and effects within the variation range are indicated in the Table as non-significant.

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Table II. Effects of Variables Expressed as β-Coefficients

Solute		$ß_{pH}$	$ß_{pr}$	$ß_{oc}$	$ß_{pH-pr}$	$ß_{pH-oc}$	$ß_{pr-oc}$
Atropine	k'_2	8.9	-8.3	1.8	-6.7	2.2	-1.5
	α	0.06	-0.10	0.20	-0.04	0.04	-0.08
Homatrop.	k'_2	7.1	-5.4	-1.2	-4.1	-0.7	1.1
	α	0.04	-0.11	-0.15	0.01*	0.01*	0.01*
4HPE	k'_2	1.9	-1.5	0.8	-1.2	0.7	0.6
	α	0.13	-0.14	0.13	-0.08	0.08	-0.09
2HPE	k'_2	21	-22	-11.6	-18	-7.4	10.9
	α	0.39	-0.42	-0.19	-0.15	0.14	0.12

pr = 2-propanol; oc = octanoate; * non-significant effect.

Table III. Optimization of Resolution and Retention Using β-Coefficients

Purpose	R_s	Variables should give		
		$ß(\alpha)$	$ß(k'_2)$	$ß(\alpha) / ß(k'_2)$
Increase of resolution	<1.5	positive	low (numerical)	high (numerical)
Reduction of retention	>>1.5	low (numerical)	negative	low (numerical)

The ß–coefficients can be applied to finding ways for improving the resolution when R_s<1.5 as well as to reduce retention for improving the detection sensitivity when R_s>>1.5. The principles are summarized in Table III. Both ß for α, ß(α), and ß for k'_2, ß (k'_2), are used. The application can be illustrated by the following examples.

Atropine has R_s=0 and k'_2=4.9 at pH 6.0 in the absence of additives. The way to an increase of the resolution is clearly indicated by the ß–coefficients: octanoate gives a maximum for ß (α) / ß (k'_2). A mobile phase with pH 6.0 and containing 3 mM octanoate gives R_s=2.0 and k'_2=3.4.

Homatropine has R_s=2.5 and k'_2=4.7 at pH 6.0. A reduction of k' by 2–propanol or octanoate seems unfavourable since the negative ß(α) indicate a fairly strong reduction of α. A small reduction of pH below 6.0 might be better as ß (α)/ß(k'_2) is very low. For the combined effects of pH and 2–propanol it is even lower but it must be difficult to find optimum conditions if two variables with opposite effects on k' are combined.

4HPE has R_s=0 and k'_2=1.1 at pH 6.0. An improvement of R_s can be achieved in different ways since both pH and octanoate give fairly high ß (α). Octanoate seems preferable since ß (k'_2) is low. However, even though α increases the resolution is incomplete owing to the low retention. On the other hand pH 7.5 gives R_s=1.8 and k'_2=5.6. and k'_2=18. A strong reduction of the retention can obviously be achieved by 2–propanol and it is accompanied by a reduction of α as can be seen from negative ß–coefficients. 3% 2–propanol gives R_s=2.0 and k'_2=1.1. A reduction of pH below 6.0 might have similar effects.

The effects of the variables on an anionic solute, 2–phenylbutyrate, could not be studied by these mobile phases as both 2–propanol and octanoate decrease k' so strongly that no stereoselectivity could be observed on any of the levels of the variables. However, it was found that an increase of pH from 6.0 to 7.5 gave an increase of α and a decrease of k'.

Effect of Cationic Additives. The studies comprised two variables, pH and a hydrophobic cation, tetrabutylammonium (TBA) and dimethyloctylamine (DMOA), respectively. Two of the solutes were cationic, 2HPE and homatropine, one anionic, 2–phenylbutyrate. The effect of the variables on the stereoselectivity is shown in Table IV.

The effects of pH are in agreement with the results for the cations given in Figures 1 and 2 and it is also obvious that the stereoselectivity increases with pH for 2–phenylbutyrate.

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Table IV. Effect of Variables on Retention and Stereoselectivity

Solute		Variables					
		pH 6.0			pH 7.5		
		–	TBA	DMOA	–	TBA	DMOA
2–phenyl–	k'_2	2.00	0.99	2.64	0.30	0.21	0.88
butyrate	α	1.51	1.59	1.97	1.95	1.84	5.70
2HPE	k'_2	4.40	1.84	2.80	31	6.0	13.1
	α	1.86	1.62	1.79	2.58	1.87	2.28
Homatrop.	k'_2	3.94	1.89	2.60	23	6.5	12.3
	α	1.24	1.25	1.27	1.42	1.49	1.48

Mobile phase: 3 mM additive + 1 % 2–propanol in phosphate buffer.

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The cationic additives have negative effects on k' of the cationic solutes whereas the influences on α are fairly small. TBA and DMOA have quite different effects on 2–phenylbutyrate. TBA decreases k'_2 and has only a minor influence on α. DMOA increases k'_2 which is the typical effect of a counter ion in an ion–pair retention process and there is a dramatic increase of α at pH 7.5. It seems that the hydrogen–bonding ability of DMOA is essential for an ion–pair retention.

When only two variables are used the experimental results are so few that an application of ß–coefficients has no advantage. The optimization of the separation is illustrated in some examples.

Homatropine has R_s=1.2 at pH 6.0. The resolution can be improved by pH and DMOA which have about the same effect on α. However, pH has a rather strong positive effect on k' whereas DMOA has a negative influence. 3 mM DMOA at pH 6.0 gives R_s=1.5 and k'_2=2.6.

2HPE has R_s=4.8 at pH 6.0. A decrease of R_s can be obtained with both DMOA and TBA but the latter has a stronger effect. 3mM TBA at pH 6.0 gives R_s=2.8 and k'_2=1.9.

2–Phenylbutyrate has R_s=2.8 at pH 6.0. A decrease of the resolution can be achieved by addition of TBA. It has a low effect on α and gives a decrease of k'. 3 mM TBA at pH 6.0 gives R_s=2.0 and k'_2=1.0.

Influence of Buffer Components. Not only hydrophobic counter ions such as octanoate but also hydrophilic buffer components can affect retention and stereoselectivity. Some examples are given in Table V.

Table V. Influence of Buffer Anions on Retention and Stereoselectivity

Solute	Buffer	Additive	k'_1	α
Homatropine	Acetate	–	1.94	1.41
– " –	Phosphate	–	2.66	1.34
– " –	Acetate	(S)–2–phenylbutyr.	4.32	1.0
– " –	Phosphate	– " –	3.03	1.40
Atropine	Acetate	–	2.11	1.06
(–)–Atropine	Phosphate	–	2.82	–
Atropine	Acetate	(S)–2–phenylbutyr.	2.74	3.90
– " –	Phosphate	– " –	3.22	3.00

Mobile phase: 0.5 mM additive and 1% 2–propanol in buffer pH 6.0, ionic strength 0.05.

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The effects of the buffer anions is apparent and it is maintained in the presence of another anionic additive which indicates that all the anionic components participate in the retention process. The effects can be rather drastic: homatropine has a fairly high separation factor with a mobile phase of (S)–2–phenylbutyrate in phosphate buffer, but the stereoselectivity is lost when the buffering is made with acetate. It is likely that the effects of the buffering anions are due to an ion–pair binding process to an uncharged site.

Influence of Chiral Additives. The effects of anionic additives on the chiral separation of cationic solutes is highly dependent not only on structure, but also on configuration as demonstrated in Table VI. The different effects of enantiomeric counter ions indicate binding by a stereoselective process probably to an uncharged chiral site.

Table VI. Influence of Chiral and Non–Chiral Phenylbutyrates on the Stereoselectivity

Additive	Atropine		Homatropine	
	k'_1	α	k'_1	α
–	2.24	1.0	1.97	1.34
(S)–2–phenylbutyrate	2.71	3.34	2.46	1.58
(R)–2–phenylbutyrate	1.81	1.57	1.94	1.08
(S)–3–phenylbutyrate	3.27	1.38	3.24	1.05
(R)–3–phenylbutyrate	3.17	1.15	2.98	1.16
4–phenylbutyrate	2.24	1.16	2.28	1.17

Mobile phase: 0.5 mM additive and 1 % 2–propanol in phosphate buffer pH 6.0.

It is of interest to note that quaternized derivatives of atropine can not be separated into enantiomers on the AGP–phase even in the presence of a chiral phenylbutyrate. Quaternized homatropine, on the other hand, gives an even higher separation factor than homatropine.

Further illustrations of the interaction of a chiral counter ion are shown in Figure 3. (R)–2–phenylbutyrate increases the retention of the second eluted enantiomer of atropine, (+)–atropine, whereas there is a decrease of the retention of the first eluted enantiomer. The two enantiomers of homatropine are also affected differently but no increase of the retention occurs.

The interaction of (R)–2–phenylbutyrate with (+)–atropine has the characteristics of an ion–pair binding process whereas the influence of the counter ion on (–)–atropine is mainly competitive. It clearly indicates that the retention processes for the two enantiomers are different and previous observations using indirect detection *(3)* have given the same conclusion. The opposite retention changes for the two enantiomers of atropine can be due to the presence of different binding sites for the diastereomeric ion pairs with (R)–2–phenylbutyrate. The existence of at least two chiral sites on the solid phase has previously been shown by the observation that octanoate as counter ion can give rise to a reversal of the retention order between cationic enantiomers *(4)*.

Drastic changes of the stereoselective separations of cationic solutes can also be obtained with chiral cationic additives. The effect of (–)–atropine as additive is demonstrated in Table VII.

Table VII. Influence of (–)–Atropine on Retention and Stereoselectivity of Cationic Solutes

(–)–Atropine (mM)	Ipratropine			Homatropine			Atropine (rac.)	
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2
0	4.60	–	1.0	2.66	3.56	1.34	2.82	–
0.55	2.42	3.54	1.46	2.01	2.56	1.27	1.57*	2.12

Mobile phase: (–)–atropine and 1 % 2–propanol in phosphate buffer pH 6.0; * system peak.

(–)–Atropine has a competitive effect on all the cations but the enantiomers are affected differently. This gives rise to a considerable improvement of the chiral separation of ipratropine, a quaternized atropine, whereas the effect on homatropine is the opposite. The results indicate binding to two sites, which are affected differently by the competitive chiral ion. The decrease of the retention of (+)–atropine gives support to the view that both sites are affected.

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Peak Width and Asymmetry Separations on a protein-based phase such as CHIRAL–AGP can be classified as high–performance affinity chromatography and binding processes occuring in such systems might have influence also on the separation of enantiomers of small molecules. Thus, it has been suggested that large plate height and peak skewness are due to slow sample dissociation from the immobilized affinity ligands *(7,8)*.

The special binding properties of the solid phase can give rise to rather unexpected effects. An example is given in Figure 4 which shows the influence of flow rate on the peak symmetry for (–)-scopolamine. The asymmetry decreases with increasing flow rate. Similar effects have been observed for other solutes but the degree of asymmetry is dependent on the structure.

The separating efficiency is also influenced by the flow rate but it is difficult to obtain estimates with good relevance on asymmetric peaks. However, improvement of the efficiency with the flow rate is indicated by the increase of the resolution.

Improvement of the peak width and the detection sensitivity can also be obtained by gradient elution. The sensitivity and the resolution are both dependent on the steepness of the gradient in solvent gradient elution *(9)*. When the stationary phase is an adsorbent a linear solvent gradient will give increased sensitivity and decreased resolution. The silica–bonded AGP has, however, different binding properties and the decrease in retention can be completely balanced by a peak compression effect resulting in an almost unchanged or sometimes even improved resolution. An example is given in Table VIII, which shows the effect of an acetonitrile gradient at pH 7.0.

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Table VIII. Comparison of Isocratic and Gradient Techniques

Elution technique	W_1	W_2	R_s
Acetonitrile 4% (isocratic)	1.88	2.49	3.4
Acetonitrile 1% + gradient 0.2%/min	1.07	1.03	4.4

Mobile phase: acetonitrile in phosphate buffer pH 7.0.
Flow rate: 0.5 ml/min. Solute: bicyclic alcohol.
W_1 and W_2 are widths of the first and second eluted peaks in minutes.

Even a pH–gradient can be used since the buffer anions are retained on the column. It has been shown that a dihydrogenphosphate gradient can improve the sensitivity for metoprolol by 50 to 60% *(10)*.

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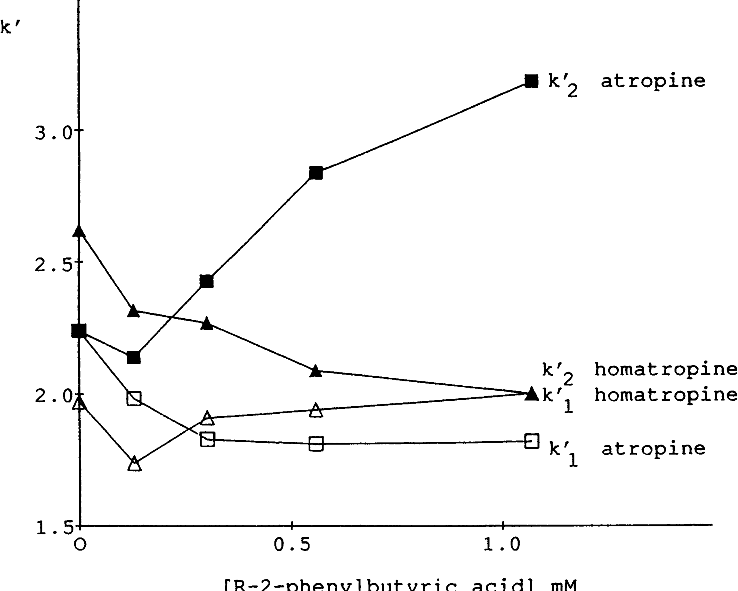

Fig. 3. (R)-2-phenylbutyric acid as additive in phosphate buffer pH 6.0 + 1 % 2-propanol

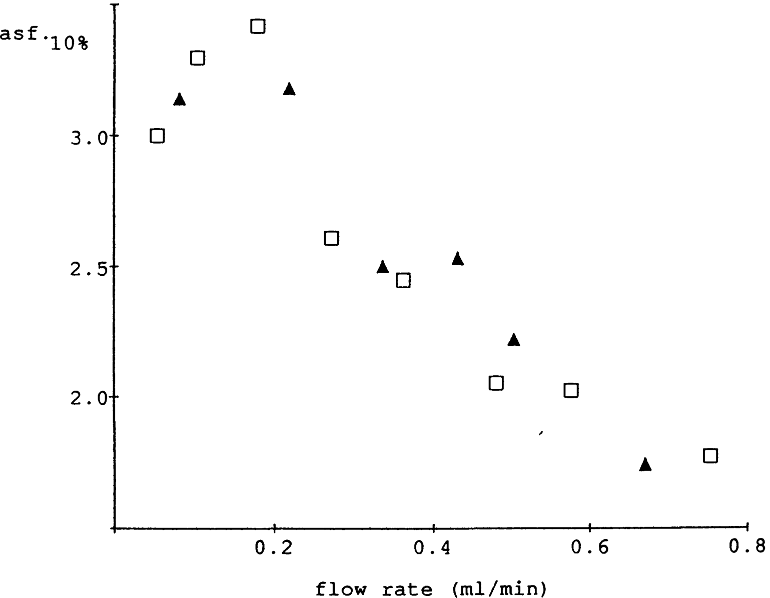

Fig. 4. Peak asymmetry (at 10 % of height): dependence on flow rate. Mobile phase: 1 % 2-propanol in buffer pH 6.0, □ acetate, ▲ phosphate. Solute: scopolamine.

Peak assymmetry due to overloading effects can be counteracted by mobile phase additives with the same charge as the solute. Chiral additives with specific interaction with the binding sites are particularly efficient. In the system presented in Table VII (–)–atropine in the mobile phase decreased the asymmetry of (+)–atropine from 2.6 to 1.7.

Conclusions.

Silica–bonded AGP retains ionic solutes by ion–exchanging and ion pairing processes and different kinds of ions from hydrophilic buffer components to hydrophobic additives can affect the binding. Chiral ions can have particularly large effects on the stereoselectivity, which indicates that the enantiomers are bound to several sites, which are affected differently by the chiral additives. Improved peak symmetry and resolution on increased flow rate indicates slow kinetics in the retention process.

Symbols.

R_s resolution: $2\,\Delta\, t_R / (W_1 + W_2)$;
α separation factor: k'_2 / k'_1;
k' capacity factor: $(t_R - t_0) / t_0$;
t_R retention time;
t_0 time for elution of a non–retained front peak;
W peak width at the base, obtained by tangents.

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Chapter 8

Chiral Recognition on Biopolymer-Based High-Pressure Liquid Chromatographic Chiral Stationary Phases

A Case for Multiple Interaction Sites

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The chiral recognition mechanisms operating on two HPLC chiral stationary phases (CSPs) based upon immobilized biopolymers, α-chymotrypsin (ACHT-CSP) and human serum albumin (HSA-CSP) have been investigated. The results of these studies indicate that the observed stereoselectivity is a measure of the differences in binding affinities at two or more sites rather than the consequence of differential affinities at a single site. The proposed multiple site/multiple mechanism model differs from the single site/single mechanism and single site/multiple mechanisms models proposed for other HPLC-CSPs.

Introduction

Stereochemical resolutions on an HPLC chiral stationary phase (CSP) involve the formation of transient diastereomeric complexes between a chiral selector on the CSP and the separate enantiomers of the solute. Enantioselectivity is then a function of the free energy difference ($\Delta\Delta G$) between the two complexes and only slight energy differences are necessary to produce an effective chiral resolution. For example, a $\Delta\Delta G$ of 410 cal/mole results in a stereoselectivity (α) of 2.00.

Within the solute/CSP complex, chiral recognition by the selector is based on the "3-point interaction" model proposed by Dalgliesh *(1)*. According to this mechanism, three interactions occur between the solute and the chiral selector and, at least one of these interactions must be dependent on the stereochemical structure of the solute. A simplified version of this process is presented in Figure 1, *(2)*.

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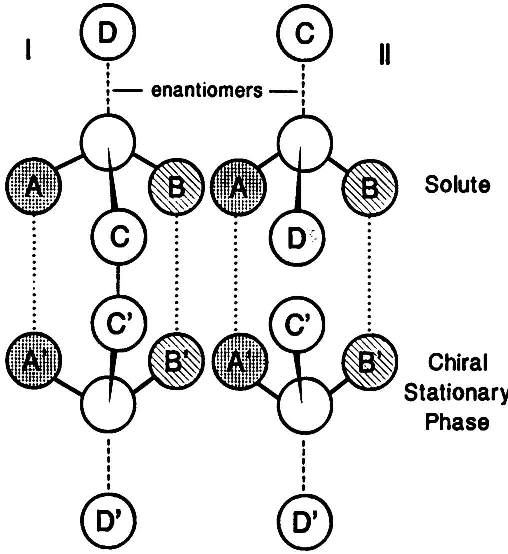

Figure 1. Illustration of the "3-point interaction" model. (Reproduced with permission from reference 2. Copyright 1984 Aster Publishing Corporation.)

In this illustration, there are three possible points of interaction between the chiral solutes (sites A, B and C) and the chiral selector (sites A′, B′ and C′). Chiral solute I interacts with the chiral selector at sites A--A′, B--B′ and C--C′, whereas its mirror image, chiral solute II does not interact at sites C--C′. If the C--C′ interaction results in the stabilization of the diastereomeric complex between Solute I and the CSP, Solute I will be retained on the column longer than Solute II. However, if the C--C′ interaction destabilizes the Solute I-CSP complex, Solute I will elute before Solute II. If C and C′ interact minimally or not at all, Solute I and II will not be resolved on this CSP.

Armstrong, et al. *(3)* have used computational chemistry techniques to demonstrate that this chiral recognition mechanism is the basis of the resolution of (R)- and (S)-propranolol on a β-cyclodextrin CSP. The major difference between the diastereomeric (R)-propranolol/β-cyclodextrin and (S)-propranolol/β-cyclodextrin complexes is the position of the secondary amine group on the propranolol molecule. In the (R)-propranolol complex, the nitrogen is ideally placed for hydrogen bonding to both a 2- and a 3-hydroxyl group on the β-cyclodextrin with respective bond distances of 3.3 and 2.8 Å. In the (S)-propranolol complex, these bond distances are 3.8 and 4.5 Å. Thus, the (R)-propranolol/β-cyclodextrin complex should be the more stable of the two diastereomeric complexes and (R)-propranolol should be the more retained enantiomer. The calculated elution order is consistent with the experimental results.

The chiral recognition process described by Armstrong, et al. *(3)* is based on a single site/single mechanism approach in which the only interaction site on the CSP is the β-cyclodextrin and both propranolol enantiomers bind in the same manner. However, this is only one of the possible chiral recognition mechanisms. Two of the other possibilities are single site/multiple mechanisms and multiple sites/multiple mechanisms, Table 1.

Table 1. Possible chiral recognition processes

Single Site/Single Mechanism
Single Site/Multiple Mechanisms
Multiple Site/Multiple Mechanisms

The single site/multiple mechanism process has been previously discussed by Pirkle, et al. *(4,5)*. In these studies the chiral stationary phase contained a single chiral selector, (R)-N-(10-undecenoyl)-α-(6,7-dimethyl-1-naphthyl)-isobutylamine, and the solutes were a homologous

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series of dinitrobenzoyl derivatives of 1-phenylalkyl-amines. The experimental results indicated that the solutes interacted with the chiral selector using two different mechanisms - one based on a dipole stacking interaction between the solute and CSP and the other based on a solute/CSP hydrogen-bonding interaction. This situation is diagrammed in Figure 2.

Two chiral recognition mechanisms have also been proposed for the stereochemical resolution of enantiomeric amide and analides on the single site (R)-N-(3,5-dintro-benzoyl)phenylglycine CSP *(6-9)*. Both of the mechanisms are based on a dipole/dipole interaction between the solute and chiral selector. The difference is due to the positioning of the solutes relative to the chiral selector within the solute/CSP complexes. Pirkle and McCune *(6)* labelled the two possibilities as "head to head" and "head to tail" dipole stacking.

The determination of the chiral recognition mechanism is further complicated when biopolymers such as cellulose and serum albumin are used as the chiral selectors. Large chiral polymers can contain multiple complexation sites exhibiting different enatioselectivities, binding mechanisms and structural requirements. Francotte and Wolf *(10)* have demonstrated this phenomenon on a cellulose triacetate I CSP. We have also demonstrated the existence of multiple interaction sites on CSPs based on α-chymotrypsin and human serum albumin. These results are discussed below.

Chiral Recognition of Amino Acid Esters on an α-Chymotrypsin CSP

Wainer, et al. *(11)* have reported the synthesis of a CSP based on the enzyme α-chymotrypsin (ACHT-CSP). The immobilization of ACHT produced a stationary phase that was capable of binding and hydrolyzing substrates of ACHT such as L-amino acid amides and esters. The enzymatic activity of the immobilized and free ACHT were equivalent *(11)*.

The ACHT-CSP was capable of resolving a number of enantiomeric compounds including D,L-tryptophan amide and N-benzoyl-D,L-leucine. The resolution of D,L-tryptophan amide was a function of the activity of the enzyme which hydrolyzed L-tryptophan amide but not the D-enantiomer. The observed chromatographic resolution was actually the separation of L-tryptophan and D-tryptophan amide *(12)*. Neither the L or D forms of N-benzoyl-leucine are substrates for ACHT and the observed chiral separation of this compound was based only on differential binding to the protein *(12)*.

Since it is assumed that substrates and pseudosubstrates bind at the active site of an enzyme, the observed enantioselectivity of the ACHT-CSP should be based on a single site/multiple mechanism chiral recognition process, i.e. the single site is the active site of the ACHT and the

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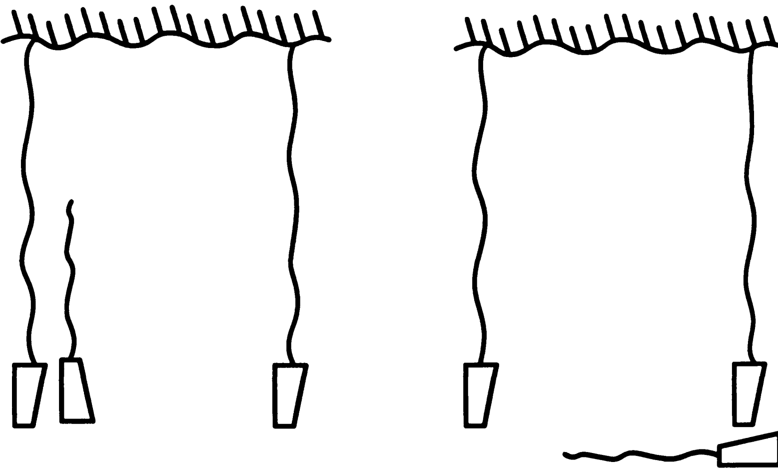

Figure 2. Two possible chiral recognition mechanisms for the stereochemical resolution of dinitrobenzoyl derivatives of enantiomeric 1-phenylalkylamines on an HPLC chiral stationary phased based upon (R)-N-(10-undecenoyl)-α-(6,7-dimethyl-1-naphthyl)-isobutylamine. (Reproduced with permission from reference 5. Copyright 1985 Elsevier.)

mechanisms are enzyme activity and solute structure. One method to determine whether a single site/multiple mechanism chiral recognition process is indeed operating on the ACHT-CSP is to block the active site of the enzyme. This can be accomplished using N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). If chiral recognition took place solely at the active site of the enzyme by either mechanism, the inactivation should result in a loss of stereoselectivity.

When the ACHT-CSP was treated with TPCK, the immobilized enzyme was deactivated *(12)*. The column was no longer able to hydrolyze L-tryptophanamide and the chiral resolutions based on enzymatic activity were lost. In addition, the TPCK inactivated ACHT-CSP was also unable to stereochemically resolve N-benzoyl-D,L-leucine and the chiral resolutions based upon solute structure were also lost *(12)*. These results support a single site/multiple mechanism chiral recognition process.

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Table 2. Enantioselectivity and chromatographic retention of amino acid esters on active and TPCK-deactivated α-chymotrypsin-based HPLC chiral stationary phases (ACHT-CSP). Data obtained from Reference 13

Solute	ACHT-CSP (active)		ACHT-CSP (TPCK)	
	k_1'[a]	α[b]	k_1'[a]	α[b]
L-Alanine benzyl ester	4.19	1.74	1.22	4.06
D-Alanine benzyl ester	7.28		4.95	
L-Tyrosine methyl ester	0.41	1.0	0.38	1.98
D-Tyrosine methyl ester	0.42		0.72	
L-Tryptophan ethyl ester	1.21	1.26	0.98	1.46
D-Tryptophan ethyl ester	1.53		1.43	

[a] Capacity factor (k′) of the first eluted enantiomer where k′ is defined as $(t - t_0)/t_0$; t = retention in seconds of the injected compound and t_0 = retention in seconds of a non-retained solute.
[b] Stereoselectivity factor (α) where $\alpha = k'_{\text{second eluted enantiomer}}/k'_{\text{first eluted enantiomer}}$.

The results obtained from the chromatography of racemic amino acid esters on the active and TPCK-inactive

forms of the ACHT-CSP are not consistent with this chiral recognition process. The results from the chromatography of three esters are presented in Table 2. Alanine is not a substrate of ACHT and the chiral resolution is due to the differential binding of the two enantiomers. L-Tyrosine methyl ester and L-tryptophan ethyl ester are substrates and are hydrolyzed by ACHT while the D-forms are not.

If a single site/multiple mechanism chiral recognition process is operating, then TPCK inactivation should result in the loss of stereoslectivity as was observed for D,L-tryptophanamide and N-benzoyl-D,L-leucine. However, the observed enantioselectivities for the esters actually increased on the inactive form of the ACHT-CSP, Table 2, *(12)*.

These results suggest that solute binding sites on the ACHT molecule exist outside of the active site of the enzyme and that the binding of amino acid esters at these sites is stereoselective. Therefore, the observed chiral resolution of the ester solutes on the ACHT-CSP must be the result of a multiple site/multiple mechanism chiral recognition process.

This conclusion was supported by the results obtained during the study of the chromatography of aspartame stereoisomers on the ACHT-CSP *(13)*. Aspartame, Nα-aspartyl-phenylalanine 1-methyl ester (APME, Figure 3), is a dipeptide which exists as 4 stereoisomers: LL-APME, DD-APME, DL-APME and LD-APME. The LL-/DD-and DL-/LD- are enantiomeric pairs and the two pairs of enantiomers are related to each other as diastereomers. The enantiomeric and diastereomeric pairs can be resolved on the ACHT-CSP *(13)*.

The effect of the molarity of the phosphate buffer in the mobile phase on retention and stereoselectivity of APME stereoisomers on the ACHT-CSP was investigated and the results are presented in Figure 4, *(13)*. It is of interest to note that the molarity of the phosphate buffer had a greater and opposite effect on the APME stereoisomers containing L-phenylalanine than those containing D-phenylalanine.

When the molarity of the phosphate buffer was raised from 0.050 to 0.500 M, the k′ for DL-APME dropped from 1.10 to 0.32 and the k′ for LL-APME fell from 1.29 to 0.33. Over the same concentration range, the k′s for LD- and DD-APME rose from 0.17 to 0.20 (LD-) and 0.18 to 0.22 (DD). The observed enantio-selectivities (α) also decreased - for LD/DL from 6.67 (0.050 M) to 1.60 (0.500 M) and for DD/LL from 7.33 (0.050 M) to 1.54 (0.500 M).

These results suggest the existence of two separate binding sites, the L-phenylalanine (L-Phe) and D-phenylalanine (D-Phe) sites. The decrease in k′ for the DL- and LL-isomers indicates that the phosphate ion either competes for binding at the L-Phe site or that the increase in the ionic strength of the mobile phase decreases the affinity

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$$HO-C(=O)-CH_2-\overset{*}{C}H(NH_2)-C(=O)-N(H)-\overset{*}{C}H(CH_2C_6H_5)-C(=O)-OCH_3$$

Figure 3. The molecular structure of aspartame.

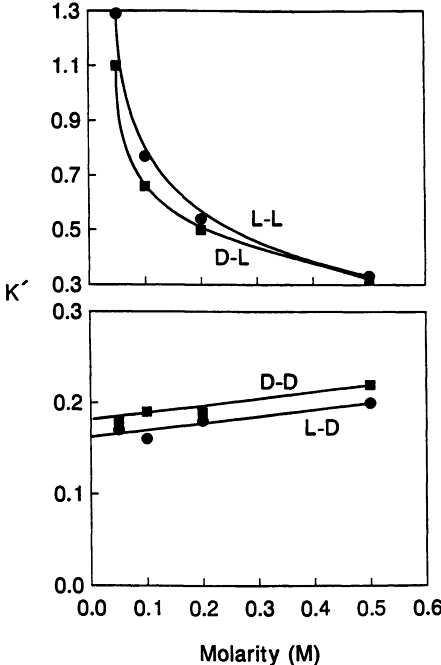

Figure 4. The effect of the molarity of the phosphate buffer in the mobile phase on retention of aspartame stereoisomers. (Reproduced with permission from reference 13. Copyright 1990 Chirality.)

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of the site for these isomers. If LD- and DD-APME were bound at the same site, their k's should be affected in the same manner by the phosphate buffer concentration. Since the observed k's of the LD- and DD-isomers increases, it appears that the phosphate ion does not compete at the D-Phe site and that a mobile phase with a high ionic strength increases the affinity of LD- and DD-APME for this site.

Chiral Recognition on a Human Serum Albumin HPLC-CSP

Human serum albumin (HSA) is a globular, hydrophobic protein which has been shown to stereoselectively bind small enantiomeric molecules *(14)*. There are two major drug-binding sites on HSA: the warfarin-azapropazone-binding area and the indole and benzodiazepine binding site which both display some stereoselectivity *(15-17)*. An additional one (digitoxin, *(18)*) to three sites (digitoxin, bilirubin and fatty acid, *(19)*) have also been proposed.

The stereoselectivity of the warfarin-azapropazone-binding area has been investigated by a number of laboratories. Sellers and Koch-Weser *(20)* have demonstrated that S-warfarin is more highly bound to HSA than R-warfarin. The indole and benzodiazepine binding site has also been the subject of a number of investigations and appears to be more stereospecific than the warfarin-azapropazone-binding area. For example, the ratio of the affinity constants of (+)-oxazepam hemisuccinate/(-)-oxazepam hemisuccinate is 49.5 *(21)*.

The different binding sites on HSA have been studied using competitive binding interactions. In this approach, a compound known to bind at a specific site on the HSA (the competitor) is added to the incubation mixture containing HSA and the molecule under investigation (the ligand). If both the ligand and the competitor bind at the same site, then there will be a reduction in the binding of the ligand to the HSA. The magnitude of the reduction will depend upon the concentration of the competitor and its affinity for the HSA.

In a chromatographic system utilizing immobilized HSA, the addition of a competitor to the mobile phase will result in a reduction of the chromatographic retention (k') of the ligand. The extent of this reduction will be a reflection of the differential binding affinities of the ligand and displacer and the sites at which these compounds bind.

The addition of a competitor to the mobile phase could also have an effect on any chiral resolutions. The extent of the impact on the stereoselectivity will depend in part upon the affinity of the competitor for the binding site of the ligand and the chiral mechanism responsible for the separation of the ligand enantiomers.

For the latter aspect, if the ligand enantiomers bind

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at the same site on the protein with the same mechanism, a single site/single mechanism situation, then the addition of a competitor should reduce the retention of both enantiomers and the stereoselectivity. If single site/ multiple mechanisms or multiple site/multiple mechanisms processes are operating, then the displacer should effect the chromatography of each isomer to a different extent.

We have reported the synthesis of an HSA-based CSP in which the protein was covalently bonded to a commercially available diol HPLC column previously activated with 1,1-carbonyldiimidazole *(22)*. The HSA-CSP can be used for chiral separations and initial studies suggested that the separations reflected binding interactions between the ligand and the native protein *(22)*.

As part of our investigation of the application and use of the HSA-CSP, we have studied the chiral recognition processes for the chiral separation of the enantiomers of (R,S)-ibuprofen (IBU) [Noctor, T.A.G.; Felix, G.; Wainer, I.W. *Chromatographia*, in press.] and a benzodiazepine derivative, (R,S)-oxazepam hemisuccinate (OXH) *(23)*. The effects of the addition of displacers to the mobile phase on the k's of (R)- and (S)-IBU were investigated using the individual IBU enantiomers and, in the case of OXH, (R)- and (S)-OXH were used. These modifiers were chosen to test for binding competition between the enantiomers indicating a single site/single mechanism process. In addition, the (R,S)-IBU were used as competitors in the chromatography of the OXH enantiomers.

The results of the addition of (R)- or (S)-IBU to the mobile phase on the retention of (R)- and (S)-IBU are presented in Table 3. (R)-IBU, which is known to be more tightly bound to plasma proteins than (S)-IBU *(24)* and the observed k's reflect this difference. The effects on the k' of (R)-IBU of the addition of (R)-IBU to the mobile phase relative to the effect of the addition of (S)-IBU also reflect the difference in binding affinities between the two IBU enantiomers. In this case, a 10 μM concentration of the (R)-isomer reduces the k' of (R)-IBU by 85% while a 10 μM concentration of (S)-IBU produces only a 44% reduction.

The fact that the addition of one enantiomer affects the retention of the other, indicates that (R)- and (S)-IBU bind at the same site on HSA and with the same mechanism. Thus, it appears that for (R,S)-IBU, chiral recognition on the HSA-CSP involves a single site/single mechanism process.

This was not the case for the OXH enantiomers *(23)*. It has been generally assumed that the enantiomers of a chiral benzodiazepine bind at the same site on HSA *(21,25)*. However, the results of the competition studies carried out on the HSA-CSP, Table 3, do not support this hypothesis. The addition of up to 0.050 mM of (S)-OXH to the mobile phase had relatively little effect on the k' of (R)-OXH

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and, conversely, the addition of up to 0.020 mM of (R)-OXH had only a slight effect on the k′ of (S)-OXH. These results indicate that on the HSA-CSP, (R)-OXH does not bind at the same site as (S)-OXH.

The effect of the IBU enantiomers on the retention of (R)- and (S)-OXH is also consistent with the assumption that the OXH enantiomers bind at different sites on HSA, Figure 5, *(23)*. IBU is known to bind at the benzodiazepine binding site on HSA which is the same site at which the IBU enantiomers bind *(21,24,25)*. Since the k′ of (S)-OXH is reduced while the k′ of (R)-OXH remains constant, these studies confirm that only (S)-OXH is bound to the benzodiazepine binding site.

Table 3. Changes in the chromatographic retention of ibuprofen enantiomers, (R)-IBU and (S)-IBU, and oxazepam hemisuccinate enantiomers, (R)-OXH and (S)-OXH, as a result of the addition of the opposite enantiomer to the mobile phase

Solute/Competitor	Competitor Concentration [mM]	k′
(S)-IBU/(R)-IBU	0.000	21.37
	0.005	18.27
	0.010	16.33
	0.050	11.69
	0.100	10.47
(R)-IBU/(S)-IBU	0.000	73.41
	0.020	66.84
	0.040	61.86
	0.100	41.17
	0.200	31.83
(R)-OXH/(S)-OXH	0.000	8.36
	0.005	8.31
	0.010	8.26
	0.020	8.17
	0.050	8.00
(S)-OXH/(R)-OXH	0.000	22.86
	0.005	22.80
	0.010	22.69
	0.020	22.20
	0.050	nd[a]

[a] Not determined due to large background noise in the chromatogram and poor peak efficiency.

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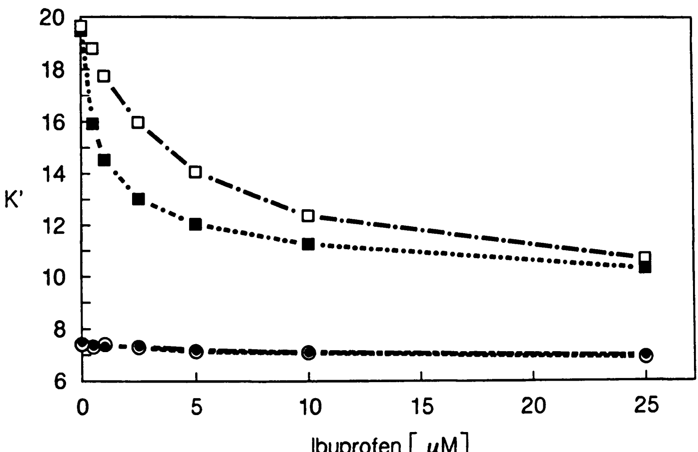

Figure 5. The effect on the k′ of (R)- and (S)-OXH of the addition of (R)- and (S)-ibuprofen to the mobile phase. Where: ■ = k′ of (S)-OXH [(R)-ibuprofen in the mobile phase]; ● = k′ of (R)-OXH [(R)-ibuprofen]; □ = k′ of (S)-OXH [(S)-ibuprofen]; ○ = k′ of (R)-OXH [(S)-ibuprofen].

These results indicate that the chiral resolution of (R)- and (S)-OXH is the result of a multiple site/multiple mechanism process. The actual binding process at each site has not been identified and is currently under investigation.

In addition, (R)-IBU, which is known to be more tightly bound to plasma proteins than (S)-IBU *(24)*, also had a greater effect on the k' of (S)-OXH. This result also confirms the hypothesis that the enantioselectivity in the chromatography of IBU on the HSA-CSP is the result of a single site/single mechanism process arising from binding at the benzodiazepine site.

Conclusion

In their study of dipole/dipole stacking chiral recognition mechanisms, Pirkle and McCune *(6)* concluded that "enantiodifferentiation is a time-weighted average of multiple processes and cannot be stringently ascribed to a single mechanism in all instances." The results presented in this review support this observation and extend it to include the fact that the chiral recognition process can include multiple sites as well as multiple mechanisms. This observation will become increasingly important as the number of biopolymer-based chiral stationary phases grows and as their applications expand.

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Chapter 9

Chiral Separations and Modes of Association of Hydrogen-Bonding Molecules

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The state of the art of chiral chromatographic resolution is briefly reviewed, with emphasis on the procedures introduced in the author's laboratory. This is followed by a discussion of proposed mechanisms of resolution in systems in which selectors and selectands interact by hydrogen bonding. Essentially, it is proposed that resolution models should be based on associates of the most stable conformations of both the stationary phase and the solute. Data available from minimal energy computations and from hydrogen bonding modes in biological systems should be used for this purpose.

The resolution of optical isomers requires a chiral medium. This can be provided in two ways: (1) Intramolecularly, through the introduction of a chiral handle by reaction with a functional group leading to the formation of diastereomers, or (2) Intermolecularly, through the provision of an external chiral environment. In the chromatographic approach, resolution is achieved in the first case on an **achiral** phase, whereas a **chiral** phase is used in the second case.

The simpler diastereomeric approach was introduced first and although it has certain inherent disadvantages is still very useful and sometimes the only solution to certain problems of resolution.

In the last decade, the use of the second approach to enantiomeric analysis has made considerable strides. Many laboratories all over the world have engaged in the development of this brand of chromatography and abundant information has, thus, become available on the resolution of optical isomers on chiral phases by various chromatographic methods, such as GC, LC, TLC, Droplet Countercurrent Chromatography, as well as solvent extraction and capillary electrophoresis. These results prove most effectively how wrong the intuition of chemists was a generation ago, when it was generally assumed that chiral selector-selectand interactions could not possibly be large enough to effect resolution.

Table 1 illustrates the range of resolution coefficients observed in various systems by different authors. Starting with very small values, as found, for example, on chiral propylene glycol (PPG, I), the coefficients are seen to increase on stationary phases consisting of L-amino acid derivatives. Far larger values are reached for the diamide selectors (III) and still higher ones for ligand exchange on chiral Cu(II) α-amino acid complexes (IV, V). Other classes of selectors are equally apt to give high chiral recognition, as demonstrated by the crown ethers derived from 2,2'-dihydroxy-1,1'-

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Table 1. Resolution Coefficients (α) for Different Types of Selector/-Selectand Systems

	SELECTOR	SELECTAND	α (temp)	$-\Delta\Delta G$** (cal)
(I)	$H.O(CH_2CH(CH_3)O)_nCH_2CH(CH_3)OH$	Norbornanols	1.01 *(1)* (70°C) GC	7
(II)		N-TFA-Leu t-Bu Ester	1.08 *(2)* (90°C) GC	55
(III)		N-TFA-Leu iPr Ester	1.34 *(3)* 130°C	235
		III: R_1=n-$C_{11}H_{23}$; R_2: iPr, R_3=t-Bu	[3.5-4.0] (extrapolated to 0°C) GC	760
(IV)		Valine	4.8 *(4)* (0°C) HPLC	860
(V)		Proline	10.9 *(5)* (20°C) HPLC	1400
(VI)		[Phe]+/ClO4-	30* *(6)* (0°C)	1860
(VII)			121 *(7)* (25°C) HPLC	2860

*Enantiomer distribution constant between two immiscible solvents.
** $-\Delta\Delta G = RT \ln \alpha$

binaphthyl (VI). A real *tour de force* was accomplished by Pirkle, who designed a multidentate solute giving α=120 on silica gel to which N-β-naphthyl-L-alanine was bonded (VII).

The extreme values cited in Table 1 (I, VII) are of special interest. At the start of research on the topic, we pursued two different lines of approach. On the one hand, chiral phases were synthesized, specifically designed to maximize the interaction between the selector and the selectand in the region close to the respective chiral centers. On the other hand, it was also decided to examine the chiral form of a stationary phase known to be generally efficient and versatile. The fundamental premise of the latter line of attack was that every chiral solvent should show some difference in its interaction with antipodic solutes and, thus, it should suffice to provide a sufficiently high number of theoretical plates to detect the separation of enantiomers. PPG (I) seemed suitable for this purpose because it is a generally useful phase and the synthesis of its chiral form had been carefully worked out. However, attempts to resolve amino acid esters and other racemic solutes on very long capillary columns were unsuccessful *(8)*. It was most gratifying to learn many years later that chiral PPG was, indeed, found to have some stereoselectivity (Table 1, I). Though the coefficients were only of the order of 1.01, this case demonstrates that many chiral solvents of only minimal stereoselectivity could indeed be marshalled to fulfill a useful analytical function, given suitable columns.

At the other end of the spectrum, the examples cited from the work of Cram and of Pirkle (VI and VII, respectively) show that non-biological solvent-solute systems can be found in which the stereoselectivity approaches that of enzymes.

In assessing the magnitude of the effects observed, it should be remembered that the ΔΔG values calculated from the retention volumes are affected in their magnitude by many solvent-solute interactions, some of which might have little or no stereoselectivity. Thus, associates must exist in the stationary phase with chiral recognition higher than indicated by the free energy data listed.

With the chiral selectors known today — many of which are commercially available — an impressive range of compounds can be resolved. Activity in the field continues to be intense, and many new chiral phases and resolution procedures are emerging at a rapid rate. Interesting recent developments include the use of proteins, such as BSA and α_1-acid-glycoprotein, bonded to solid supports as selectors, as well as that of carbohydrates, such as cyclodextrins and various derivatives of polysaccharides and cellulose. Also, considerable progress has been made in preparative scale chromatographic resolutions.

Mechanism of Resolution

In parallel with these practical achievements, advances have also been reported in the elucidation of the resolution mechanism.

The chromatographic resolution process proceeds through the rapid and reversible formation of diastereomeric solute-solvent associates. If the difference between the free energies of solution of these diastereomeric species is sufficient, separation of the optical isomers will result.

Modern chiral selectors were designed to incorporate structural features permitting such associations through a variety of intermolecular forces, e.g. hydrogen bonding, charge transfer complexation, coordinative bonds to metals, ion-ion and dipole-dipole attractions and non-bonded interactions.

Solute and solvent molecules may, in general, interact with each other in many different ways. To understand the mechanism of resolution, evidence has to be produced for the presence of a selector-selectand association, which may satisfactorily explain the stereoselectivity observed. By definition, such intermediate diastereomeric species are readily dissociable and, thus, their study poses a difficult problem.

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Therefore, indirect approaches have to be used in order to produce evidence for the structure of the relevant solute-solvent complexes. This will be illustrated below by the discussion of proposed mechanisms of resolutions for three different types of selectors interacting with selectands through hydrogen bonding.

N-Lauroyl-(S)(or R)-α–(1-Naphthyl)Ethylamine). An example of a selector-selectand pair linked by hydrogen bonds, which was chosen for the investigation of the mechanism of chiral recoginition, is N-lauroyl-(S)(or R)-α–(1-naphthyl)ethylamine interacting with lower molecular weight N-acyl-α-arylethylamines. By X-ray spectroscopy *(9)*, it was found that the selector arranges itself in the crystalline state, as well as in the melt, in a 5-Å translational array, as shown in Fig. 1, with the hydrogen bonds in the plane of the paper and the naphthyl rings oriented perpendicularly to it.

To explain the mechanism of resolution it was assumed that the selectand intercalates into the hydrogen bonded array of the selector matrix. According to the configuration of the solute, two diastereomeric arrangements having different stabilities result. When selector and selectand have the same configuration (Fig. 1a), the original motif of hydrogen bonding is preserved, the aromatic groups make plane-to-plane contacts and the hydrogen atom linked to the asymmetric carbon is wedged between two aromatic rings as in the original stack. The methyl group, on the other hand, points away from the stack. It should be pointed out that if atoms had been drawn with their Van der Waals radii, it would have become immediately apparent by inspection of Fig. 1 that the hydrogen linked to the asymmetric carbon fully occupies the space between the rings.

For unequal configuration of solvent and solute (Fig. 1b), intercalation with maintenance of selector conformation requires that the hydrogen and the methyl groups linked to the asymmetric carbon of the solute be interchanged. The methyl group, thus wedged between the two naphthyl groups, would lead to severe overcrowding, i.e. there is a worse fit between the host and the guest in this case than in the case of the RR combination. These predictions of the model are in agreement with the experimentally found difference in selector-selectand interactions. Other evidence supporting the intercalation model, including energy computations of relevant diastereomeric selector-selectand associates were reported by Weinstein S. et al *(9)*.

Diamides (III). Like N-lauroyl)-α–(1-naphthyl)ethylamine, the diamides interact with selectands essentially through hydrogen bonding. Indeed, if the amide nitrogens are methylated, no resolution occurs.

Studies of the conformation of the diamides by X-ray diffraction, NMR and IR spectroscopy, and by minimal energy computations led to the conclusion that the pleated sheet β-structure [Fig. 2] and the R-α-helix (Fig. 3) are the most stable conformations. The proposals for the mechanism of resolution for this type of selectors are based on the idea that these same conformations of the diamides are also those which are involved in the stereoselective solvent-solute associates.

Thus, for the resolution of the α-amino acid esters a model, as shown in Fig. 2, is proposed. The diamide is seen to hydrogen bond through its "C_7" side to the selectand, which has only a "C_5" moeity *(10)*. The alkyls attached to the asymmetric centers are oriented parallel to each other, when the configuration of both partners are the same. For the opposite configuration of the selectand, the alkyl groups will be differently oriented towards each other, resulting in a difference in the energy of formation of the associates.

No direct evidence of this model is as yet available. However, a number of observations support this proposal. Thus, it has been found that the presence of a "C_7" conformation in a selector enhances stereoselectivity. Similarly, for proline as selectand, the N-TFA isopropyl ester which does not possess an -NH- group is resolved only with a very small coefficient, whereas, the N-TFA-t-butylamide, which has a "C_7" moiety, is resolved with a large coefficient.

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R
R
R
R
a

R
S
R
R
b

Figure 1. A chiral guest molecule *N*-trifluoroacetyl-α-phenylethylamine inserted into the H-bonded stack of (*R*)-*N*-lauroyl-α-(1-naphthyl)ethylamine. Selector and selectand may have the same (a) or an unequal (b) configuration.

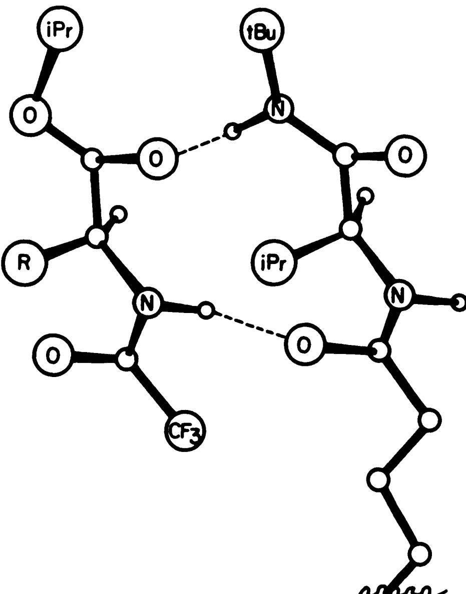

Figure 2. Hydrogen bonded association of a N-acyl-L-valine t-butyl amide phase in its pleated sheet β–conformation with the N-TFA isopropyl ester of an L-α–amino acid.

Figure 3. Association of the diamide III in its R-α-helix conformation with 1,2–diphenylethyleneglycol dipentafluoropropionate.

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Furthermore, the influence of the size of the α–substituents in both selector and selectands on the resolution is in agreement with the model.

The concept can be extended to compounds other than derivatives of α-amino acids and not having a "C_5" side in their molecule, e.g. N-acyl-β- and γ-amino acid esters and N-acyl-O-acyl-2-amino alkan-1-ols. The enantiomers of the latter two classes of compounds elute in the inverse order, as compared with the α-amino acid derivatives. It is proposed that a clue to this behavior is hydrogen bonding of the selectands to the selector through the "C_7" side found in both types of derivatives *(10)*.

Solutes, such as aromatic diketones, aromatic diesters and N-TFA proline esters do not have both a CO and an -NH- function and, hence, the above mechanism is not applicable. It has been proposed by Koppenhoefer and Bayer *(11)* that an alternative relatively stable conformation of the diamides, the R-α-helix (Fig. 3), is involved in the resolution process of these compounds. The corresponding associate for diamide-1,2-diphenylethyleneglycoldiester is shown with the two >CO groups of the selectand hydrogen bonding to two -NH- groups of the diamide. In a number of cases *(11)* these models led to a correct interpretation of the observed order of elution as judged by the best fit (minimal eclipsing) in the respective diasteromeric associates illustrated by the example given in Fig. 3.

N,N'-2,6-Diaminopyridinediylbis[S-2-Phenylbutanamide]. A different example of a naturally occurring mode of hydrogen bonded association leading to the separation of enantiomers has been reported recently by Feibush, et al. *(12)*. These authors studied the resolution of drugs such as hydantoins, barbiturates, glutaramides, etc., which all contain an -OC-NH-CO- moeity in their molecule.

In the design of a suitable selector, they used the concept that the selector-selectand associate should involve a system of hydrogen bonds as found in complementary nucleic base pairs. N,N'-2,6-diaminopyridinediylbis[S-2-phenylbutan-amide] (VIII, Fig. 4), linked to silica gel through an n-undecyloxy handle, was chosen for this purpose and indeed showed stereoselectivity for the above compounds.

The authors succeeded in crystallizing a 1:1 complex of an achiral analog of VIII (the 2,6-di-n-butanamide) with the achiral 4-ethyl-4-methyl-2,6-piperidinedione. X–ray analysis showed that the two compounds interacted to form a system of two hydrogen bonded rings similar to that in the cytosine-guanidine pair. By analogy it was assumed that the chiral selector VIII forms the same array of hydrogen bonds with the selectands studied.

The X-ray data also established that the amide groups are in an extended conformation and, further, it was deduced from NMR studies that the substituents at the α–carbons [-NH-CO-CH(Et)φ] were oriented as shown in Fig. 4, with the hydrogen directed towards the selectand.

On the basis of this model, it is possible to interpret the experimental facts. Thus, for the case shown in Fig. 4 [selectand = 1-(1-cyclohexen-yl)-1,5-dimethyl-barbiturate], it can be readily seen that the configuration of the S-selectand fits the selector better than the R-isomer, as expected from the observed order of elution. The two large substituents, respectively, at the 1-position of the S-selectand (cyclohexenyl) and the α-carbon of the amide group of the selector (phenyl) are, indeed *trans* to each other. The model also satisfactorily accommodates most of the other observations reported.

Conclusions

The proliferation of modern chromatographic systems capable of separating enantiomers has completely changed the concepts which prevailed in the area 20-25 years ago. It is now recognized that whenever a chiral solvent-solute pair can associate in a manner, such as the respective asymmetric centers can interact with each other, chiral differentiation has a good chance of occurring.

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Figure 4. Structure of the complex between N,N'-2,6-diaminopyridinediylbis[S-2-phenylbutanamide] (VIII) with 1-(1-cyclohexenyl)-1,5-dimethylbarbiturate. Configuration of selectand — S-isomer: R_1 = methyl, R_2 = cyclohexenyl. R-isomer: R_1 = cyclohexenyl, R_2 = methyl.

In this report, emphasis was put on the modes of association in hydrogen bonded systems and, particularly, of the diamides of α-amino acids (III) and of 2,6-diaminopyridine (VIII). It was assumed that the conformations involved in the selector-selectand complex are those computed to have minimum energies and/or are known to occur frequently in corresponding biological molecules. Good evidence exists for the occurrence and the determining stereoselective role of such associates in some cases, while in others the corresponding models still remain speculative. Further support for these models could be forthcoming from studies by spectroscopic methods, such as two-dimensional NMR, and from molecular graphics.

The similarity of such selector-selectand associations with those of biological molecules suggests that a systematic study of the mechanisms of resolution of the relatively simple chromatographic systems might be relevant to the understanding of the far more complex recognition phenomena occurring in nature.

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Chapter 10

Liquid Chromatographic Separation of Enantiomers by Hydrogen-Bond Association

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This paper describes the liquid chromatographic separation of enantiomer by small bimolecular association, the driving force of which being the action of bidentate hydrogen bonds. This separation can be carried out by chiral mobile and stationary phases (CSPs) functionalized with chiral tartramide and amino acid diamides.

Many different enantiomers could be separated by hydrogen-bond association on CSPs derived from these components. The strategy for maximizing the separability of enantiomers, which is related to elimination of nondiscriminating interactions of CSPs, and the structure and thermodynamic features of diastereomeric hydrogen-bond associates are discussed in the following.

Enantiomer separation was also possible in aqueous media when hydrogen bonding functionality was within a hydrophobic environment such as the liquid-solid interface in CSPs and micellar interior core. Attention is directed to the hydrophobic features of interfacial phase and micellar hydrophobic core related to enantiomer separation.

The recognition of molecular chirality by liquid chromatography has given rise to a novel type of separation with chiral selector molecules carrying the intended binding affinity for the enantiomeric selectand molecules (*1-3*). This method revealing the selector and selectand relationship resembles group-specific affinity chromatography (AC) by which a large set of biomolecules with common structural elements can be recognized (*4*).

In various aspects of molecular recognition applying liquid chromatography, chiral separation imposes stereochemical dependence on molecular association and distinguishes enantiomers as two distinct peaks. The different chromatographic mobilities of these peaks are due to diastereomeric interactions that occur along with various transient interactions between the column system incorporating the chiral selector and the pair of enantiomeric selectands. Diastereomeric interaction thus makes possible different thermodynamic stabilities between the enantiomers in chromatographic terms. This is emphasized in the approach by CSPs including selectors immobilized onto solid support and chiral polymers.

Chiral selectors are either small molecules comparable to enantiomeric selectands or macromolecules such as proteins. Proteins (*1-4*) have been used for the separation

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of enantiomers as the macromolecular selector immobilized on the silica surface, which appears mediate between AC and the chiral chromatography relevant to enantiomer separation. These affinity matrices indicate a slow kinetic desorption-adsorption process (*5*) due to strong and specific binding not excluded from the separation process with such biomolecules. This drawback can be overcome by transient molecular associations between small molecular species whose driving force is the action of hydrogen bonds, ionic bonds, and π-π interactions. This type of chromatography should make possible various interactions and conformations in both the selector and selectand to intervene in diastereomeric association, possibly accompanied in some cases by lower selectivity due to their weighted average.

Chiral separation through hydrogen-bond associations between small molecular species (*6, 7*) is discussed in the following, with emphasis, in the first half, on diastereomeric complex structures formed between the chiral selector and selectand, and, in the second half, the introduction of hydrogen-bond association into aqueous phase.

Chiral hydrogen bonding selectors from optically active tartaric acid and amino acids were introduced into chromatographic phase systems by three means: CSPs, chiral mobile phase additives (CMPAs), and a solute zone injected into a column, this probably being a exceptional means. In the last case when enantiomerically enriched mixtures are injected, the additional enantiomer itself exceeding racemic composition can act as the chiral selector (*8-11*). The driving force leading to chiral separation is also through diastereomeric homologous and heterogous associations of enantiomers in the migrating solute zone.

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Scope of Hydrogen-Bond Association on Enantiomer Separation

Enantiomer Separation with CSPs. It has been possible to separate many different enantiomers by hydrogen-bond association on various CSPs containing those which optically active tartramide and amino acid diamides are immobilized onto the silica gel surface.

Stability difference due to CSP-enantiomer interactions is the major factor determining the degree of preferential retention in one of a pair of enantiomers. Thus, CSPs are a sophisticated means for determining sense of stability difference between diastereomeric complexes, that is, for determining which enantiomer forms the most stable complex with the chiral selector. However, since achiral interactions which do not contribute to enantioselectivity between CSP and enantiomers must be considered in the overall retention along with the above chiral interactions, retention and the separability of enantiomers should thus be affected by these interactions (*12-14*). This is particularly important in our hydrogen bonding systems since nondiscriminating interactions involving the sorption process with the surface silanol possibly compete with the recognition force to a considerable extent and lessen enantioselectivity. Thus, to clearly demonstrate enantioselectivity based on the intrinsic specificity of the chiral selector, achiral interactions should be prevented between CSP and enantiomers, so as to maximize separability (*14*).

In consideration of the above, two different approaches were used: chiral selector moiety was partitioned off from the silica gel surface by a long spacer and the surface was exhaustively trimethylsilylated to remove bare silanol. Highly reactive monochlorosilane derivatives were also used as a silylating reagent, thus permitting a sufficient degree of surface modification and, most important, without regenerating any nondiscriminating sites such as additional silanol groups on the modified surface.

Using CSPs derived from optically active *N,N'*-dialkyltartramide (**1**)(*14*) and amino acid diamides (**2-4**)(*15*) by the above strategy, it was possible to expand and extend the resolution spectrum of hydrogen-bond association for many different enantiomers. This was not evident at the start of this study when the tartramide selector

was linked to the silica gel surface via a propylene unit through reaction of the corresponding triethoxysilyl derivatives (*16*), as in the case of CSP1, and *N*-acyl-L-valine was linked covalently (*17-20*) or glutaryl-L-valine *tert*-butylamide monocarboxylic acid ionically (*21*) to the aminopropyl silica gel, as with CSP2.

	R
1	$-(CH_2)_{11}-Si(CH_3)_2-O-Si$
1b	*i* - Pr

	R1	R2
2	*i* - Pr	$-(CH_2)_{10}-Si(CH_3)_2-O-Si$
3	*sec* - Bu	
4	*tert* - Bu	
2b	*i* - Pr	Me

CSP1. Of our two series of CSPs, CSP1 was found particularly capable of separating the following broader categories of enantiomers possessing at least two hydrogen bonding sites: *O*-phenylcarbamoyl(X) derivatives of 2-hydroxy carbonyl compounds (ester and ketones) (a), 3-hydroxy carbonyl derivatives (ester, ketone, and *N*-alkylamides) (b), *N*-phenylcarbamoyl(X) derivatives of 2-amino alcohols (c), amino esters (d), and primary amines (e), glutarimides (f), barbiturates (g), 2-hydroxy ketoximes (h), 2,2'-dihydroxy-1,1'-binaphtyl (i), (2-hydroxyphenyl)phenylcarbinol and 2-pyridylphenylcarbinol (j), and 1,2-diols (k). The separation factors (αs) observed typically ranged from 1.1 to 1.8. These values are not very high but sufficient for the complete resolution of two enantiomeric peaks since the hydrogen-bond association assures fast sorption-desorption as a prerequisite for maximum column efficiency in CSP-enantiomer interactions.

Derivatization involving phenylcarbamoylation may possibly provide better hydrogen bonding sites to enantiomers belonging to categories (a), (c), and (d), this being in addition to their intrinsic functionalities, and providing second hydrogen bonding sites to the essentially monofunctional primary amines (e). Glutarimides (f) having imido functionality made possible one of the most effective separations on CSP1 as well as barbiturates (g), as shown in Figure 1. The separation of these heterocyclic drugs was also accomplished by a CSP derived from *N,N'*-2,6-pyridinediyl bis(2-phenylbutanamide), which mimics multiple hydrogen bonding between complementary bases in double-strand DNA (*22*).

CSP2. CSP2-4 differing in their amino acid side-chains and, in order to entrap enantiomers through hydrogen bonding, depend entirely on two amide functionalities. They are the simplest of all those so far examined and are produced through an association mode that mimics mutual hydrogen bonding between amide functions in linear peptides. It thus follows that they provide highly specific enantioselectivity for amino acid enantiomers. Exhaustive elimination of achiral interactions from CSP-enantiomer interactions led to maximum separability of the amino acid derivatives, and made CSP effective for other series of enantiomers. These CSPs share partially in some cases the following categories of enantiomers: (a), (b), (c), (d), (e), and (k), in the scope of CSP1.

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For CSP2, *N*-4-nitrobenzoylated amino acid isopropyl esters provided the most effective αs, from 1.33 to 3.27, exceeding those obtained with the corresponding *N*-phenylcarbamoyl derivatives except for the proline derivative. The enantiomers of the leucine derivative could be separated the most efficiently ($\alpha = 3.27$), their elution order being such that the L enantiomer was more strongly retained than the D enantiomer; the former thus forms a more stable associate than the latter on CSP2, as shown in Figure 2. This separation indicated a difference in the free energy of diastereomeric complexes ($\Delta\Delta G^o$), 0.70 kcal/mol, as determined from the equation, $\Delta\Delta G^o = -RT\ln \alpha$ (T = 296K).

Changes in the amino acid side-chains of chiral moiety on CSP2-4 affected enantiomer separation. CSP3 derived from L-leucine provided higher α values for 2-hydroxy carbonyl and amino acid derivatives than those obtained on CSP2. CSP4 from L-*tert*-leucine gave α for 2-amino alcohol derivatives exceeding those on CSP2. A modest resolution of all categories of enantiomers was, however, possible on CSP2.

Enantiomer Separation with CMPAs. Treatment to eliminate nondiscriminating interactions provided the maximum separability between enantiomers and, as expected, accompanied by lessening their retentivity. This lessened considerably the separation between individual amino acid derivatives on CSP2. The solution to this problem is provided by application of a chiral mobile phase additive (CMPA), a possible soluble analogue of CSP. Amino acid derivatives the same as those in the CSP study were separated with *N*-acetyl-L-valine *tert*-butylamide (**2b**)(*23-25*), a soluble analogue of CSP2, by which was possible the instantaneous separation of individual amino acid components and their enantiomers in the complex mixtures (*25*). This separation can be explained in terms of the intervention of the solute-silica gel phase equilibrium in the retention process.

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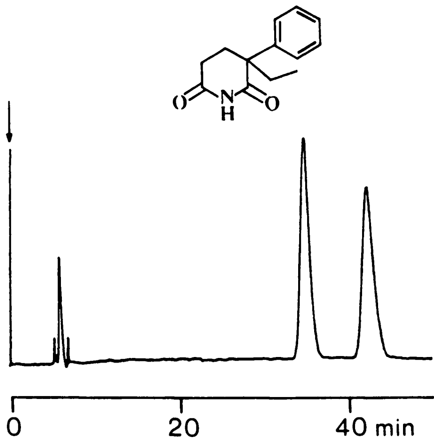

Figure 1. Optical Resolution of Glutethimide of CSP1. Conditions: column, 50 X 0.1 (i.d.) cm; mobile phase solvent, 4%(v/v) 2-propanol in *n*-hexane; column temperature, 20°C; flow rate, 60 μL/min; detection, UV at 254 nm. k' of the first-eluted enantiomer and α are 5.65 and 1.25, respectively. (Reproduced from ref. *14*. Copyright 1987 American Chemical Society.)

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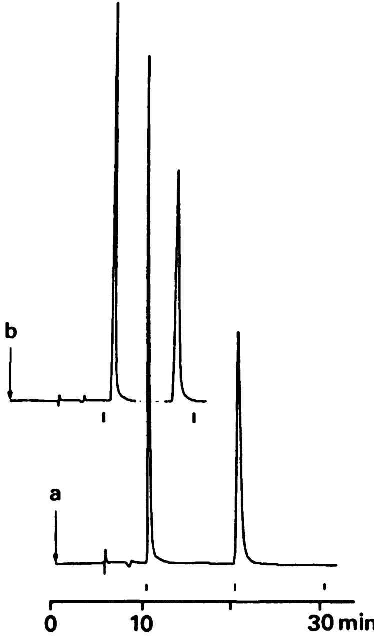

Figure 2. Optical Resolution of Racemic *N*-(4-Nitrobenzoyl)amino Acid Isopropyl Esters on CSP2. Conditions: column, 50 X 0.1 (i.d.) cm; mobile phase solvent, 1%(v/v) 2-propanol in *n*-hexane; column temperature, ambient; flow rate, 60 μL/min; detection, UV at 254 nm. k' of the first-eluted D enantiomer and α for each derivative are: 0.71 (α 3.27) for the leucine (a) and 0.81 (α 2.33) for the alanine derivatives (b).

It may possibly be, thus, more difficult to apply CMPA than CSP owing to the complex equilibrium involving CMPA, enantiomeric solute, and solid support. The separation by CMPAs should of course be possible by incorporating the equilibrium between the enantiomers and CMPA into the ordinary solute partitioning equilibrium. This would facilitate manipulation of retention and improve enantioselectivity in the chromatography. Various enantiomers have been found separable using (*R,R*)-*N,N'*-diisopropyltartramide (**1b**), a soluble analogue of **CSP1**, to an extent comparable to that observed on this CSP (*26, 27*).

Structure and Dynamics in Diastereomeric Associations

Based on the presume of elimination of nondiscriminating interactions, consideration is directed to the manner in which diastereomeric association to produce different degrees of stability occurs between chiral selector molecules bound to the silica surface and the enantiomeric selectands. NMR has been shown the best means for clarifying the structures of such transient diastereomeric complexes, and, in some cases, stability differences since, as clearly shown by Pirkle *et al.* (*28*), chromatography indicates essentially weighted average of the contributions of all possible complexes in the same manner as does NMR.

Our systems are based on simultaneous bidentate hydrogen bonding. The chiral diamide system from amino acids (**CSP2-4**) affords the "donor-and-acceptor" bidentate motif. While that from tartramide (**CSP1**) is variously transfigured to afford "acceptor-and-acceptor" and "donor-and-acceptor" motifs toward enantiomers. This may be ascribed to the flexible disposition of two sets of amide and hydroxyl functionalities supported by the C_2 symmetric chiral backbone, relative to the rigid disposition of the two amides adjacent to the asymmetric carbon on the amino acid diamides and also to conformational changes involving formation and/or scission of intramolecular hydrogen bonds between these functionalities. Such transfigurations of the hydrogen bonding motif via conformational reorganization may probably be the reason for the broader spectrum of enantiomer separation by **CSP1**, exceeding **CSP2-4**. Conformational reorganization regarded as "induced fit" has been also observed in the amino acid diamide system.

Diastereomeric Association Relevant to Tartaric Acid Derivatives. The "acceptor-and-acceptor" binding motif of (*R,R*)-**1b** toward 1,2-diols was clarified by examining the X-ray crystal structure of a complex consisting of (*R,R*)-**1b** and (*S,S*)-9,10-dimethyl-9,10-dihydrophenanthrene-9,10-diol (**5**)(*29*), as illustrated in Figure 3. This bimolecular complex is comprised of two sets of hydrogen bonds between gauche hydroxyls of (*S,S*)-**5** and two amide carbonyls of (*R,R*)-**1b**, and twists about the C_2 axis identical with that of each component so as to minimize steric interactions between two components. In the complex, (*R,R*)-**1b** forms an intramolecular hydrogen bonded ring consisnting of an amide proton and a hydroxyl oxygen adjacent to the amide unit on either side of two asymmetric carbons. The relative orientation of these rings displays a propeller-like twist, the rotation sense of which is the reverse of that shown by gauche hydroxyls of (*S,S*)-**5**: one with counterclockwise and the other with clockwise rotation.

The racemic *trans*-**5** was one of the most effectively separated 1,2-diols in all these series on **CSP1**, and from the preferential retention of (*S,S*)-**5** the most stable associate was concluded to be formed between the (*R,R*)-**1b** analogue on **CSP1** and (*S,S*)-**5** rather than its *R,R* enantiomer (*29*). The participation of two hydroxyls of **5** in the hydrogen bonding system with (*R,R*)-**1b** was observed by ^{1}H-NMR; the chemical shift of (*S,S*)-**5** for both of which shifted downfield to a greater extent than that of (*R,R*)-**5** when (*R,R*)-**1b** was added to a $CDCl_3$ solution of racemic *trans*-**5**. Conformational adjustment to allow for bidentate hydrogen bonding of the two amide

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Figure 3. X-ray Crystal Structure of the Associate of (*R*,*R*)-**1b** with (*S*,*S*)-**5**. (Reproduced from ref. *29*. Copyright 1988 American Chemical Society.)

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carbonyls of (*R*,*R*)-**1b** with **5** in solution may thus possibly occur when the two hydroxyls of (*R*,*R*)-**1b** are restrained through formation of their intramolecular hydrogen bonding on the opposite side. An intramolecular hydrogen bond between two hydroxyls of (*R*,*R*)-**1b** could not actually be detected in the crystal structure but this formation in solution should be possible through their gauche relationship.

The (*R*,*R*)-**1b** conformer reorganized by the hydrogen bonding network can be superimposed on methylenated derivative **6**. This 1,3-dioxolane derivative provides two amide carbonyls for bidentate hydrogen bonding with (*S*,*S*)-**5** in the same sense of rotation as that of (*R*,*R*)-**1b**, provided the hydrogen bonded ring is formed between an amide proton and oxolane oxygen on either side. Following the addition of (*R*,*R*)-**6** to racemic *trans*-**5**, the ^{1}H-NMR resonance of the two hydroxyls of (*S*,*S*)-**5** shifted, as would be expected in the case of (*R*,*R*)-**1b**, downfield to an extent greater than that noted for (*R*,*R*)-**5**. Our observation on complex stability appears to be supported by the finding that 1,3-dioxolane derivative **6**-immobilized CSP (**6b**) retained (*S*,*S*)-**5** the most strongly. Thus, (*R*,*R*)-**6** should associate enantioselectively with (*S*,*S*)-**5** through the same bidentate hydrogen bonding motif as that for the system of (*R*,*R*)-**1b** and **5**.

(*R*,*R*)-**6** (*S*,*S*)-**5**

	R
6	*i*-Pr
6b	$-(CH_2)_{11}-Si(CH_3)_2-O-Si$

A "donor-and-acceptor" motif of (*R*,*R*)-**1b** can be produced by rotating one set of asymmetric carbon-carbonyl carbon bonds in the present conformer. This alternative conformer substituting hydroxy oxygen-amide proton by hydroxyl proton-amide carbonyl hydrogen bonding in the back-side network could be considered from that actually observed in the X-ray crystal structure of self-associated (*R*,*R*)-**1b** (Dobashi, Y.; Dobashi, A.; Iitaka, Y.; Hara, S. unpublished data.).

The association mode between (*R*,*R*)-**1b** and (*S*,*S*)-**5** led to the concept of "complementary twist" in molecular design for chiral recognition. Based on this concept, (*R*,*R*)-4,4'-bi[5-(*N*-isopropylimino)-1,3-dioxolane] (**7**) was designed as a novel chiral selector such that two *N*-isopropylimino groups serve as hydrogen-bond acceptors (*30*).

This chiral selector showed greater enantioselectivity for *trans*-**5**. (*R*,*R*)-**7** can duplicate the conformational state of (*R*,*R*)-**1b** in association with (*S*,*S*)-**5**; that is, two bonds between dioxolane oxygens and asymmetric carbons have a gauche relationship possibly as a result of a preferable conformation of (*R*,*R*)-**7** which gives rise to an anti relationship between two imino nitrogens each having bulky isopropyl substituent. Due to minimum steric interaction between the two components, two association sites in combination, one with counterclockwise and the other with clockwise rotation, should be also complementary in this system. A large shift differ-

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ence was noted in the ^{1}H-NMR resonance of the two hydroxyl protons of *trans*-**5**. Taking advantage of this, each enantiomer of *trans*-**5** was titrated by (*R*,*R*)-**7**. Based on the assumption of the formation of a 1:1 associate, the association constant was determined as 25 for the (*R*,*R*)-**7**-(*S*,*S*)-**5** and 5.8 for (*R*,*R*)-**7**-(*R*,*R*)-**5** system (296K). The former associate is thus shown to possess a greater stability of 0.86 kcal/mol than the latter in consideration of the difference in free energy. Justification for the associated structure is demonstrated by the involvement of the imino nitrogen of (*R*,*R*)-**7** as a hydrogen accepting site.

(*R*,*R*)-**7** (*S*,*S*)-**5**

Diastereomeric Association Relevant to Amino Acid Derivatives. Enantiomeric mixtures of *N*-acetylvaline *tert*-butyl ester (**8**) were found to form diastereomeric dimers interlinked via NH--O=C (ester) hydrogen bonds in a "donor-and-acceptor" motif (*31*) and differing in stability. This difference was elicited from a split of the amide proton of **8** into two signals for the D and L enantiomers, termed self-induced NMR nonequivalence (*32-34*), as observed in enantiomerically enriched mixtures. The chemical shift difference in these signals increases as far as the enantiomeric composition differs from that of the racemic mixtures.

Self-Induced NMR Nonequivalence. An NMR study confirmed the amide proton and each carbonyl carbon of both the ester and amide group to be hydrogen bonding sites, based on concentration dependence of these chemical shifts. Of the two carbonyl carbons of **8**, the signal of the ester carbonyl carbon showed nonequivalence, as also noted essentially for the amide proton, though this signal shifted downfield to a lesser extent than that of the amide carbonyl carbon with an increase in concentration. It is thus evident that the association motif of **8** is comprised of two types of intermolecular hydrogen bonds: NH--O=C (amide) and NH--O=C (ester). The diastereomeric species produced by the bidentate latter bonds are likely the cause of NMR nonequivalence. Hydrogen-bond dimers interlinked via the former bond at dimer equilibria would not contribute to NMR nonequivalence due to an only singular interaction; they function rather to lessen nonequivalence under the weighted average of all possible equilibrium species.

Self-induced NMR nonequivalence is not essentially dependent on stability differences in diastereomeric species. However, it can be found from the relationship between the upfield deviation of each enantiomer from the chemical shift of the pure enantiomer and enantiomeric composition in enriched mixtures, according to mathematical treatment of nonequivalence as conducted by Kabachnik *et al.* (*35*). The ratio of the equilibrium constants of homochiral and heterochiral dimerization (K_{homo}/K_{hetero}) of **8** was determined at the following temperatures: 1.25 (20°C), 1.45 (0°C), and 1.96 (-20°C) in 0.1 M of CCl_4 solution (*11*). The homochiral (L-L) dimer is thus shown to possess a greater stability than the heterochiral (L-D) dimer.

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The chiral interactions described in the previous section is the interaction that is capable of bringing about stability differences in diastereomeric complexes. Thus, the NH--O=C (ester) hydrogen bond of **8** should be such a chiral interaction responsible for enantiomeric separation.

L-8

L-8

Self-Induced Chiral Separation. The stability difference between diastereomeric dimers of **8** was the most important factor for the separation of enantiomeric **8** without any foreign chiral substances in nonaqueous bare silica gel chromatography. This was observed when injecting ^{14}C-radiolabeled racemic **8**, previously diluted with unlabeled L-**8**, into a column and detecting the radioactivity (*11*), as shown in Figure 4.

The L enantiomer in the solute zone enriched with L enantiomer was retained the least on the stationary surface due to additional stability imparted to the homochiral dimer in the bulk mobile phase. This consequently gave rise to frontal zone spreading of the L enantiomer to greater extent than that of the D counterpart under the overloading conditions, resulting in their separation.

When the L enantiomer is continuously loaded on the column, the situation in the bulk mobile phase may possibly be maintained provided that recognition inducement by association between the solute and additive adsorbed onto the silica gel surface is cancelled out. Using the above L-**8** solution as CMPA, ^{14}C-labeled racemic **8** was separated into two distinct peaks on a silica gel column and the elution order was the same as that in the self-induced chiral separation (k' of the first-eluted L enantiomer and α, 3.94 and 1.20, respectively, with 10%(v/v) $CHCl_3$ in *n*-hexane containing 0.1 M of L-**8**.). The above assumption was validated at least in part by the finding that CSP, whose *C*-terminal *N*-*tert*-butylamide of CSP2 is substituted by the *tert*-butyl ester, retained the L enantiomer of **8** the most strongly (k' of the first-elute D enantiomer and α, 1.45 and 1.09, respectively, with 1%(v/v) 2-propanol in *n*-hexane.). It should be pointed out that this chromatographic observation is consistent with different stability in **8** indicated by the NMR study.

Diastereomeric Complex Structure on CSP2. In the previous section, CSP terminated with the L-amino acid diamides (**2-4**) was found capable of separating enantiomeric *N*-acylated amino acid esters more efficiently than any other types of enantiomer. A chiral recognition model to account for the separation on CSP2 is proposed based on IR and NMR studies using a solution of *N*-acetyl-L-valine *tert*-butylamide (**2b**), a soluble analogue of CSP2, and *N*-4-nitrobenzoylleucine isopropyl ester (**9**) (Dobashi, A.; Dobashi, Y.; Fukutomi, R.; Hara, S. unpublished data.). A more stable (L-L) diastereomeric complex is induced through the simultaneous forma-

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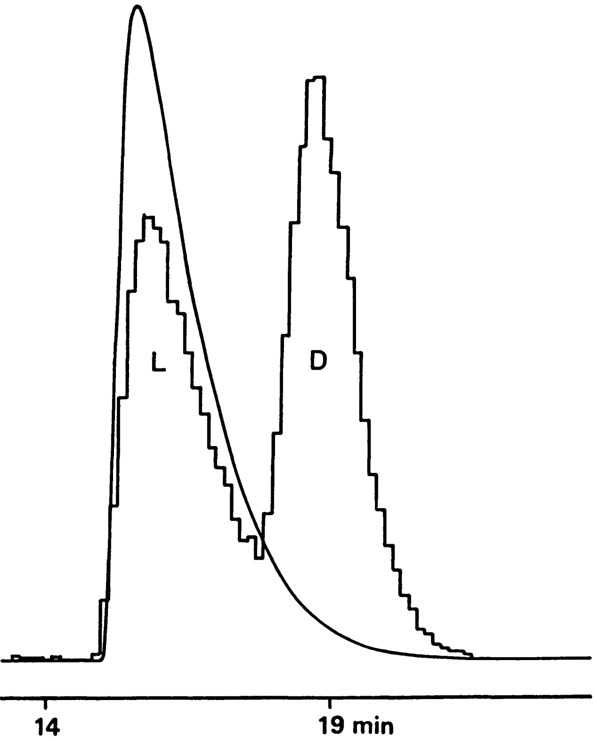

Figure 4. Separation of Racemic [$^{14}COCH_3$]-**8** Diluted with L-**8** on a Silica Gel Column. Conditions: mobile phase solvent, 7.5%(v/v) 2-propanol in *n*-hexane; column temperature, 9.5° C; flow rate, 1 mL/min; detection, UV at 230 nm and ^{14}C radioactivity by scintillation counting with a flow cell; a mixing ratio of the racemic (2.4 mM in $CHCl_3$; 11.7 μCi/mL) and L-enantiomeric solution (0.095 M in $CHCl_3$) by weight (1:128) and injection volume of the mixture (20 μL) (k'_L 3.15, k'_D 3.89; α 1.23).

tion of bidentate NH--O=C hydrogen bonds between **2b** and **9**, and hydrogen bonding sites in **2b** are the *N*-terminal carbonyl group and *C*-terminal amide proton, designated as C_7 side.

L-**9**
R=*sec*-Bu

2b

The pair of enantiomers of **9** showed distinct NMR resonance in the presence of the soluble analogue **2b** by external comparison. In equimolar amount mixtures of **2b** and L-**9** in $CDCl_3$, the resonance of the nitrobenzamide proton H_3 shifted downfield more than the corresponding mixture of D-**9**. This chemical shift difference indicates diastereomeric associates to be formed instantaneously between **2b** and enantiomeric **9** in possible binary association equilibria containing the self-association of **2b** and that of **9**. This difference would thus appear to **2b** in a manner similar to **9**.

Of the two amide protons of **2b**, the chemical shift difference of the *C*-terminal amide proton H_2 was greater than that of *N*-terminal amide H_1 throughout the concentration range examined. But of the two carbonyl carbons, i.e., *N*-terminal C_1 and *C*-terminal amide carbonyl carbon C_2, the resonance of C_1 showed greater chemical shift difference between the diastereomeric mixtures. For **9**, such a difference was observed only on ester carbonyl carbon C_3. From these results, the chemical shift differences may be considered to be induced through formation of diastereomeric dimers, in which associative interactions are bidentate NH--O=C hydrogen bonds between the C_7 side of **2b** and C_5 side of **9**, at least in the most stable associated complex (chemical shift differences observed by external comparison of the equimolar amount of mixtures of **2b** and **9** are as follows: *N*-terminal (H_1) and *C*-terminal amide proton (H_2) of **2b**, 0.067 and 0.108 ppm, respectively, and nitrobenzamide proton (H_3) of **9**, 0.097 ppm in 0.1 M each solution; *N*-terminal(C_1) and *C*-terminal amide carbonyl carbon (C_2) of **2b**, 0.073 and 0.028 ppm, respectively, and ester carbonyl carbon (C_3) of **9**, 0.053 ppm in 0.05 M each solution ($CDCl_3$).).

Amino acid diamides form an intramolecular hydrogen bond on the C_7 side as indicated by IR measurements on the NH stretching band in solution (*36, 37*); that is, intramolecularly hydrogen bonded and nonbonded conformers, designated as folded and extended forms, respectively, are in equilibrium. When the enantiomeric amino acid derivative **9** approaches the chiral valine diamide selector on CSP**2**, the intramolecular hydrogen bond on the C_7 side (C_7 chelation) is preferably displaced by bidentate intermolecular hydrogen bonds with the enantiomeric selectand. The formation of intermolecular hydrogen-bond associates may possibly be accelerated through formation of an intramolecular hydrogen bond on the C_5 side (C_5 chelation) situated behind the selector. By such hydrogen bonding rearrangement, the enantiomeric selectand is retained on CSP**2**.

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CSP terminated with *N*-methyl-L-valine diamide (**2c**), lacking the C_5 hydrogen bonding side, confirmed the assumption that a "donor-and-acceptor" hydrogen bonding motif in the C_7 side of the selector is the key factor for determining the preferential retention of enantiomers. CSP2c was capable of separating the amino acid derivatives in the same elution order as that observed on CSP2 though the αs were less (for instance, k' of the first-eluted D enantiomer and α for the leucine derivative, 2.50 and 1.73, respectively, under the same conditions as those used on CSP2.). However, the soluble analogue of CSP2c itself, *N*-acetyl-*N*-methylvaline *tert*-butylamide, was not retained on CSP2c. This chromatographic observation indicates that the C_7 chelation cannot be scissored without participation of the C_5 chelation, and the hydrogen-bond association on **2b** may possibly be controlled by its intramolecular C_5 chelation.

It has been said that the structure we have proposed does not adequately account for the observed stability difference since either enantiomer of the amino acid derivatives would be capable of forming these bidentate hydrogen bonds in the case using the classical chiral diamide phase, (*N*-formyl-L-valyl)aminopropyl silica gel. An alternative amide dipole stacking model is thus proposed, as a "face-to-face" rather than "edge-to-edge" approach (*28, 38*). Counterevidence is not shown at least by the NMR study.

Computer-aided chemistry should be capable of providing an answer to the relative free energies of diastereomeric complexes (*39-43*) in the face of the subtle problem as to whether the enantioselectivity observed in chromatography can be predicted in our hydrogen bonding model containing various conformational analogues, and whether dipole stacking should be considered as higher energy intermolecular complexes for this prediction assuming that these diastereomeric complexes would be not the major but yet significant species leading to differences in stability.

Chiral Separation under Aqueous Media

Hydrophobic interactions contribute most to molecular association in aqueous media (*44*). The chiral separation of enantiomers possibly occurs when other binding forces such as hydrogen bonding enter the hydrophobic microenvironment well shielded from bulk aqueous phase, along with hydrophobic interactions.

Aqueous Phase Operation of CSP2. CSP2 terminated with L-valine diamide affords such an environment in which hydrogen-bond association may be induced for the resolution of enantiomeric *N*-acylated amino acid esters in reversed phase liquid chromatography (Dobashi, A; Dobashi, Y.; Ono, T.; Ishida, K.; Hara, S. unpublished data.). CSP2 has a long decamethylene spacer by which the chiral moiety is partitioned off from the silica surface. This spacer unit contributes to formation of a hydrophobic interfacial phase, as well as amino acid side-chains and the *tert*-butyl group of the chiral moiety.

Formation of the hydrophobic interfacial phase and the influence of overlaying solvents, i.e., water-organic solvent mixtures, on CSP2 can be detected by the fluorescence of pyrene sorbed onto this phase, as observed in chemically bonded packings such as octadecyl-bonded silica gel (*45-47*). The intensity ratio of pyrene fluorescence peak at 383 nm relative to that at 373 nm indicates microenvironment polarity around pyrene as probe (*48*).

Microenvironment polarity in the chiral layer was noted to depend on the particular organic solvent used and its concentration. With water-methanol mixtures, octadecyl-bonded phase has been shown to reach a minimum polarity at about 50%(v/v) methanol (intensity ratio, about 1.3, this being less than that in alkane solution, about 1.7), followed by increase in methanol concentration. The phase polarity increased again with the methanol concentration exceeding 50%. Intercalation of

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methanol in the bonded layer is the reason for this increase. For CSP2, no such difference transition was noted in the two regions of solvent composition. The intensity ratio, following a slight initial increase from 1.08 (0.8%(v/v)) to 1.10 (10%(v/v) methanol), gradually decreased with increase in methanol and was still 1.08 at 30%(v/v) methanol. Collapse of the hydrocarbon-bonded phase on exposing the probe to the mobile phase and silica surface would appear to account for reduction in phase polarity for high water fractions (*47*). Possibly, CSP2 produces a hydrophobic environment with sufficient volume capable of adequately shielding pyrene from solvent and silica surface polarity even in a region where the water fraction is high.

Figure 5 shows a typical resolution of racemic *N*-4-nitrobenzoylamino acid isopropyl ester on CSP2 without the trimethylsilylation. The L enantiomer, rather than the corresponding D enantiomer, was retained the most in a series of amino acid derivatives. The elution order was identical to that in nonaqueous phase experiments though the α was less. Hydrophobic interactions are the major factor determining the degree of retention of a solute enantiomer on CSP2. The elution order of each amino acid derivative was thus determined by the degree of hydrophobicity of the amino acid side-chain in the solute.

The trimethylsilylation of CSP2 led to greater enantiomer retention, as expected from observation that exhaustive trimethylsilylation of the chiral bonded phase actually caused the intensity ratio to increase throughout the region examined (increase: 0.08 at 0.8%(v/v) methanol), but slightly lessened the α (for instance, k' of the first-eluted D enantiomer and α for the leucine derivative, 3.65 and 1.28, respectively, with 40%(v/v) water-methanol mixture). Reduced enantioselectivity under aqueous mobile phases may be explained by the intervention of achiral hydrophobic interactions in the overall retention process, as discussed in the previous section, and also by competition of mobile phase components penetrating the interfacial phase with diastereomeric association. The following trimethylsilylation brought about adverse effects though not in the nonaqueous phase operation and this is partial support for the above explanation.

A less polar organic solvent, such as tetrahydrofuran (THF), must become distributed to a greater degree within the phase than the more polar methanol, consequently, creating greater polarity. At 25%(v/v) THF, the intensity ratio was 0.90. The hydrophobic features of interfacial phase to bring about enantioselective hydrogen bonding between the terminated chiral selector and enantiomers should be hindered by increased intercalation of the less polar THF. Chiral separation was thus prevented with 40%(v/v) water-THF mixtures in all cases.

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Chiral Separation by Micelles. The interfacial phase formed with the water-methanol mobile phase continued to have a lower polarity which depended on solvent composition to a lesser extent than did that of the water-THF mobile phase. The former thus provides a favorable hydrophobic interfacial phase for inducing diastereomeric hydrogen-bond association so that consequently, chiral separation of enantiomers becomes possible.

Prompted by this consideration, chiral amide functionality was incorporated into surfactant molecules so as to clarify the enantioselective entanglement of the micelle with enantiomers. Chiral surfactant molecules associate hydrophobically to form micelles in aqueous solution (*44*), in a manner similar to the formation of interfacial phase on the bonded phase. The hydrogen bonding affinity of their amide units thus becomes effective for chiral separation in such a micellar hydrophobic core. For observation of this, the micellization of chiral surfactants, *N*-dodecanoyl-L-amino acid sodium salts was carried out (*49, 50*).

Micellar enantioselectivity was observed by electrokinetic capillary chromatography (*51-53*), base on the distribution of the solute between the micelle and bulk aqueous phase, without any solid support to hold the stationary phase. Micellar electroki-

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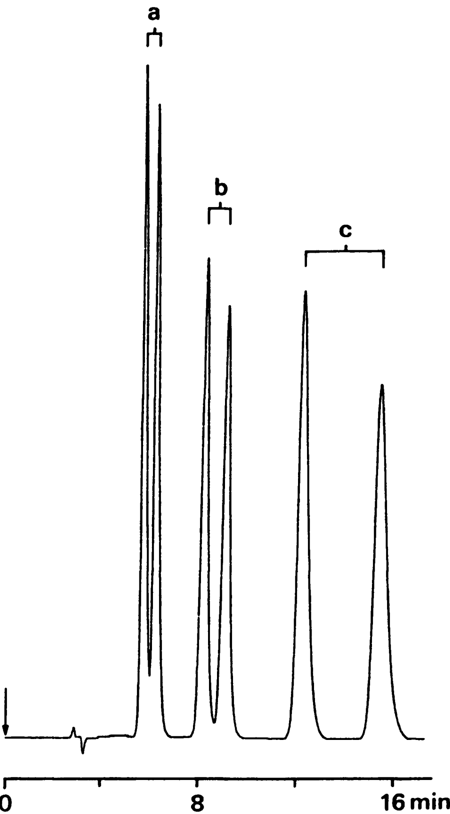

Figure 5. Separation of a Mixture Containing Three Enantiomeric Pairs of *N*-(4-Nitrobenzoyl)amino Acid Isopropyl Esters on CSP2 without the Trimethylsilylation. Conditions: column, 25 X 0.46 (i.d.) cm; mobile phase solvent, 40%(v/v) water in methanol; flow rate, 1 mL/min; column temperature, ambient (*ca* 23°C); detection, UV at 254 nm. k' of the first-eluted D enantiomer and α for each derivative are: 1.00 (α 1.17) for the alanine (a), 1.89 (α 1.17) for the valine (b), and 2.88 (α 1.34) for the leucine derivatives (c).

netic chromatography should thus indicate the distinct binding affinity of chiral micelles toward enantiomers.

Figure 6 shows the results for the resolution of enantiomeric *N*-3,5-dinitrobenzoylated amino acid isopropyl esters with a chiral micellar solution consisting of sodium *N*-dodecanoyl-L-valinate (**10**). The 3,5-dinitrobenzoyl derivatives were separated the most effectively in all solutes examined, containing the corresponding 4-nitrobenzoyl and benzoyl derivatives. Esterification of *N*-acylated amino acids and the aromatic moiety in the acyl substituents are essential for enantiomer separation. In the elution order of the amino acid derivatives separated, the D enantiomer eluted faster than the corresponding L enantiomer in all cases, indicating the chiral micelle to bind to the L enantiomer having the same configuration as its chiral component to a greater extent than the D counterpart. The elution order of each series of amino acid derivatives was dictated by the extent of increase in hydrophobicity of the amino acid side-chains, following entanglement with the micellar interior core.

Amide functionality in the chiral micelles may be as a hydrogen bonding site for entrapping enantiomers into their ordered shallow hydrophobic region. The extent of water penetration into micellar systems was examined by ^{1}H-NMR. An increase in the concentration of **10** above cmc (6 mM in pure water) caused the chemical shift of the amide proton to shift upfield from 7.64 (1 mM) to 7.35 ppm (0.1 M). The amide group is thus buried in the micellar core and shielded from water, thus making it capable of serving as a hydrogen bonding site.

A micelle consisting of sodium *N*-dodecanoyl-L-alaninate (**11**) was then used in place of the micelle of **10** in order to assess the influence of the steric bulkiness of amino acid side-chains in the chiral surfactants on the extent of enantiomer resolution (*50*). A micellar solution of **11** provided smaller αs for all examined solutes in the micelle of **10**. For instance, the leucine derivative providing the most effective separation decreased from 1.31 to 1.17. The intensity ratio of pyrene fluorescence is responsible for the microenvironment polarity of the micellar interior core, as well as that of the interfacial phase. The pyrene intensity ratio for the micellar core was the same as that for the micelle of **10** (1.10). The same order of intensity ratio for two different micellar systems can be interpreted as essentially the same extent of water penetration into both chiral micelles. The lesser enantioselectivity in the micellar system of **11** may thus possibly be due to the lesser perturbation of the micellar structure, as a result of the smaller steric bulkiness of **11** when an amino acid derivative having a configuration opposite to that of the surfactant is intercalated between densely packed pure enantiomeric surfactants.

The micelle with nonpolar hydrocarbon tails oriented inward to form an interior core region provides spatially flexible hydrophobic environment to entangle enantiomers, in contrast to macrocyclic compounds having spatially restricted hydrophobic cavities such as cyclophanes and cyclodextrins (*54-56*). Enantioselective inclusion complexation into the chiral cavities of cyclodextrins has been studied extensively. In such a case, enantiomer becomes bound by hydrogen bonds to hydroxyl groups at the rim of the cyclodextrin cavity.

In addition to ordinary surfactants having nonpolar hydrocarbon tails, there are other types of chiral surfactants having hydrophobic and hydrophilic faces; these are bile salts such as taurodeoxycholate by whose micellar aggregates various enantiomers containing dansylated amino acids can be separated on electrokinetic capillary chromatography (*57*). Though each bile salt shows aggregation behavior differing from that of any of the above ordinary surfactants, chiral separation by their micelles may probably be based on hydrogen bonding and hydrophobic entanglement in combination as well.

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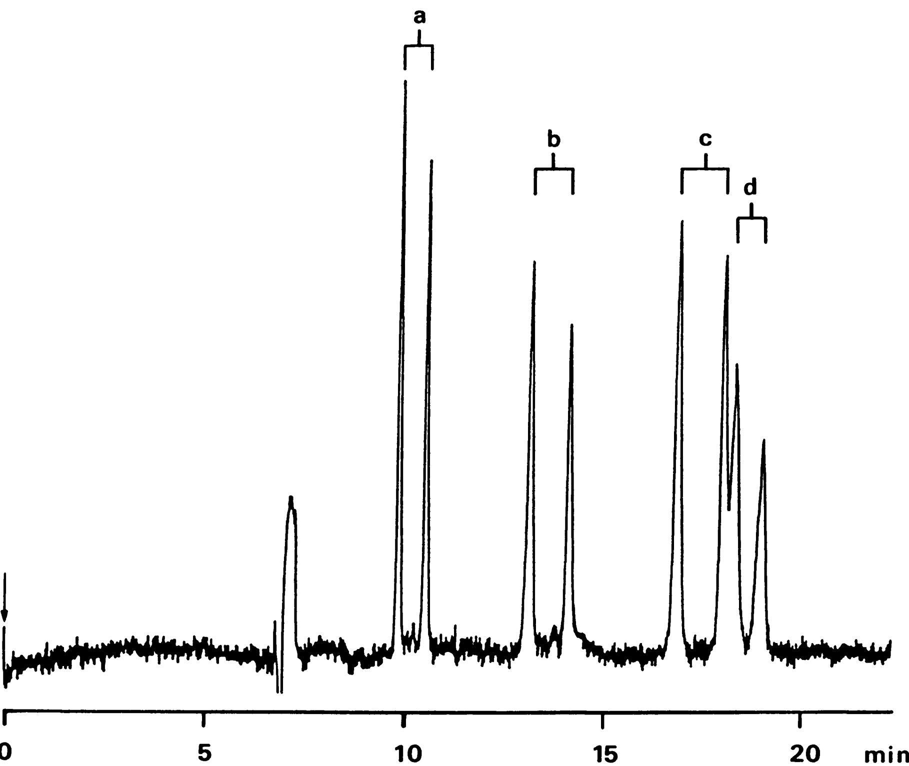

Figure 6. Separation of a Mixture of Four Enantiomeric Pairs of *N*-(3,5-Dinitrobenzoyl)amino Acid Isopropyl Esters by Electrokinetic Capillary Chromatography. Conditions: column, fused silica tubing (Scientific Glass Engineering) 50 cm in length (50 μm i.d.) for effecting the separation; micellar solution, 0.025 M sodium *N*-dodecanoyl-L-valinate (**10**) in 0.025 M borate-0.05 M phosphate buffer (pH 7.0); total applied voltage, *ca* 15.3 kV; current, 40 μA; on-column detection, UV at 230 nm; temperature, ambient (*ca.* 20°C). k' of the first-eluted D enantiomer and α for each derivative are: 0.648 (α 1.31) for the alanine (a), 1.83 (α 1.27) for the valine (b), 5.74 (α 1.31) for the leucine (c), 7.35 (α 1.20) for the phenylalanine derivatives (d). k' for a solute was calculated by $k'=(t_R-t_0)/[t_0(1-(t_R/t_{MC}))]$ where t_R is the retention time of the solute,t_0 that determined with methanol (7.20 min), and t_{MC}, that with the dye Sudan III (24.6 min) (*52*).

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Chapter 11

High-Pressure Liquid Chromatographic Resolution of Optical Isomers

Influence of Mobile and π-Donor Chiral Stationary Phases

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Increase in mobile phase strength of binary mobile phases, with racemic 3,5-dinitrobenzoylphenylethyl amide as solute enantiomer pair, reduced retention but did not affect the selectivity of (R)-naphthyl((R)-NU) or (R)-Phenyl((R)-PU-) urea chiral stationary phases. Modifiers, methanol and acetonitrile, improved resolution but did not affect selectivity. With single-component mobile phases, selectivity was determined by the specific solvent while retention diminished with increase in solvent strength. Sterically hindered solutes showed reduced retention and selectivity. (R)-NU showed much higher retention and selectivity than (R)-PU for all solute types and mobile phase compositions. Phenylalanine was less retained than alanine on both CSPs with all mobile phases, although their selectivity did not follow a fixed pattern. While leucine was well resolved on both CSPs, retention being greater on (R)-NU, isoleucine was poorly resolved on (R)-PU but was well resolved into four components on (R)-NU. All amino acids were chromatographed as the 3,5-dinitrobenzoyl methyl esters.

Molecules which are not superimposable on their mirror images are called optical isomers or enantiomers,and are said to possess chirality or handedness. Chirality exists because of: (a) asymmetry due to differentially substituted tetravalent atoms (such as carbon,silicon, and phosphorous); (b) dissymmetry due to hindered rotation about a single bond; and (c) dissymmetry due to helicity of the molecule.

The decade of the 1980's has seen the rapid rise and growth of optical isomers separation by high performance liquid chromatography (HPLC). The rapid progress in this area is due to factors which include:(a) an increasing awareness of the necessity to determine the enantiomeric purity of chemical and drug substances; (b) more ready availability of reliable chiral stationary phases (CSPs) and other techniques for the resolution of optical isomers (by HPLC); and (c)

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increased understanding of the mechanisms by which resolution is achieved by the various techniques.

In 1980, in Sweden, of the 666 drugs on the market, 174 were racemic (1). In the U.S.A. in 1982, of the 30 new drug entities approved, 22 contained chiral centers, and 11 were racemic mixtures (2). Since different enantiomers of drug substances exhibit differences in their pharmacokinetic and pharmacodynamic properties when administered separately and when mixed in different proportions, it becme increasingly important to develop analytical methodologies for determining the enantiomeric composition of a drug substance before and after its administration. Also, methodologies for producing pure enantiomers in large enough quantities for other studies were required. To meet these challenges, three basic approaches have evolved: (a) precolumn derivatization of the enantiomers using chiral derivatizing reagents to form diastereomers prior to separation on an achiral HPLC column (3); (b) addition of a chiral molecule to the mobile phase to form transient diastereomeric complexes with the enantiomer molecules during elution on an achiral column; and (c) the use of dedicated chiral stationary phases (CSPs) for the direct resolution of enantiomers with or without prior achiral derivatization. All of these approaches have advantages and disadvantages. However,dedicated chiral stationary phases proved to be more useful,more widely studied and understood, and provide a wider choice of phases and conditions for resolving an extremely wide range of optical isomers (4-6).

Today, there are more than 45 commercially available, carefully characterized and quality-controlled dedicated CSPs, the first of which was introduced in 1981 (7-9). These were classified into five general types (Type I-V) according to the manner in which the solute-CSP complexes are formed and separation is ultimately achieved (6, 10). Using this classification, it is possible to select the appropriate CSP for resolving a specific enantiomer pair. In addition, chromatographic data were used to establish a pattern for chiral recognition between the CSP and solute enantiomers (11-18).

This discussion will address primarily Type I CSPs, or "brush" phases due to the manner in which they project from the silica support. They are designed to have a pi-acid or a pi-base moiety to facilitate pi-pi interactions with appropriate solute molecules for increased enantiomeric resolution (11 ,19 ,20) which is achieved by the formation of a transient diastereomeric complex between the solute molecule and the CSP. The difference in strength between the (S)-enantiomer-CSP and the (R)-enantiomer-CSP complexes determines the extent of chiral recognition, that is, the selectivity of the separation (13, 14), and reflects the synergism of all of the forces interplaying in both the stationary and the liquid phases - hydrogen bonding, dipole-dipole, pi-pi, and steric interactions. Three points of interactions are needed between the solute and the CSP for chiral recognition (21,22). Systems involving two points of interactions (23 24) and one point of interaction (27,28) have also been demonstrated.

When Type I CSPs are used, solute molecules containing basic or acidic moieties are generally derivatized. Basic moieties,particularly amines, are converted to a pi-base (naphthoyl derivative) or a pi-acid (3,5-dinitrobenzoyl derivative) resulting in sites for hydrogen

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bonding, pi-pi, and dipole-dipole interactions with the CSP. Also, increased sensitivity, better resolution and more control over the chromatographic process are achieved. One major requirement for chiral recognition is that the amide dipole must usually be located reasonably close to the chiral center of the analyte molecule.

Studies of competing solute-CSP/solvent-CSP interactions on amide linked CSPs (17,27,28) indicate that retention and selectivity cannot be correlated directly to mobile phase polarity but are dependent on the structure of the solvent and its selectivity group which influence the association energy between the solute and the CSP (29, 30). Furthermore, dipole-dipole and hydrogen bonding mechanisms,which may act in opposite stereochemical sense, are in constant competition and the predominant mechanism is determined by the structures of the CSP and the analyte enantiomers (13, 14).

Investigations of urea-linked pi-basic CSPs (18, 28, 31, 32) indicate that: (a) they usually resolve analytes resolvable on the amide-linked CSPs;(b) they possess higher selectivity for the analyte enantiomers than the amide CSP presumably due to the rigid urea backbone; (c) the analytes appear to be resolved by a hydrogen bonding mechanism which competes effectively against the dipole mechanism due to the hydrophoebic nature of the urea moiety; and (d) retention and selectivity are easier to correlate with changes in mobile phase composition for a number of mobile phases.

This paper examines the effect of the differences in size and strength of the pi-basic moieties on the urea-linked CSPs and on factors which affect the strength of the interactions between the analyte and the CSP. The differences are expressed as differences in retention and selectivity of the analyte enantiomers and on peak shape. Also, guidelines for selecting mobile phases and derivative types are formulated from the data.

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Experimental

Materials. The Supelcosil LC-(R)-Naphthyl Urea and Supelcosil LC-(R)-Phenyl Urea chiral columns, the chiral solute test mixture racemic-3,5-dinitrobenzoylphenylethylamine (DNBPEA) and methanolic HCl were gifts from Supelco (Bellefonte, PA, U.S.A.). Racemic leucine, isoleucine, phenylalanine, and alanine were purchased from Sigma (St.Louis, Missouri U.S.A.). All solvents were HPLC grade. Methyl ester derivatives of 3,5-Dinitrobenzoyl amino acids were prepared per (28).

Mobile Phase Mixtures. All binary and ternary mobile phases were hand-mixed and filtered through 0.2 um Millipore filters and degassed under vacuum before use. All mobile phase changes were made by equilibrating the columns with a minimum of 100 ml of mobile phase. Chromatography was carried out at ambient temperatures. Tables I and II list all ofthe binary and ternary mobile phases used in the study. Individual experimental conditions are listed with the figures.

Discussion

Chromatographic Parameters. The results are expressed in terms of the

TABLE I. Binary Mobile Phases

R-Naphthyl Urea CSP		R-Phenyl Urea CSP	
Composition (v/v)	P*	Composition (v/v)	P
a. Hexane/2-propanol			
90:10	0.48	95:5	0.29
80:20	0.86	90:10	0.48
75:25	1.05	83:17	0.75
70:30	1.24	75:25	1.05
50:50	2.00	70:30	1.24
		50:50	2.00
b. Hexane/Methylene Chloride			
50:50	1.60	50:50	1.60
45:55	1.75	40:60	1.90
40:60	1.90	33:67	2.14
35:65	2.05	25:75	2.35
33:67	2.11	21:79	2.47
30:70	2.20		
25:75	2.35		
c. Hexane/Chloroform			
40:60	2.50	50:50	2.10
38:62	2.58	45:55	2.30
33:67	2.78	33:67	2.78
25:75	3.10	25:75	3.10
14:86	3.54	20:80	3.30
06:94	3.86		

d. Hexane/alcohols

Components	Composition (v/v)	P*
Hexane:ethanol	80:20	0.94
Hexane:n-propanol	80:20	0.88
Hexane:2-propanol	80:20	0.86
Hexane:n-butanol	80:20	0.86
Hexane:t-butanol	80:20	0.90
hexane:n-octanol	80:20	0.90

P* = Mobile Phase Strength

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TABLE II. Ternary Mobile Phases

R-Naphthyl Urea CSP			R-Phenyl Urea CSP		
Composition (v/v)	P*	dP	Composition (v/v)	P	dP
a. Hexane/2-propanol/acetonitrile					
75:25:0	1.05	0.00	75:25:0	1.05	0.00
75:25:0.2	1.06	0.01	75:25:0.33	1.07	0.016
75:25:0.5	1.07	0.02	75:25:0.67	1.08	0.032
75:25:1.0	1.09	0.04	75:25:1.25	1.11	0.059
75:25:1.5	1.12	0.07	75:25:2.50	1.17	0.116
75:25:2.0	1.14	0.09	75:25:5.00	1.28	0.230
75:25:3.0	1.18	0.13	75:25:10.0	1.48	0.430
b. Hexane/2-propanol/methanol					
75:25:0	1.05	0.00	75:25:0	1.05	0.00
75:25:0.2	1.06	0.01	75:25:0.31	1.06	0.013
75:25:0.5	1.07	0.02	75:25:0.62	1.08	0.025
75:25:0.6	1.08	0.03	75:25:1.25	1.10	0.050
75:25:1.0	1.09	0.04	75:25:2.50	1.15	0.099
75:25:2.0	1.13	0.08	75:25:5.00	1.24	0.193
c. Hexane/Chloroform/Acetonitrile (dPx10-3)					
36:64:0	2.66	0.00			
36:64:0.003	2.66	0.094			
36:64:0.006	2.57	0.018	Corresponding values for R-Phenyl urea are too small to be significant		
36:64:0.012	2.61	0.038			
36:64:0.025	2.63	0.078			
36:64:0.050	2.71	1.567			
36:64:0.090	2.85	2.820			
d. Hexane/chloroform/methanol (dPx10-3)					
36:64:0	2.55	0.00			
36:64:0.003	2.56	0.073	Corresponding values for R-Phenyl urea are too small to be significant		
36:64:0.006	2.57	0.146			
36:64:0.012	2.58	0.293			
36:64:0.024	2.61	0.585			
36:64:0.048	2.67	1.170			

P* = Mobile Phase Strength

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average capacity factor, **k'(av)**, and selectivity factor, alpha (α). Derivation of these and other parameters is done in **Table III** and from **Figure 1**.

Structures. Both the LC-(R)-Naphthyl Urea (R)-NU (**Figure 2a**) and the LC-(R)-Phenyl Urea (R)-PU (**Figure 2b**) phases are brush-type, urea-linked chiral stationary phases (CSPs) covalently bonded to silica.These phases differ in the strength and bulk of their pi-basic group. With its more bulky naphthyl group, (R)-NU has a stronger pi-basic character than (R)-PU. All other features of the phases are similar. Therefore, differences in retention and selectivity of the analytes are assumed to be due primarily to differences between the naphthyl and the phenyl moieties.

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TABLE III. Derivation of Chromatographic Parameters

to = retention time of an unretained substance
t1 = retention time of the first enantiomer
t2 = retention time of the second enantiomer

$$k'1 = (t1-to)/to \quad (1)$$

$$k'2 = (t2-to)/to \quad (2)$$

$$k'(av) = (k'1 + k'2)/2 \quad (3)$$

$$\alpha = (k'2/k'1) \quad (4)$$

$$dk' = k'[BMP] - k'[TMP] \quad (5)$$

$$dP = P[TMP] - P[BMP] \quad (6)$$

BMP = binary mobile phase
TMP = ternary mobile phase

where P = mobile phase strength (Ref. 29 ,30)

$$= A.X1 + B.X2 + C.X3 + \ldots \quad (7)$$

(A, B, C, ..., represent mobile phase polarity of solvent A, B, C, etc. in the mixture; and X1, X2, X3, etc. represent volume fraction of each component respectively).

Nature of the Analytes. The analyte enantiomers were racemic 3,5-dinitrobenzoylphenylethyl amide (DNBPEA); and 3,5-dinitrobenzoyl methyl ester derivatives of racemic phenylalanine (DNBPME), alanine (DNBAME), isoleucine (DNBIME), and leucine (DNBLME) (**Figures 2c-g**). These analytes were chosen to test the importance of steric, dipole-dipole, and hydrogen bonding interactions between the solute and the CSPs. All carboxy moieties of the amino acids were derivatized to the methyl ester (ME) to maintain consistency in the acid portion of

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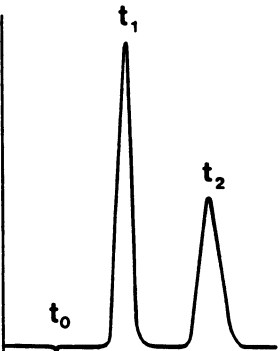

Figure 1. Chromatographic Parameters: to = time for an unretained substance to elute; t1 = time for the first enantiomer to elute; and t2 = time for the second enantiomer to elute from the column.

Figure 2a.

Figure 2b.

Figure 2c.

Figure 2d.

Figure 2e.

Figure 2f.

Figure 2g.

Figure 2. Molecular structures of: (a) (R)-Phenylurea CSP; (b) (R)-Naphthylurea CSP; (c) 3,5-dinitrobenzoylphenylethyl amide (DNBPEA); (d) 3,5-dinitrobenzoylphenylalanine methyl ester (DNBPAME); (e) 3,5-dinitrobenzoylalanine methylester (DNBAME); (f) 3,5-dinitrobenzoylisoleucine methyl ester (DNBILME); and (g) 3,5-dinitrobenzoylleucine methylester (DNBLME).

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the amino acids and to reduce the polarity at the acid end of the solute molecules.

Nature of the Mobile Phase. The mobile phases consisted of solvents which interact through hydrogen bonding, dipole-dipole or steri interactions, or in combinations of these interactions, with the solute and the CSP. Mobile phases were ternary, binary and single-component. Each component was chosen to examine specific solvent-CSP interactions.

Binary Mobile Phases (BMP). These mobile phases were prepared with hexane as the inactive component and 2-propanol, chloroform or dichloromethane as the polar component, representing reciprocal hydrogen bonding, non-reciprocal hydrogen bonding and dipole interactions, respectively. **Figure 3** shows the plots of k'(av) vs. P and **Figure 4** shows the plots of vs. P for DNBPEA on (R)-NU (Curves A,B,C) and (R)-PU (curves A',B',C') with hexane/ 2-propanol (A and A'), hexane/chloroform (B and B') and hexane/dichloromethane (C and C') respectively as the mobile phase. The dramatic differences in retention and selectivity due to the greater pi-donating ability of (R)-NU over (R)-PU are evident from the plots. It should be noted that "selectivity" was compared from data with different k' in some instances since the latter could not be obtained for both CSPs with a single mobile phase. The following are observed from **Figure 3**: (a) retention of the solute enantiomers decreases on both CSPs with increase in mobile phase strength; (b) the percent decrease in retenion is similar on both CSPs for the same mobile phase strength;(c) at much lower mobile phase strength, retention is markedly lower on (R)-PU than on (R)-NU; and (d) on both CSPs, mobile phase strength increases in the order 2-propanol > chloroform > dichloromethane, or in terms of solvent-CSP interactions:reciprocal hydrogen bonding>non-reciprocal hydrogen bonding > dipole interactions.

It is evident from the foregoing that the difference in strength of the BMP required to elute DNBPEA with the same retention time is dependent on the structure of the solvent molecules as well as on their solvent properties. At the same time, however, the strength of the pi-interaction between the solute analyte and the CSP dictates their overall retention. From **Figure 4** the following are observed: (a) selectivity is lower on (R)-PU than on (R)-NU with all mobile phases; (b) selectivity remains unchanged with increase in mobile phase strength for a given binary mobile phase; and (c) selectivity on (R)-PU is similar with the three binary mobile phases while on (R)-NU selectivity increases in the order 2-propanol>dichloromethane> chloroform. These observations suggest that selectivity is not affected by the overall strength of the mobile phase, but rather by the specific solvent properties of the mobile phase components. The greater selectivity seen with (R)-NU over (R)-PU is due to the greater pi-basic character of the naphthyl over the phenyl moiety.

Steric Effects of Solvent Components. **Figure 5** shows a plot of k'(av) vs. Carbon Number for a series of linear and branched alcohols in hexane/alcohol BMPs with DNBPEA as the solute analyte and (R)-NU

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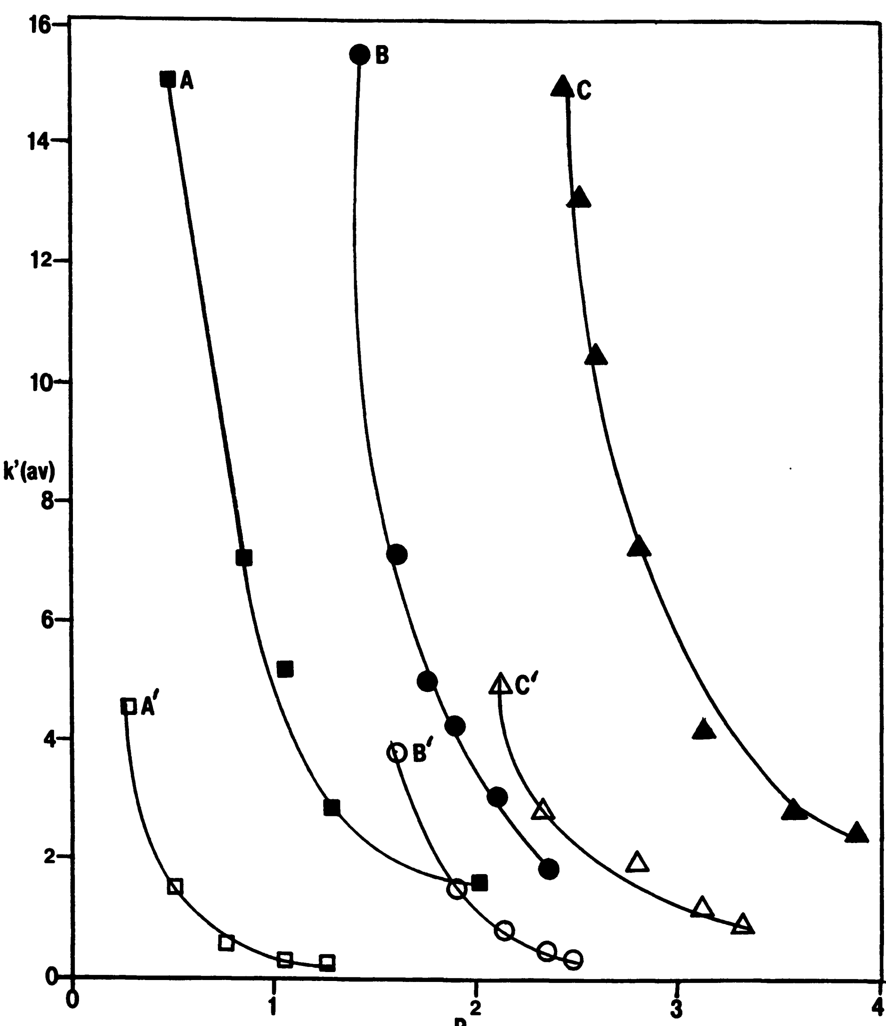

Figure 3 Change in retention, k'(av), of DNBPEA with increase in mobile phase strength (P) of binary mobile phases on (R)-NU (curves A,B,C) and (R)-PU (curves A', B', C'). Binary Mobile Phases: A & A' = hexane/2-propanol; B & B'= hexane/chloroform; and C & C' = hexane/dichloromethane.

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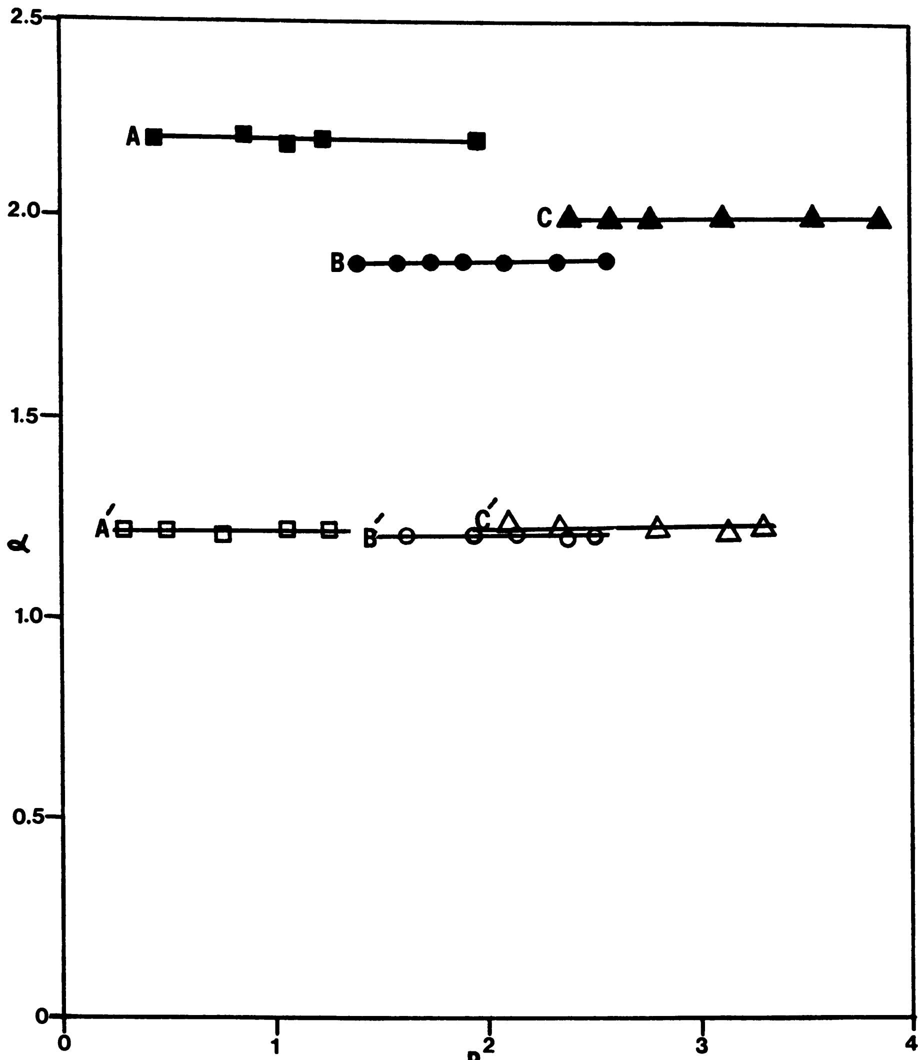

Figure 4. Selectivity, α, of DNBPEA with increase in mobile phase strength (P) of BMPs on (R)-NU (curves A,B,C) and (R)-PU (curves A' B', C'). BMPs:A & A'= hexane/2-propanol;B & B'= hexane/chloroform; and C & C'= hexane/ dichloromethane.

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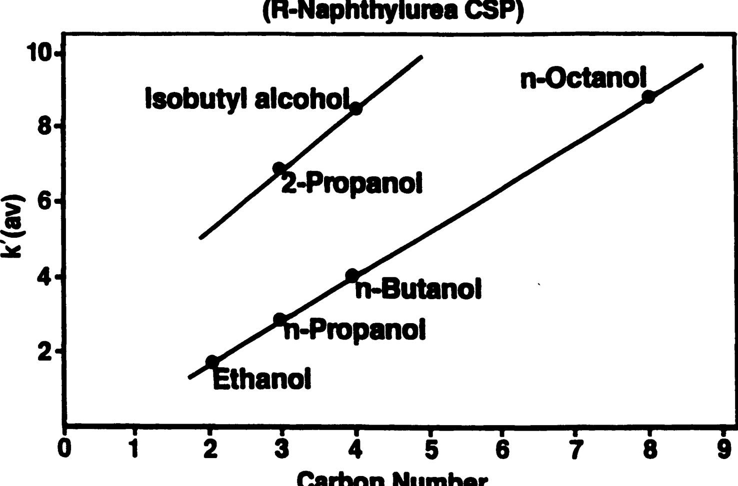

Figure 5. The effect of steric bulk of alcohols in binary mobile phases on retention, k'(av), of DNBPEA, on(R)-NU with increase in carbon number of the alcohol. (Reproduced with permission from reference 18. Copyright 1989 Elsevier.)

as the CSP. With the linear alcohols retention increases linearly with carbon number while branched alcohols have a similar effect on retention as linear alcohols with twice the number of carbon atoms. These results are directly related to the steric bulk of the alcohols since all the mobile phases were prepared with the same mobile phase strength. A similar trend was seen with (R)-PU although retention was much lower in all cases. For both CSPs, selectivity was greater with the branched alcohols than with the corresponding linear alcohol, indicating that the steric bulk of mobile phase components influences the selectivity of the CSP. With the more bulky alcohols, peak shape is poorer indicating that the sterically hindered alcohols are also responsible for poorer mass transfer as well (**Figure** 6)

Ternary Mobile Phases (TMP). Very small changes in modifier concentration have a tremendous effect on retention and resolution on both CSPs, the effect being much more dramatic on (R)-PU than on (R)-NU. When methanol or acetonitrile is used as modifier in hexane/ 2-propanol **BMP**, retention decreases rapidly with minute additions of the modifier. Higher modifier concentrations cause retention to decrease less rapidly. **Figure** 7 is a plot of **dk' vs. dP** and **k'(av)** **vs. dP** (definitions found in **Table** III.) for DNBPEA on (R)-NU and **Figure** 8 for DNBPEA on (R)-PU. The effect of the modifier alone is expressed as the difference in **k'(av)**, **dk'**, between the **TMP** and the **BMP**, as a function of the change in mobile phase strength (**dP**) due to the modifier. From this it is evident that the modifier is primarily responsible for the observed reduction in retention since **dk'** is the mirror image of **k'(av)**. Both modifiers, acetonitrile and methanol, have a similar effect on the **BMP** (hexane/ 2-propanol), although the effect is more pronounced with methanol than with acetonitrile. This may be because methanol is a strong hydrogen bonding solvent and disrupts solute/CSP interactions more greatly. With (R)-PU, the modifier effect is more dramatic, by at least five-fold. This could be due to the weaker pi-interaction with the solute analyte.

Similar but more dramatic trends are seen with hexane/dichloromethane or hexane/chloroform (**Figure** 9) modified with methanol or acetonitrile on (R)-NU, with DNBPEA as the analyte. In both cases, methanol is the stronger modifier. However, the change in retention is even more dramatic than with hexane/2-propanol. Because 2-propanol is a much stronger hydrogen bonding solvent than either chloroform or dichloromethane, it can compete more strongly with the modifiers in solvent-CSP interactions, thereby reducing the effect of the latter. The modifier effect is even greater when (R)-PU is used as the CSP, suggesting that the strength of the pi-interaction is also a factor in solute-CSP interaction. From the foregoing, it is obvious that when working with these CSPs, one must be careful to exclude or control traces of polar contaminants in mobile phase components in order to obtain reproducible solute retentions.

Single-component Mobile Phases. Pure solvents, chloroform and dichloromethane were used to study solvent-CSP interactions using DNBPAME and DNBAME as solute enantiomers. From **Table IV** it is observed that alanine is more strongly retained than phenylalanine on both

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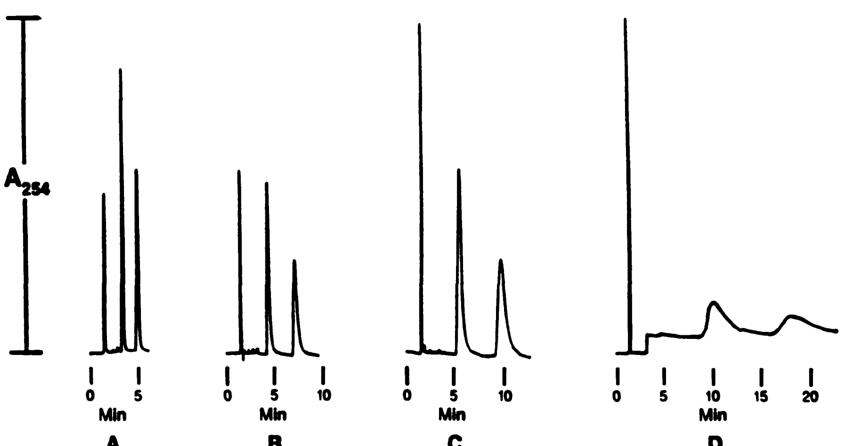

Figure 6. The effect of Steric bulk of alcohols in binary mobile phases on peak shape of DNBPEA on(R)-NU CSP. A = hexane/ethanol; B = Hexane/n-propanol; C = n-butanol; and D = hexane/n-octanol. (Reproduced with permission from reference 18. Copyright 1989 Elsevier.)

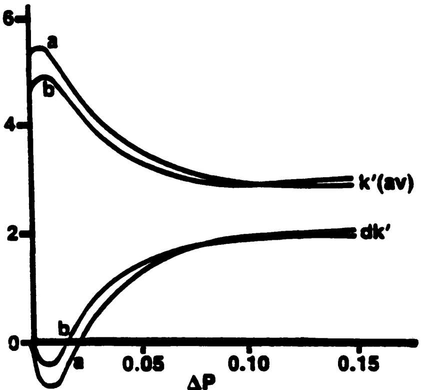

Figure 7. Reduction in retention, k'(av), of DNBPEA on (R)-NU by the addition of methanol (curve b) and acetonitrile (curve a) to hexane/2-propanol (75/25 v/v) mobile phase. The modifier effect is reflected by the corresponding change in retention, dk', with change in mobile phase strength, dP. (Reproduced with permission from reference 18. Copyright 1989 Elsevier.)

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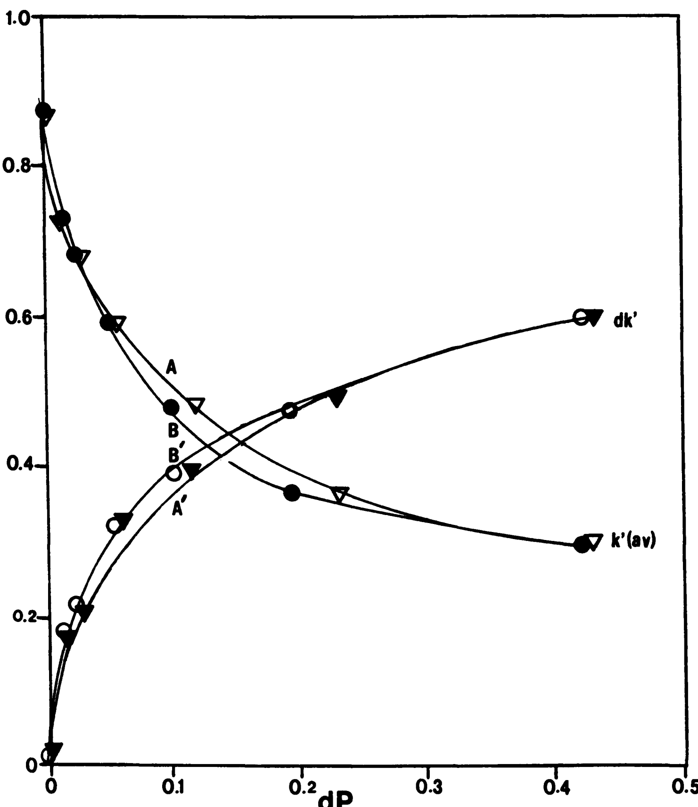

Figure 8. Reduction in retention, k'(av), of DNBPEA on (R)-PU by the addition of methanol (curve B) and acetonitrile (curve A) to hexane-2-propanol (75/25 v/v) mobile phase. The modifier effect is reflected by the corresponding change in retention, dk', with change in mobile phase strength, dP.

CSPs. Morover, both analytes are retained longer on (R)-NU than on (R)-PU. Chloroform causes less retention of the solutes than does dichloromethane due to its greater solvent strength. The strength of dichloromethane can be increased by the addition of a modifier such as acetonitrile. Methanol is too strong a modifier to be used at any concentration and displaces the analyte molecules more readily than dipolar solvent from the CSPs. The more hindered solute, phenylalanine, is displaced more easily.

Nature of the Derivative. The test analytes chosen for the study were derivatized with 3,5-dinitrobenzoyl chloride to arm them with the pi-acid 3,5-dinitrobenzoyl moiety. The pi-acid function serves to orient the analyte molecules to interact in a specific manner with the CSPs through pi-pi interaction with the pi-basic functionalities. This allows the other parts of the solute molecules to orient themselves with the CSPs to facilitate other interactions which influence chiral recognition and retention of the analyte enantiomers. The type

Table IV. Influence of Steric Bulk of solute enantiomers on Retention and selectivity with BMPs and TMPs

Mobile Phase	p*	(R)-NU		(R)-PU	
		k'(av)		k'(av)	
A. Phenylalanine					
Chloroform (100%)	4.10	0.37	1.56	0.16	1.28
Dichloromethane (100%)	3.10	1.10	1.14	0.09	2.86
Dichloromethane/ Acetonitrile (100/1)	3.13	0.79	1.16	0.07	1.19
B. Alanine					
Chloroform (100%)	4.10	1.06	1.58	Eluted in SF	
Dichloromethane (100%)	3.10	3.23	1.28	0.40	1.33
Dichloromethane/ Acetonitrile (100/1)	3.13	2.29	1.40	0.31	1.36

* p=Solvent polarity; Ref. [29, 30].

of derivative formed is very important for chiral recognition on both CSPs. Figures 10a-10c show the chromatograms of three derivatives of phenylalanine. The 3,5-dinitrobenzoyl derivative is very well resolved followed by the p-nitrophenyl-N-acetyl derivative. The phenylthiohydantoin (PTH) derivative, which lacks a pi-acid group, is not resolved. However, on (R)-PU, PTH derivatives of alanine, valine and threonine have been resolved (32). These observations support the need for pi-pi interactions for orienting the solute with the CSP for chiral recognition, and the strength of chiral interaction is deter-

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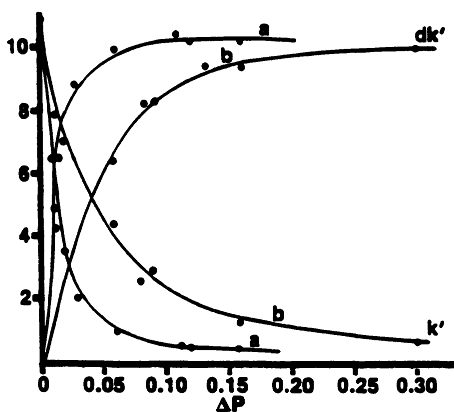

Figure 9. Reduction in retention, k'(av), of DNBPEA on (R)-NU by the addition of methanol (curve a) and acetonitrile (curve b) to hexane/chloroform mobile phase (200/325). The modifier effect is reflected by the corresponding change in retention, dk', with change in mobile phase strength, dP. (Reproduced with permission from reference 18. Copyright 1989 Elsevier.)

Phenylalanine Derivatives
(R-Naphthylurea CSP)

A B C

0 5 | 0 5 10 | 0 5 10
Min.

Figure 10. Effect of derivative type on selectivity of (R)-NU CSP: A = 3,5-dinitrobenzoyl; B = p-nitro-N-acetyl; and C = Phenylthiohydantoin derivatives, all with hexane/dichloromethane/methanol (100/30/1.5 v/v). (Reproduced with permission from ref. 33. Copyright 1989 Supelco, Inc.).

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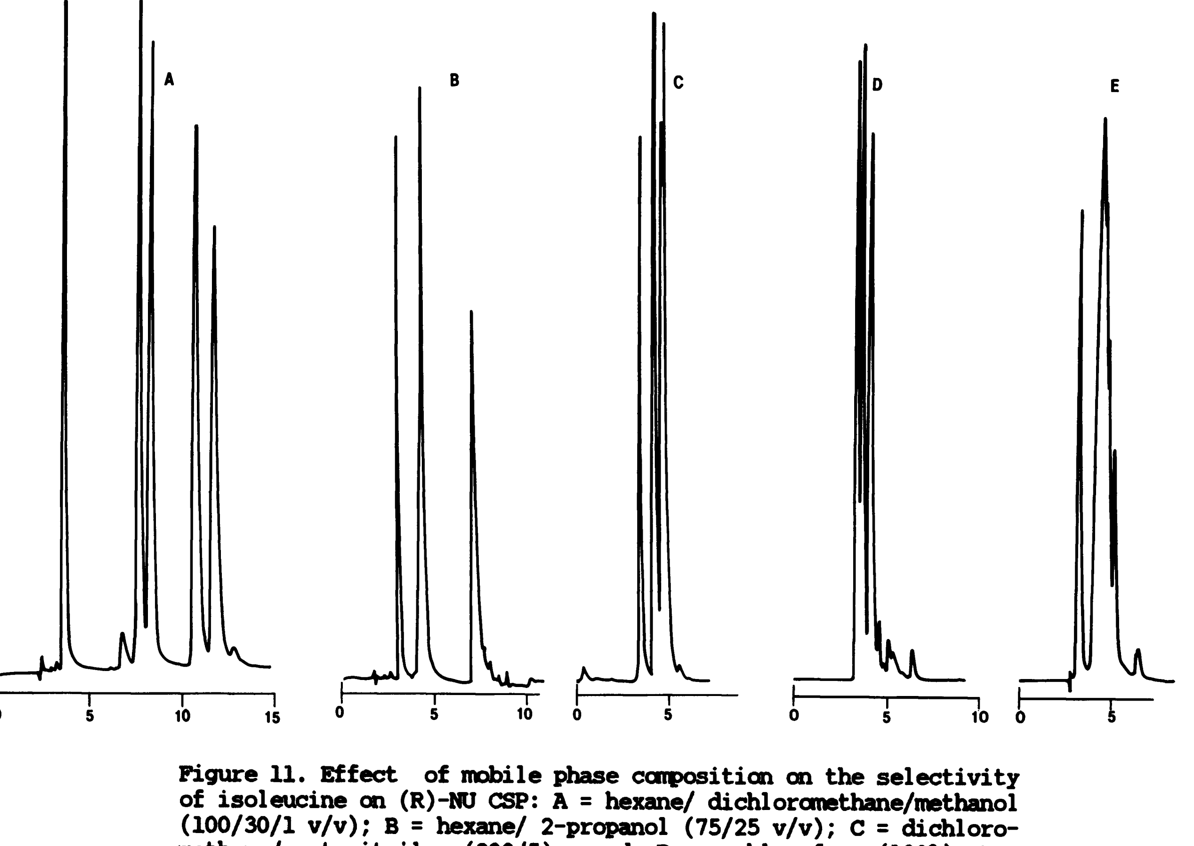

Figure 11. Effect of mobile phase composition on the selectivity of isoleucine on (R)-NU CSP: A = hexane/ dichloromethane/methanol (100/30/1 v/v); B = hexane/ 2-propanol (75/25 v/v); C = dichloromethane/acetonitrile (200/5); and D = chloroform (100%); E = elution of isoleucine on (R)-PU with mobile phase A.

mined to a great extent by the strength of pi-pi interaction between the solute and the CSP.

The Influence of Mobile Phase and Solute Structure on Chiral Recognition. Mobile phase components greatly influence chiral recognition as shown in Figures 11a-11d, which are chromatograms of isoleucine on (R)-NU with different mobile phases. In Figure 11a, isoleucine, which has two chiral centers, is resolved into four components when hexane/dichloromethane/methanol is used as the mobile phase. However, when hexane/2-propanol is used as the mobile phase, only two peaks are observed (Figure 11b). A mixture of dichloromethane/acetonitrile attempts to resolve it into three components (Figure 11c), while chloroform (100%) resolves it into two poorly retained components (Figure 11d). These observations indicate that unless the appropriate mobile phase is used, resolution of optical isomers may not occur. Figure 11e shows the chromatogram of isoleucine on (R)-PU with hexane/dichloromethane/methanol as the mobile phase. Resolution is poor, indicating that the strength of the pi-pi interaction is also important for enantiodifferentiation. Leucine, which has a smaller side chain than isoleucine, was resolved on both (R)-NU and (R)-PU with the same mobile phase, indicating that steric interactions affect selectivity by hindering proper orientation of the solute molecule around the chiral center.

Conclusions

Pi-pi acid-base interactions between the solute and the CSP are important for retention and selectivity in the resolution of optical isomers on brush-type CSPs. The stronger pi-base CSP have larger retention and selectivity values. Also, the structure of the solute analyte molecules helps to determine retention and selectivity; the more hindered analytes are retained less and display lower selectivity. The derivative type determines the primary interaction of the analyte with the CSP thereby aiding chiral recognition. Finally, the choice of mobile phase helps to determine both retention and enantiodiffferntiation. While some general rules can be forwarded for controlling retention, there is no fixed pattern for determining selectivity for widely varying solute-CSP pairs.

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Chapter 12

Direct Stereochemical High-Pressure Liquid Chromatographic Separation of Aminoglutethimide and Its Major Metabolite

Its Applications to Biological Fluids

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A direct, isocratic, sensitive and precise liquid chromatographic method is developed for the enantiomeric separation of aminoglutethimide (AG) and its acetylated metabolite (AcAG) using cellulose tris-3,5-dimethylphenyl carbamate (Chiralcel OD) column and cellulose tris-(4-methylphenyl beonzoate) ester (Chiralcel OJ) column in series. The enantiomeric elution order is determined by chromatographing the racemate aminoglutethimide and racemate acetylated aminoglutethimide separately and their enantiomers under the similar conditions. This method has been applied to determine and identify the enantiomers of AG and AcAG in the urine sample collected from a metastatic breast cancer patient after 24 hours administration of (±)AG. Large amounts of (+)-R-aminoglutethimide are excreted unchanged in the urine together with a smaller quantities of its (+)-R-acetylated metabolites, while most of the (-)-S-aminoglutethimide is metabolically converted into (-)-S-acetylated aminoglutethimide.

In most cases chiral drug enantiomers have different biological and pharmacological activities due to different interactions on the receptors (which also have chiral centers), posses different pharmacokinetics and metabolic pathways. Drug chirality is becoming an important issue facing the pharmaceutical industry and pharmaceutical research in developing of new drugs (1,2).

A rapid survey of the 700 most frequently prescribed drugs shows that 75% of them are marketed and administered as racemic mixture. It is therefore important during the development of drugs to be able to isolate the enantiomers (if chiral centers exist) to assess which is responsible for the potency, the toxicity and for the side effects.

For these reasons, there is a great demand in the pharmaceutical industry for the developments of effective analytical techniques and preparative separation of a variety of enantiomeric compounds that are known to have different physiological activities.

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Different chromatographic techniques have been developed over the past fifteen to twenty years to separate stereoisomeric compounds. But the efficiency, speed, wide applicability, and reproducibility of high performance liquid chromatography (HPLC) have made it the method of choice.

There are now more than 40 chiral stationary phases (CSP's) available commercially for direct separation and resolution of wide range of drug enantiomers by HPLC(3-6). One highly promising is the cellulose-based CSPs (O-Type) described by Ichida, et. al.,(7). The primary mechanism of these phases are the formation of the solute-CSP complex through attractive interactions but where inclusion complexes also play an important role. Chiralcel OD (Cellulose tris-3,5-dimethylphenyl carbamate) (Scheme 1) and Chiralcel OJ [Cellulose tris-(4-methylphenyl benzoate) ester] (Scheme 2), are two of these phases which were used in series in this study for enantiomeric separation of aminoglutethimide (AG) and its major metabolite acetylated aminoglutethimide (AcAG) using a single chromatographic conditions.

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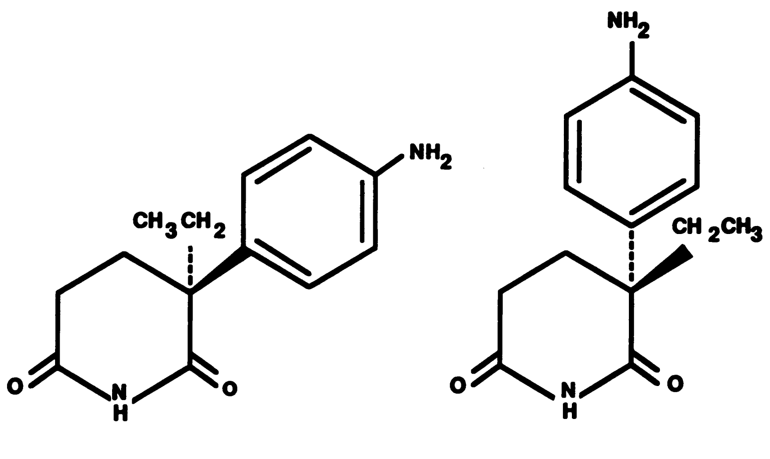
Aminoglutethimide, chemically known as (±)-3-(aminophenyl)-3-ethyl-2,6-piperidinedione (AG) (Scheme 3) is currently clinically used for the treatment of metastatic breast cancer and adrenocortical tumors and Cushing's syndrome(8,9). Metabolic studies in man have shown that, after single doses of AG, large amounts are excreted unchanged in the urine together with smaller quantities of its acetylated metabolite, N-acetyl aminoglutethimide (AcAG), accounting for 4-25% of an oral dose of AG(10,11).

Kamblawi et. al.(12) developed a simple HPLC method for the quan--titative determination of AG and AcAG in urine in order to study the inter-individual variation in the excretion of AG and its acetylation profile in man and other species. Several methods have been developed for the assay of AG in biological fluids and has been reviewed by Aboul-Enein(13). It was reported that the (+)-R-isomer had the most stereodogenesis inhibitory activity (two to three times more potent than the racemate), while the (-)-S-isomer had very little activity at dose levels 10-folds higher(14). Resolution of aminoglutethimide, therefore, provided the compound (+)-R-aminoglutethimide with essentially all of the steroid synthesis inhibiting activity of the racemate.

Resolution of racemic aminoglutethimide adopting chemical methods was reported in earlier works(14). A direct resolution of racemic aminoglutethimide (AG) and its acetylated metabolite (AcAG) was obtained using a 100-mm long α_1-acid glycoprotein column(15). Racemic aminoglutethimide and its acetylated metabolite were also separated using chiralcel OD and chiralcel OJ column(16). Maximum and symmetrical stereochemical resolution (R) obtained for AG was on Chiralcel OD column. Aminoglutethimide was also resolved on Chiralcel OJ column, but the peak obtained was not symmetrical.

Scheme 1. The structure of the chiral stationary phase (OD-CSP) used in this study.

Scheme 2. The structure of the chiral stationary phase (OJ-CSP) used in this study.



Scheme 3. The absolute configuration of (-)-S and (+)-R aminoglutethimide.

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Maximum and symmetrical stereochemical resolution (R) with baseline separation was obtained for acetylated aminoglutethimide on Chiralcel OJ column using hexane and 2-propanol (50:50). Acetylated aminoglutethimide was also resolved on Chiralcel OD column, but the elution order was reversed. Taking in consideration of all the above-cited observations it was decided to develop a method where aminoglutethimide (AG) and its major metabolite (AcAG) could be simultaneously separated and analysed in biological fluids e.g. urine, using one single isocratic solvent system, thus making it more rapid and convenient is described in this paper.

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EXPERIMENTAL

Apparatus

The Waters (Waters Associates, Milford, Mass., 01757, U.S.A.) LC system consisted of a Model M-45 pump, a U6K injector, and a Lamda-Max Model 481 LC spectrophotometer UV detector operated at 257nm. The stationary phase of Chiralcel OD analytical column of cellulose tris-3,5-dimethylphenyl carbamate and Chiralcel OJ analytical column of cellulose tris-(4-methylphenyl benzoate) ester (25cm x 0.46cm, I.D., Daicel Chemical Industries, Tokyo, Japan) coated on silica gel with particle size 10 um were used.

Chemicals

Racemic aminoglutethimide (±AG) (Lot No. 800383), (+)-R-aminoglutethimide (+AG) (CGS-2396), and a racemic acetylated aminoglutethimide (±AcAG) (Ba 17873) were supplied by Ciba Geigy, Basle, Switzerland. HPLC grade hexane was obained from Fisher Scientific, New Jersey, U.S.A. HPLC grade 2-propanol was obtained from Romil Chemicals, Ltd., England. (+)-R-AAG was prepared as previously described method by Aboul-Enein and Islam (15).

Sample Pretreatment: Urine

Fifty ml of urine sample was collected from a female patient with metastatic breast cancer, receiving AG for treatment. It was diluted to 100ml with water, and then extracted with methylene dichloride (25ml x 3). The volume was reduced under reduced pressure and dried with nitrogen flow. The residue was redissolved in an appropriate volume of methanol and injected to the column.

Chromatographic Conditions

The maximum and symmetrical stereochemical resolution of AG and AcAG enantiomers in urine were obtained using hexane and 2-propanol (50:50) on Chiralcel OD followed by Chiralcel OJ

columns in series. The flow rate was 0.7ml/min and chart speed was 0.25cm/min. Temperature was maintained at 23°C. Detection was obtained at UV 257nm with sensitivity range 0.01 AUFS. The sample amount injected was 5 nmole for racemate AG, 2.5 nmole for (+)-R-AG enantiomer, 1 nmole for racemate AcAG, 0.5 nmole for (+)-R-AcAG enantiomer.

Determination of Enantiomeric Elution Order

The enantiomeric elution order was determined by chromatographing the racemate AG and racemate AcAG separately and their respective enantiomers under the similar conditions. Thus racemate AcAG was found to be eluted earlier than racemate AG. In case of racemate AG, the peak that eluted earlier was identified as (-)-S-AG and in case of racemate AcAG, the peak that eluted earlier was found to be (+)-R-AcAG.

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RESULTS & DISCUSSION

Aboul-Enein and Islam(15) recently described a direct stereochemical resolution of racemic aminoglutethimide and its acetylated metabolite using a 100-mm long α_1-acid glycoprotein column. However, the resolution obtained with this column was not symmetrical and without base line separation for AcAG. The method was also found to be lengthy, and required a careful consideration of a number of parameters such as preparation of phosphate buffer and pH adjustment, salt effect, concentration of organic modifier and temperature control. Two new methods for stereochemical resolution of AG and AcAG using Chiralcel OD and Chiralcel OJ column are described by Aboul-Enein and Islam(16). It was found that the maximum stereochemical resolution obtained for AG was on Chiralcel OD column while for AcAG was on Chiralcel OJ column using different solvent systems. This paper describes another developed method where AG and AcAG could be resolved and identified simultaneously in biological fluids using Chiralcel OD and Chiralcel OJ columns in series using one isocratic solvent system, thus making it more rapid and convenient. Using this method, enantiomers of AG and AcAG were also identified in the urine sample collected from a female metastatic breast cancer patient. The optimization of separation were achieved using different concentration of 2-propanol as a mobile phase in room temperature. The chromatogram of enantiomer separation of aminoglutethimide (AG) on Chiralcel OD and Chiralcel OJ in series is shown in Fig. 1. Compared with the chromatogram of (+)-R-aminoglutethimide (Fig. 2) the peak that eluted with a lower capacity factor was identified as (-)-S-AG and the peak that eluted with a higher capacity factor was identified as (+)-R-AG. Maximum stereochemical resolution (R) obtained was 13.68. The chromatogram of enantiomer separation of AcAG on Chiralcel OD and Chiralcel OJ in series is shown in Figure 3. Compared with the chromatogram of (+)-R-AcAG (Fig. 4), the peak that eluted with a higher capacity factor was identified as (-)-S-AcAG. Maximum stereochemical resolution obtained was 4.39.

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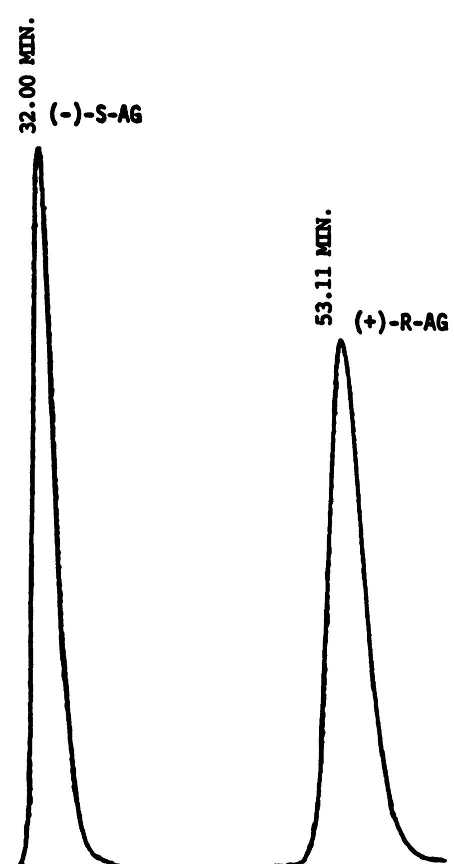

Fig. 1. Enantiomeric separation of racemic AG. Columns: Chiralcel OD and Chiralcel OJ (250 x 4.6 mm, I.D.) in series; mobile phase: hexane and 2-propanol (50:50); flow rate: 0.7 ml/min.; chart speed: 0.25 cm/min.; temperature: 23°C; detector: UV 257 nm; sensitivity: 0.01 AUFS; sample amount: 5 nmole.

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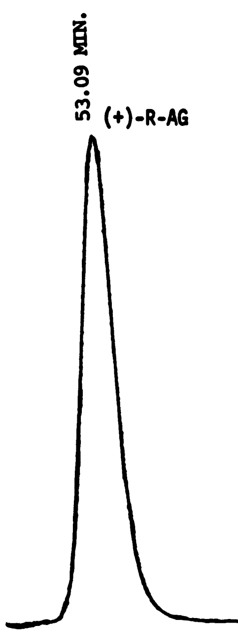

Fig. 2. Chromatogram of (+)-R-AG. Conditions were same as in Figure 1, except the sample amount was 2.5 nmole.

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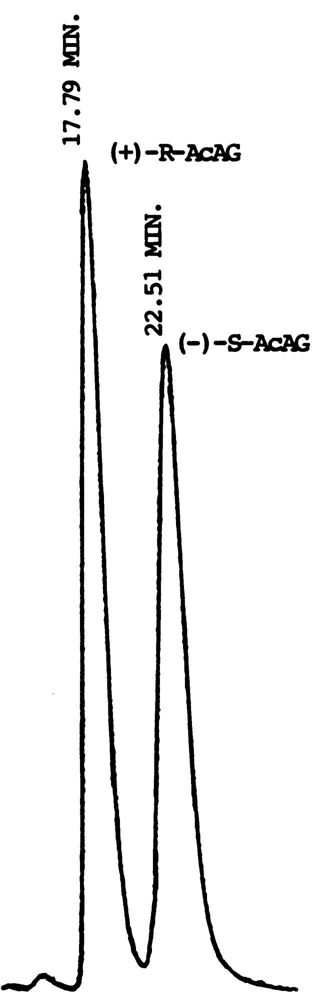

Fig. 3. Enantiomeric separation of racemic AcAG. Conditions were same as in Figure 1, except sample amount injected was 1 nmole.

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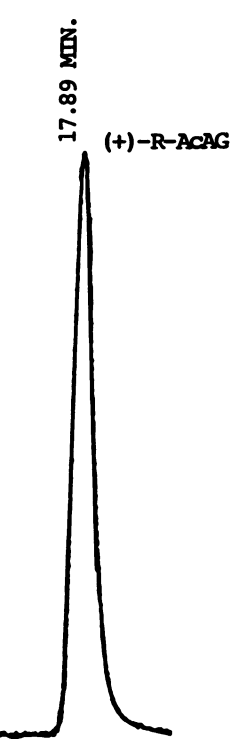

Fig. 4. Chromatogram of (+)-R-AcAG. Conditions were same as in Figure 1, except sample amount injected was 0.5 nmole.

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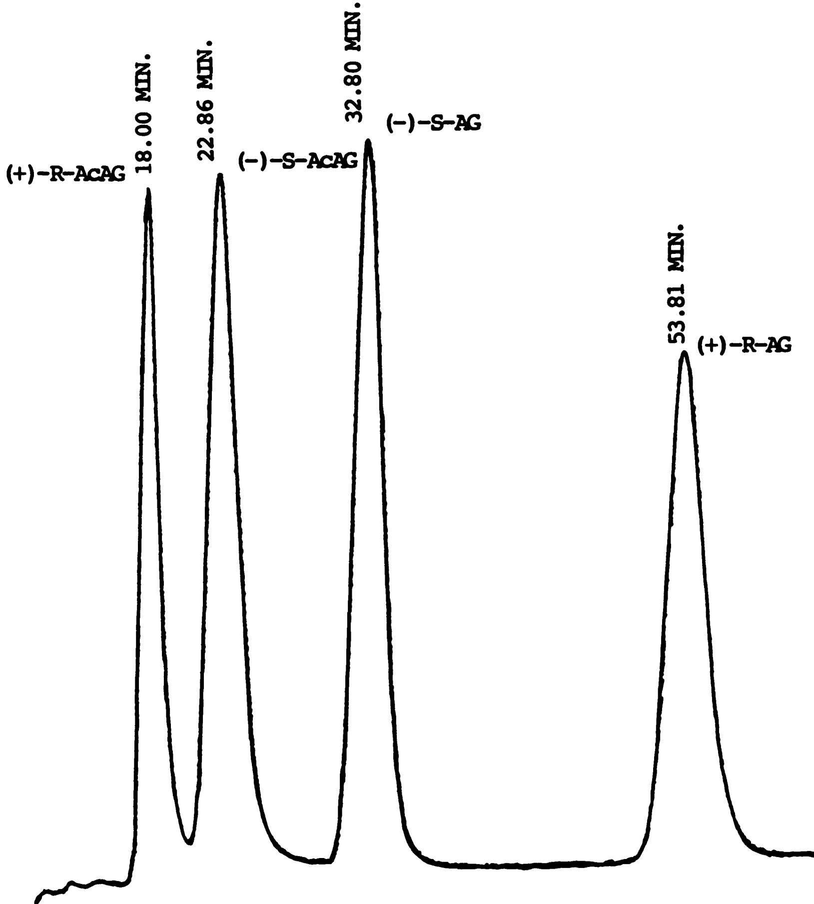

Fig. 5. Enantiomeric separation of mixture of racemic AG & AcAG. Conditions were same as in Figure 1, except sample amount injected was 5 nmole for AG and 1 nmole for AcAG.

Enantiomeric separation of mixture of racemic AG and AcAG is shown in Fig. 5. All the four peaks could be identified separately by comparing it with the chromatograms of Figs. 1-4. The enantiomeric separation of a methylene chloride extract of racemic AG and AcAG in a urine sample collected from a metastatic breast cancer patient after 24 hours administration of AG is shown in Figure 6. All the peaks were easily identified from retention times comparing them with Fig. 5.

CONCLUSION

Direct stereochemical separation of aminoglutethimide (AG) and its acetylated metabolite (AcAg) was achieved on commercially available cellulose tris-3,5-dimethylphenyl carbamate (Chiralcel OD) and cellulose tris-(methylphenyl benzoate) ester (Chiralcel

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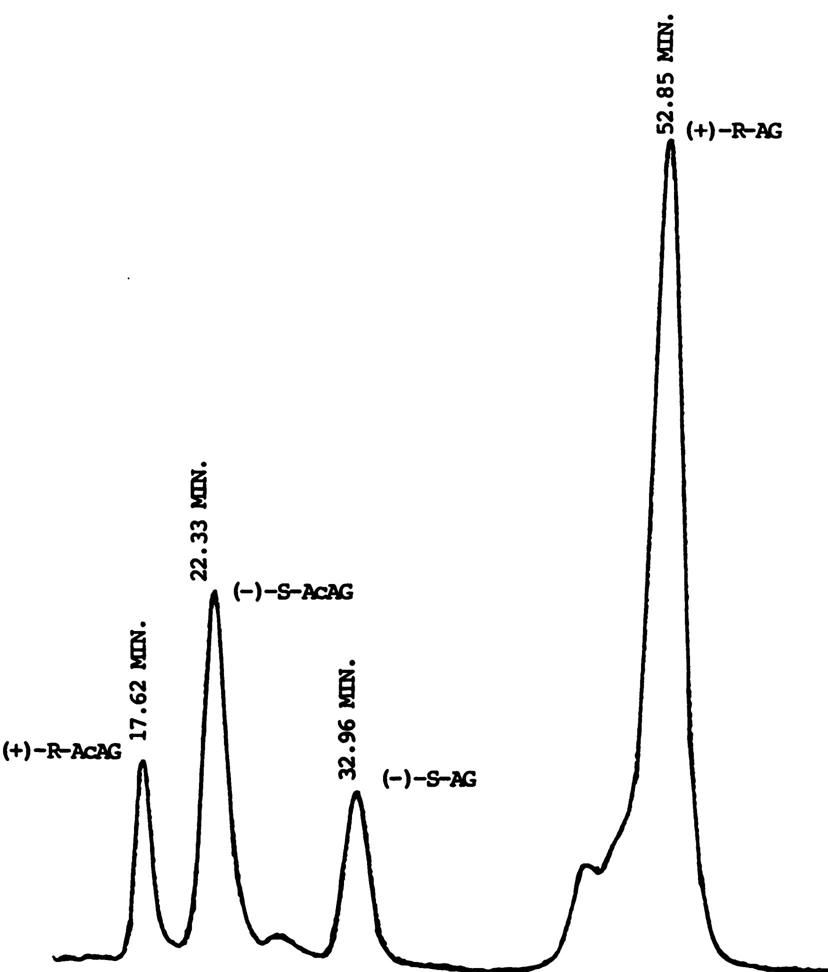

Fig. 6. Chromatogram obtained from the methylene chloride extract of 50 ml of urine sample collected from a metastatic breast cancer patient after 24 hours administration of racemic AG. Chromatographic conditions were same as in Fig. 1.

OJ) columns in series using one single isocratic solvent system, hexane and 2-propanol (50:50) at 23°C with flow rate 0.7ml/min. The method was applied to determine and identify the enantiomers of AG and AcAG in the urine sample collected from a metastatic breast cancer patient after 24 hours administration of AG. The effects of stereoselectivity on drug action and metabolic disposition has been recently reviewed(17). It was observed from this determination that the metabolism of AG racemate in human is stereospecific. A large amount of (+)-R-AG is excreted unchanged in the urine together with a smaller quantities of (+)-R-AcAG, while most of the (-)-S-AG is metabolically converted into

(-)-S-AcAG. This simple method could also be applied to determine and quantitate the AG enantiomers and its acetylated metabolite AcAG in biological fluids. Furthermore, this method could be used for determination of the acetylation phenotype of patients receiving AG.

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Chapter 13

Amino Acid Racemization

A Tool for Dating?

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In living tissue, all amino acids have L configuration, but after the death of an organism they undergo racemization which is a time-dependent process and therefore can be used for dating. The ratio of D to L amino acids found in a fossil is a measure of time elapsed since death. D/L ratios of protein hydrolysates can be determined by various enantioselective chromatographic techniques. Amino acid racemization is an inexpensive dating method and requires little material, e.g. 100 mg of mollusk shell, but the racemization rate depends on temperature, the type of material, and the biological species involved. A straightforward description of the kinetics is not possible. Absolute dating of a fossil is difficult or impossible but amino acid racemization is one more dating technique amongst many others. Suitable materials are mollusk shells, egg shells, foraminifera, and teeth.

With a very few exceptions, all the amino acids used by nature for protein and peptide biosynthesis are in the L (S) configuration. This, however, is not the thermodynamically stable state (as life itself does not represent thermodynamic equilibrium) which is only represented by the racemate, i.e. the 1:1 mixture of D and L enantiomers. This means that after the death of an organism, all the amino acids - free, peptide or protein bound - start to undergo racemization. This is also true for closed-system parts of the living body, mainly certain sections of teeth. Because racemization is a rather slow process, it was an obvious way to determine the age of a fosil by measuring its content of D (R) amino acids. This was first done in 1968, when Hare and Abelson analysed modern, late Pleistocene, and

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Miocene shells and found increased D/L ratios with age (*1*). Since then, quite a number of applications of dating various materials by amino acid racemization (AAR) have been carried out, thereby enriching knowledge in geology, archeology, and anthropology.

Amino Acid Racemization

The Obstacles. Although the principle of AAR is very simple, its use for dating is less straightforward. The AAR rate is influenced by temperature and by the species of organism involved. The kinetics is not always linear or linearizable, especially not for higher D/L values, and it is different for each amino acid present. Laboratory experiments performed at higher temperatures are not really satisfying models as it seems that the extrapolation of high-temperature kinetic patterns to low temperature kinetics in fossils, i.e. to much longer time scales, is questionable. AAR only gives reliable dating results if the material investigated acts as a closed system because otherwise small peptides and free amino acids can be brought in or removed by the surroundings, mainly groundwater.

As every chemical reaction, the rate of AAR of course depends on temperature. A 100,000 year old mollusk shell found in mid-latitudes has a D/L ratio (of some amino acids) of e.g. 0.4, but less than 0.1 in Northern Canada. This makes the method unsuitable for absolute dating even in cases where the detailed and true kinetics would be known because the temperature history of a fossil is rarely ever known precisely enough, especially when long time intervals are dealt with. Because the rate/temperature relationship is not linear, the use of simple mean environmental temperatures is not admissible; instead of this an "effective diagenetic temperature" has to be taken into consideration where only slightly elevated temperature intervals are weighed markedly higher than cooler intervals. With an energy of activation of approximately 30 kcal/mol (120 kJ/mol) (*2*), racemization rate doubles every 4 K at ambient temperatures. The problem becomes obvious by remembering that the mean annual temperature in central Europe or the northern USA was about 5 K lower than present during the various ice ages, with warmer interglacial periods. Fortunately, the situation is much better than it might seem. First, relative dating can be of enormous value especially in geology where it is often possible to distinguish between clearly different aminozones (strata bearing material, e.g. mollusk shells, of nearly uniform D/L values) where other dating techniques and especially traditional field criteria fail in recognizing significant time gaps or unconformities (*3*). Second, if any specimen showing a certain D/L value can be dated by some other method, e.g. C-14, this sample can be used to establish the effective diagenetic temperature of the site where it was found.

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Although AAR is a simple reaction

$$\begin{array}{c}H\\|\\H_2N\text{-}C\text{-}R\\|\\COOH\end{array} \rightleftharpoons \begin{array}{c}H_2N \quad R\\ \backslash\,/\\ C^-\\|\\COOH\end{array} + H^+ \rightleftharpoons \begin{array}{c}H\\|\\R\text{-}C\text{-}NH_2\\|\\COOH\end{array}$$

planar (*4*)

it does not necessarily follow simple linear first-order kinetics, see e.g. (*5*). The reason, therefore, is that protein degradation by hydrolysis and racemization are closely linked together. Both racemization rate and racemization degree are highly variable depending on whether a mid-chain, N-terminal, C-terminal or free amino acid is involved in either protein, polypeptide or dipeptide (*6*). Moreover, both chain-degradation and racemization strongly depend on the type of amino acid involved. This is the reason for the highly different AAR rate of different materials and even of different species (*7,8*): every protein shows its own degradation/racemization pattern depending on its amino acid composition, and all the proteins of a sample sum up to its apparent kinetics.

The kinetics of racemization, i.e. of a first-order reversible reaction, is described by (*9*)

$$\ln \frac{1 + (D/L)}{1 - K(D/L)} = (1+K)\,kt$$

with equilibrium constant $K = 1$ for enantiomers, or 0.77 for the diastereomers allo-/isoleucine, first order rate constant k, and time t. This equation is often used for the description of laboratory heating experiments although the data may not fit well in all cases.

Concerning laboratory heating experiments it is possible that the apparent kinetics obtained by them differs from the natural kinetics at much lower temperatures because the protein degradation processes are not identical.

The Advantages. No extraordinary instrumentation is necessary for AAR analysis since it can be done with either today's widespread gas or liquid chromatography, making the method accessible in principle to almost every laboratory. However, one should bear in mind that the method is trace analysis and that amino acids are abundant. Much less material, e.g. 100 mg of shell (allowing ten or more injections), is necessary than for classical C-14 radiocarbon dating which is especially important when working with small mollusk or snail shells or with teeth. The analysis is much cheaper than with accelerator C-14 dating. By choosing the appropriate amino acid(s), a time range from several hundred years back to one million years (also depending of the effective diagenetic temperature, of course) can be covered. In contrast to this, C-14 can only be used back to 50,000 years, and U-series radioisotope dating is suitable back to 300,000 years.

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Nevertheless, there is no doubt that AAR dating is only one out of a great choice of dating methods basing on very different approaches (radioisotopes, stable isotopes, electron spin resonance, thermoluminescence, fission tracks, palaeomagnetism, pollen analysis, dendrochronology). Only the careful combination of all those suitable for a certain dating problem will be successful. Excellent field work is always a prerequisite. In the case of mollusk shells, one has to be sure that the genera found at the place are identified with certainty.

The Use of Several Amino Acids. AAR dating began with the use of only one single amino acid, isoleucine, and still many laboratories only use this one. The reason, therefore, is that isoleucine has two centers of asymmetry, and by racemization (strictly speaking epimerization) it converts to allo-isoleucine. The allo/iso diastereomers can be separated by nonchiral chromatographic methods. Perhaps the use of isoleucine is the worst choice of all the possible amino acids because diastereomers are chemically not identical and different derivatization or detector response properties cannot be excluded. This would impair the comparison of allo/iso values obtained by different laboratories using different methods of analysis; in an interlaboratory study the results for isoleucine reported by some laboratories deviated by more than 15% from the mean value (*10*). It is also possible that allo- and isoleucine behave differently in geological and geochemical processes. Since now excellent gas and liquid chromatographic methods for the simultaneous determination of several amino acid enantiomers are available, the practice of using only isoleucine should be abandoned.

The use of several amino acids (not more than about ten of them are present in a hydrolyzate) gives the opportunity to have a more sound basis for age estimation. Moreover, since racemization rates vary from one amino acid to another, as can be seen from Figure 1, the one(s) appropriate for a given dating problem can be used. Aspartic acid is racemizing at the highest rate thus making this amino acid suitable for dating of young fossils whereas isoleucine is slower by orders of magnitude.

The Precision of the Method. Routine laboratory precision can be better than 5% (related to D/L values) but a precision of better than 10% is more common. Accuracy, as found in round robin analyses, seems to be poorer (*10*). Unfortunately, as yet, there is no accepted official standard since the United States National Institute of Standards and Technology (formerly the National Bureau of Standards) or a similar institution not yet defined one. Unofficial standards can be obtained from Wehmiller (*10*).

If ages have to be estimated from D/L values, the uncertainty is greater than the analytical error and can reach a 25% age uncertainty for a 10% analytical uncertainty for D/L values greater than 0.45 (if D/L is lower, also the age uncertainty is lower) (*10*). A thorough study of this problem has been published by McCoy (*11*). As a general rule, the resolution of AAR dating can be assumed to be 20-25% of the estimated age (7).

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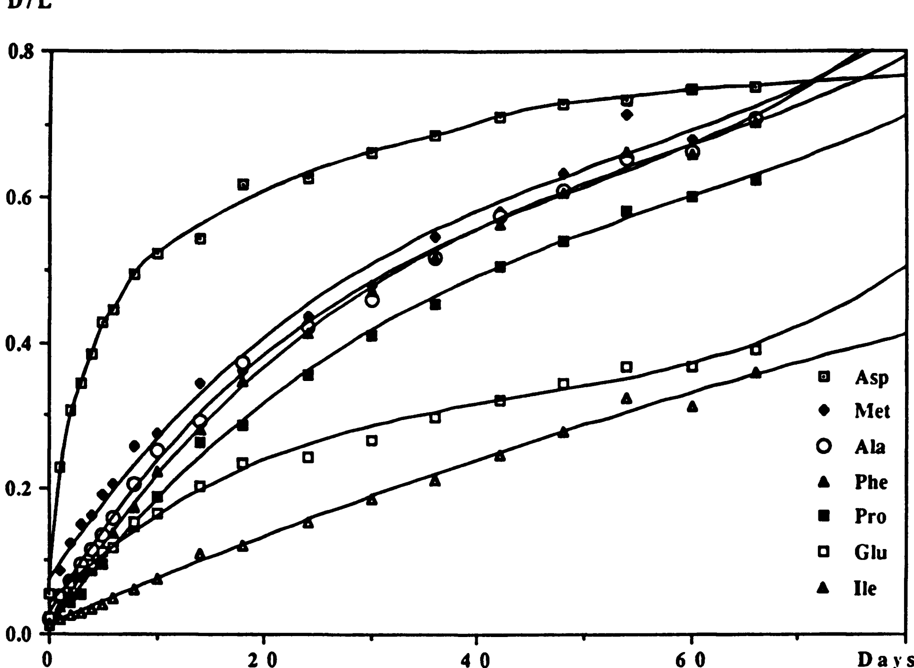

Figure 1. The progress of racemization with time as found in a laboratory heating experiment of *Trochoidea seetzeni* land snail shells (Meyer, V.R.; Goodfriend, G.A. Weizmann Institute of Science, Rehovot, in preparation). The shells are modern ("living") ones from the Negev desert (Israel), heating temperature was 106.5°C. Each data point is the mean of six analyses, the points are fitted with a third degree polynom.

Concerning relative dating, "two sites of similar thermal history are of significantly different age if their mean D/L ratios do not overlap at two standard deviations", if D/L is higher than approximately 0.1 (*8*).

Some Applications of AAR Dating

Only a few examples can be given here.

Marine Mollusk Shells. At present, one of the challenging scientific questions is reconstruction of the climatic history during the Quaternary (the last ca. 2 million years) with its frequent temperature changes which were the cause of a series of glacial periods and warmer interglacials. In periods of warm climate, the global sea level was considerably higher than during ice ages due to the variable volume of water preserved as ice. In regions of tectonic uplifting, ancient shorelines can often be reconstructed and if they contain fossil shells AAR dating can be used to determine their age. Studies have been performed along the Atlantic coast of Patagonia (Argentina), where results were compared with electron spin resonance data (*12*). In the USA, numerous sites along or nearby the Atlantic coastline have been investigated, e.g. in North Carolina with the comparison of pollen data (*3*).

An extended and impressive study was published by Miller and Mangerud in 1985 (*8*). Fossil mollusks from shallow water marine sediments of the North Sea and the Norwegian Sea were analyzed for their ratios of allo-/isoleucine. Interglacial (i.e. high sea level) sediments could be correlated and assigned to the various interglacials. For the first time it was possible to prove that all the "classical" Eemian sites from Northwestern Europe are the same age indeed (the Eemian was the last interglacial). The mean allo/iso ratios were as follows:

Sequence	*Age (12)* *ka before present*	*allo/iso*
modern		0.013 ± 0.002
late glacial	20	0.060 ± 0.010
middle Weichselian glaciation	50	0.090 ± 0.010
Eemian interglacial	120	0.18 ± 0.02
Saalian glaciation	260	
Holsteinian interglacial	400	0.29 ± 0.02
Elsterian glaciation	470	
late Cromerian interglacials	500	0.46 ± 0.04

Extensive pre-Eemian marine sediments along the Southwest coast of Denmark previously correlated with the Holsteinian were shown to be of late Cromerian age.

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Terrestrial Land Snails. Together with C-14 radiocarbon dating, AAR was used for the reconstruction of the extinction history of Jamaican snails (*14*) and for the determination of colluvial and fluvial sedimentation in the Negev desert (Israel) during the past 10,000 years (*15*).

Egg Shells. In many archeological sites egg shells are common and, as mollusk shells, they seem to preserve their content of amino acids excellently. Recently, AAR dating from different sites in Africa showed highly promising results which may help to understand the spreading of early men over this continent (*16*).

Teeth. Teeth are built up from different materials, the inner dentin (where primary and secondary dentin can be distinguished, primary dentin being older than secondary) and the outer enamel. Whereas enamel was successfully used in archeological dating (*17*), perhaps the use of dentin for the dating of "living" (i.e. not fossil) teeth is more interesting. At least part of the dentin is a closed system without turnover of proteins after the growth of a tooth. During lifetime aspartic acid, as the amino acid with the highest racemization rate, is racemizing to a considerably high degree because the temperature of 37°C is rather high (D/L about 0.05 for a 60 years old tooth). Due to physiologic conditions there is no uncertainty about temperature fluctuations, but body temperature is slightly variable among individuals thus limiting the precision of the method. Age estimation can be precise within $\pm$ 1-3 years. Therefore AAR can be one more tool to estimate the age of a person (*18*) or of a cadaver in forensic science (*19*).

Bones. Unfortunately, bones are not closed systems which means that they both loose amino acids and are prone to contamination, especially from groundwater. Most researchers think they are not suitable for AAR dating at all and only a few scientists are still working in this field. The most recent review is from 1985 by Bada (*20*). Perhaps AAR can be used if the state of preservation is not poor which could be defined by quantitative analysis of the amino acid content. The ratios of HyPro/Asp and Gly/Asp must not be lower than 1.5 and 5, respectively, for successful AAR dating (Elster, H.; Gil-Av, E.; Weiner, S. *J. Archeol. Sci.*, in press).

Wood. Wood also seems to be somewhat problematic for AAR dating and the kinetics is not yet well understood. Investigations were made by using aspartic acid for dating of fossil spruce from northern Canada (i.e. from cold climate) (*21*).

Sample Preparation. Careful mechanical cleaning and cutting of a suitable sample is always needed. Prior to all the chromatography-oriented steps, all kinds of shells as well as teeth are simply hydrolyzed in 6N hydrochloric acid, e.g. at 105°C for 22 hours. Racemization induced by this treatment is low but has to be taken into consideration. Bone samples need to be dialyzed first.

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The Analytical Methods

Gas Chromatography. Capillary gas chromatography offers excellent resolution power, but sample preparation is time-consuming because desalting (by ion-exchange or precipitation) is necessary and separation without prior derivatization is not possible. In earlier days derivatization with expensive (+)-2-butanol (and trifluoroacetic acid anhydride) was necessary to obtain diastereomers which could be separated on OV 225 or Carbowax 20M. Now chiral stationary phases, Chirasil-Val (*22*) being the most popular one, are available which allow the direct separation of enantiomers so derivatization can be done with isopropanol. The separation on Chirasil-Val of the amino acids found in a land snail shell hydrolysate is shown in Figure 2.

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High Performance Liquid Chromatography. Until now the use of HPLC in AAR dating has been restricted to achiral amino acid separation thus giving only information about allo/isoleucine. Today, several chiral HPLC methods are available and there is no question they could be used successfully in this field. The big advantage of HPLC over GC is that desalting is not necessary. A very attractive approach is the use of (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) as chiral and commercially available derivatization reagent which allows separation of all the protein amino acids (i.e. more than necessary in AAR dating) in one run on a standard C_8 or C_{18} reversed phase (*23*). The derivatization can be automated. This is also true for derivatization with chiral thiols and o-phthalaldehyde (*24,25*). Several chiral stationary phases allow the direct enantioseparation of amino acids without prior derivatization; they are of the ligand exchange (*26*) or the crown ether type (Crownpack from Daicel, 8-1 Kasumigaseki 3-chome, Chiyoda-ku, Tokyo 100, Japan). None of these phases has a high enough separation performance which would allow the separation of all of the amino acid pairs of interest in one run; however, in cases where only one amino acid is studied (e.g. aspartic acid in young samples) they could be of interest.

Thin Layer Chromatography. Perhaps TLC is less attractive because quantitiative analysis is not straightforward; however, it should not be forgotten. At least it could be of interest for the semi-quantitative screening of samples. TLC offers the advantage that one does not have to worry about contamination and fouling of the chromatographic system due to impurities. TLC plates on the basis of chiral ligand exchange are commercially available and allow the direct analysis of amino acids without prior derivatization (*27*). A review on the various possibilities for the TLC resolution of amino acid enantiomers was published by Bhushan (*28*).

Capillary Electrophoresis. CE is the newest of the methods discussed here and it looks very promising. This method could combine the advantages of both gas and liquid chromatography - high resolution power and ease of sample preparation (*29-31*). The chiral selector is added to the buffer solution in the capillary. This is a rapidly growing field which ho-

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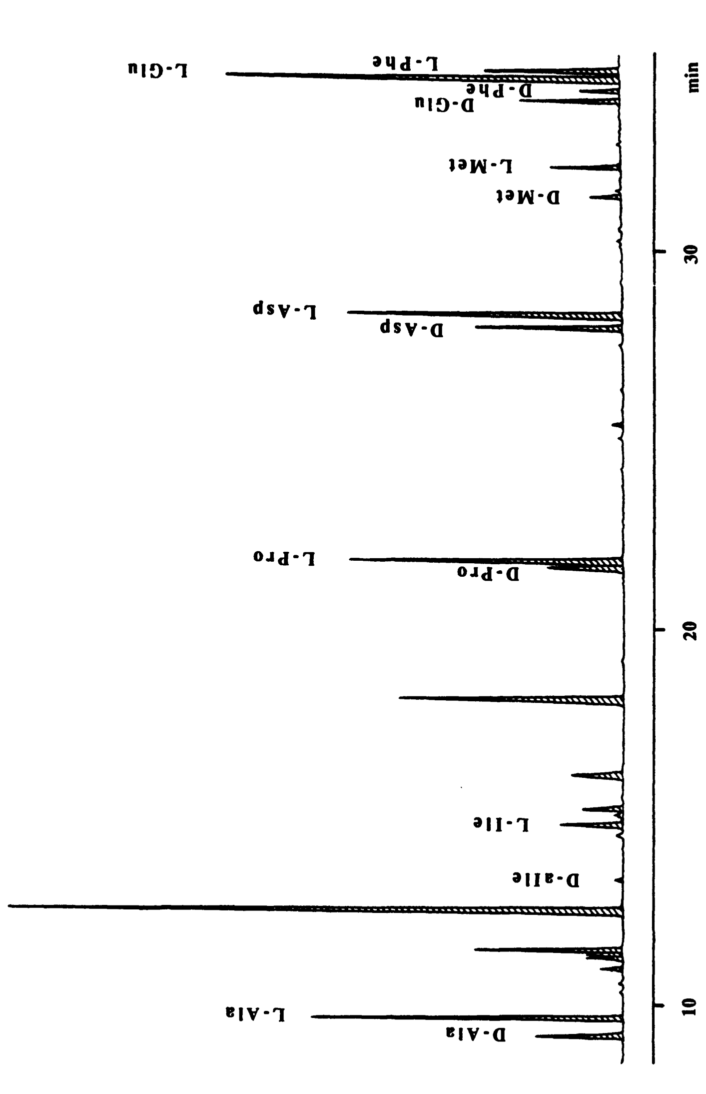

Figure 2. Capillary gas chromatogram on Chirasel-Val of laboratory-heated land snail-shell (*Trochoidea seetzeni*) hydrolysate as *N*-trifuoroacetyl-isopropyl derivative (V. R. Meyer and G. A. Goodfriend, Weizmann Institute of Science, Rehovot). Seven amino acid pairs can be used for quantitative analysis; several more, including the nonchiral glycine, are present but cannot be used because of coelution with impurities or because of the small size of the peaks. Asp is the sum of aspartic acid and asparagine. Glu is the sum of glutamic acid and glutamine. Capillary: 0.25 mm x 50 m; stationary phase: Chirasil-Val III (Alltech); carrier gas: helium, 120 kPa; detector: NPD; temperature program: hold 50 °C for 1 min, 50 °C/min to 115 °C, hold 15 mins, 4 °C/min to 200 °C, hold 10 mins.

pefully can be introduced soon into AAR dating. The instrumental set-up in principle is simple, however, not inexpensive because the peaks are so extremely narrow that detection and quantitation is a challenge.

Conclusion: AAR - A Tool for Dating!

It should have become clear that AAR is not the panacea for dating problems but that it is one more tool of high value if used properly. This, however, is true for every dating method; they all have their obstacles and limitations. Interdisciplinary work is always a prerequisite. With the introduction of highly efficient analytical methods, especially chiral stationary phases and derivatization reagents for chromatography and the recently developed capillary electrophoresis, AAR could soon become a mature dating technique.

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