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New bio-analytical separations utilizing chiral mobile phase additives in thin layer chromatography and chiral stationary phases in high performance liquid chromatography

Jo Dee Duncan

Department: Chemistry

New Bio-Analytical Separations Utilizing Chiral Mobile Phase Additives in Thin Layer Chromatography and Chiral Stationary Phases in High Performance Liquid Chromatography

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Dr. Daniel Armstrong

Dr. Oliver Manuel

Dr. James Stoffer

Dr. Gary Bertrand

Dr. Colin Benjamin

ABSTRACT

The problem addressed by this dissertation is the separation of optical isomers in commercial as well as biological samples. The chromatographic separation of enantiomers is an important and rapidly developing field of study.

Chiral separations of pharmaceutical compounds and important organic intermediates in high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) were achieved. Two methods were employed for the direct liquid chromatographic resolution of chiral analytes: chiral stationary phases (CSPs) and chiral mobile phase additives (CMAs). Native and derivatized β-cyclodextrins (β-CD) were used as chiral stationary phases in reverse phase and normal phase HPLC, respectively. This study marked the first use of derivatized β-CDs for chiral separations in normal phase media. N-carbobenzoxy-glycyl-L-proline and (1R)-(-)-ammonium-10-camphorsulfonate were utilized as CMAs in normal phase TLC for the resolution of several aromatic amino alcohols. Maltosyl-β-CD and hydroxypropyl-β-CD were employed as CMAs in reverse phase TLC. A study was conducted with hydroxypropyl-β-CD to determine how the degree of substitution of a derivatized CD could effect development time, the viscosity of the solution and the enantioselectivity.

In addition, studies were initiated to determine the presence of trace levels of D-amino acids in: amniotic fluid, blood serum and urine. The blood and urine of healthy young adults were analyzed and found to contain trace to percent levels of D-amino acids. The human amniotic fluid samples did not have detectable levels of D-amino acids.

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I would like to dedicate this work to my friends and family. I would like to thank members of my research group for donating their body fluids.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (cont'd)

LIST OF ILLUSTRATIONS (cont'd)

LIST OF TABLES

LIST OF TABLES (cont'd)

I. INTRODUCTION

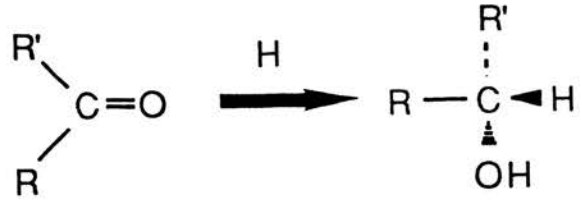
A substantial amount of research has been conducted in the area of chiral separations due to the demand for efficient, precise and dependable methods for the determination of optical purity and/or preparative scale separation of optical isomers (enantiomers). One reason for this effort is the fact that two enantiomeric forms of a molecule can have different effects in biological systems (1), and as the need for information on the often widely variant activities of enantiomers of pharmacologically important compounds increases, the demand for chiral separation methods will continue. Differences in biological activity of drugs, agrochemicals, etc., may originate due to dissimilarities in: protein binding and transport, mechanism of action, metabolism rates, changes in activity due to metabolism, clearance rates, and persistence in the environment. Another important use for enantioselective separations is to check reaction progress and purity (% composition of each enantiomer, quality control, etc.) during asymmetric syntheses.

The chromatographic separation of enantiomers is an important and rapidly developing field of study. There has been a dramatic increase in the number and nature of racemic separations obtained by chromatographic methods. The development of fast and productive chiral separation methods continues to escalate. These separation methods involve the use of chiral counter ions/chiral mobile phase additives (CMA) in thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), and of chiral stationary phases (CSP) in TLC, HPLC and gas chromatography (GC).

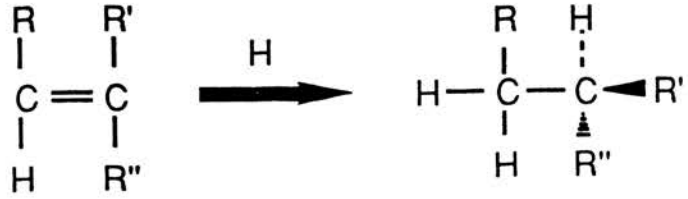
Consequently, manufacturers in pharmaceutical, pesticide, and food and beverage industries now have the means to tailor the preparation of products which contain optically active compounds. It has been reported that approximately 57% of the active pharmaceutical compounds prescribed in the United States contain at least one stereogenic center (2, 3). The biological activity of two enantiomeric forms of an active drug can be significantly different. For example, it was found that the L-form of thalidomide is a powerful teratogen and the D-form is a sedative and soporific drug (4, 5). The administration of the racemate D, L-thalidomide to pregnant women during the 1960's was associated with a large number of newborn abnormalities. This drug was withdrawn from the market a few years later. Another example of a drug which exhibits enantioselective biological activity is propranolol. Propranolol is a β-adrenergic blocker. The (S) enantiomer of propranolol is about 100 times as potent as the (R) isomer (2, 6). Such differences in pharmacological activity between enantiomers is often the rule rather than the exception. However, in the period 1983-1985, 88% of the new chiral drugs introduced were marketed as racemates (3). Many individuals believe that racemic pharmaceuticals will soon be considered as drugs with 50% impurity (2, 3, 5).

Regulatory authorities have begun requesting data on stereochemistry as knowledge of the biological importance of chirality increases. The awareness that the efficacy and toxicology of enantiomers may differ from each other and from the racemic mixture continues to grow. Studies indicate that metabolism can produce chiral metabolites from non-chiral molecules (7). Some reaction examples are shown in Figure 1.

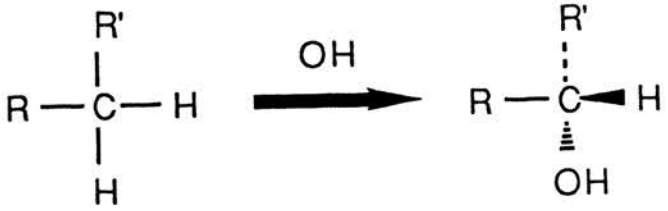
Ketone reduction



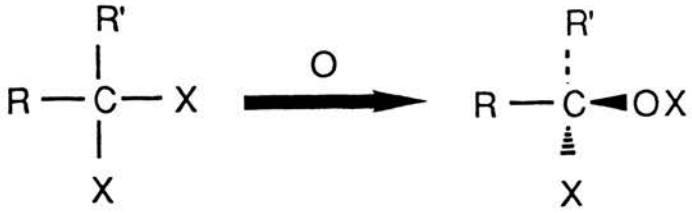
Reduction of a double bond



Hydroxylation



Oxidation



Oxidation of tertiary amine

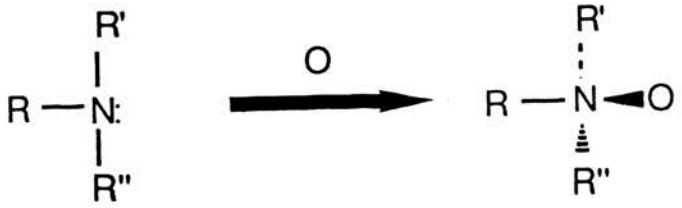


Figure 1. Examples of reactions which could produce chiral metabolites

Recently, the Food and Drug Administration (FDA) has issued guidelines on the submission of New Drug Applications (NDAs) which addresses the question of stereochemistry in the manufacturing of drug substances (8). The Food, Drug and Cosmetic (FD & C) Act requires a complete description of the "methods used in the manufacture of the drug" which includes testing to demonstrate its identity, strength, quality and purity. Consequently, the decision of whether to market the racemate in preference to a pure enantiomer must now be justified by the submission of relevant information.

The significance of stereoisomerism in relation to biological activity has been recognized since Pasteur reported the first separation of enantiomers. The question to be answered was 'Why are living systems chirally discriminating?'. The answer was that living systems are composed of chiral molecules. Therefore the human body is a highly stereospecific environment and in nature, biological asymmetry at the molecular level is the norm, not the exception.

All naturally occurring amino acids are L-amino acids and most natural sugars are D-sugars. There are twenty primary protein constituents which consist of 19 L-α-amino acids and one cyclic L-α-imino acid, proline (9). Phospholipids, steroids, nucleotides, nucleosides, and bile salts are other examples of chiral molecules of biological necessity.

Over 50 years ago Kögl and Erxleben (10) reported the presence of D-amino acids in the hydrosylates of tumor proteins. Although most other investigators believed that the D-amino acids were produced via racemization during the acid hydrolysis step, the controversy went on for over a decade (11, 12). Currently, there is not a lot of conclusive evidence for the occurrence of D-amino acids in the proteins of man. However,

there is undisputed evidence for the occurrence of both free and bound D-amino acids in a variety of micro-organisms (13). Given the central importance of amino acids in all living systems and their use in the diagnosis of certain disorders or diseases in man, it seems logical to investigate all possible aspects of these compounds

Amino acid analysis has been used in biochemistry for the determination of free amino acids and to study proteins, *i.e.*,- purity, quantitation, composition, sequencing, etc. The separation of amino acids on cation exchange resins has been the preferred technique for their quantitation in both hydrosylates and physiological fluids since the classic studies of Moore and Stein (14, 15). The ease of quantitation was greatly enhanced by the development of the automatic analyzer (16). Although ion-exchange chromatography has been the main method for amino acid analysis, recently the technology associated with HPLC has been applied with increasing success. The comprehensive studies on the chromatography of amino acids performed by Hamilton (17-19) in the late 1950s was echoed in HPLC literature. Modern HPLC equipment has recently achieved the level of sophistication which was standard in amino acid analyzers a decade ago. It should be emphasized that ion exchange chromatography of amino acids depicts one specific application of the general analytical procedure of liquid column chromatography and that high performance (or high pressure) liquid chromatography is the terminology applied to the technique as currently optimized.

The interest in HPLC lies in the speed, economy, and ease of this technique in comparison to that of conventional ion-exchange methodology. The development of HPLC technology for amino acid analysis is lacking for underivatized samples but it is possible to attain fast sensitive

separations for many amino acid derivatives (20, 21). There have been a few reports in the literature concerning the analysis of free amino acids using reversed-phase HPLC (22). Nonpolar stationary phases have been successful in attaining separations of underivatized amino acids but the resolution is not comparable to that obtained in conventional ion-exchange chromatography.

Resolved samples of chiral amino acids are now required for the majority of amino acid analyses. Chiral amino acids can be obtained from the following: (a) natural source, (b) asymmetric synthesis, or (c) resolution of a D, L amino acid obtained through synthesis.

There are two methods for the direct liquid chromatographic analysis of chiral compounds: chiral stationary phases and chiral mobile phase additives. The formation of reversible diastereomeric complexes with the CMA or the CSP is the basis for the chromatographic resolution of optical isomers. Enantiomers have identical chemical and physical properties in an isotropic environment but diastereomers may display dissimilar properties in isotropic environments; this difference makes it possible to separate diastereomers. Differences in the distribution properties of the diastereomeric complex may be due to differences in the binding strength to the adsorbent and/or to dissimilarities in the complex solvation or solubility in the mobile phase.

There have been several reports on using CMAs in chromatographic analysis. Numerous racemic mixtures can be resolved on conventional achiral LC columns by utilizing the proper chiral additive in the mobile phase. The first to report this beneficial innovation were Gil-Av and co-workers (23) and Karger and associates (24) utilizing ligand exchange chromatography (LEC). In LEC, a transition metal ion such as Cu(II) or

Zn(II) must be added to the mobile phase. The CMA consisted of a chiral bidentate ligand with a hydrophobic moiety. The ligand strongly associates with the reverse-phase media due to its hydrophobic segment. Suitable racemic analytes, particularly amino acids, form ternary complexes which are retained by the stationary phase.

Chiral separations have also been obtained using suitable CMAs with achiral stationary phases in reversed-phase (25) and normal-phase (26) modes. The selectivities of these CMAs sometimes differ from those of available CSPs. Most of the recent work done in normal phase HPLC involved the use of chiral counter ions. Pettersson and Schill reported the separation of enantiomers by "ion-pair" chromatography using quinine, camphorsulfonic acid, albumin, and n-dibutyl tartrate as chiral counter ions (27). Pettersson and Josefsson reported the separation of racemic aromatic amino alcohols in ion interaction chromatographic systems using N-carbobenzoxy-glycyl-L-proline (ZGP) (28). Knox and Jurad reported the separation of optical isomers by zwitterion-pair chromatography; the chiral agent in this case was L-leucyl-L-leucyl-L-leucine (29).

Research involving the use of CMAs in HPLC has revealed several important parameters: the nature of the solid phase, the concentration and nature of the CMA and the amount of polar modifier in the mobile phase (27). Consequently, the use of CMAs is a viable but relatively unexplored approach to chiral separations.

Cyclodextrins (CD) have proven useful as chiral stationary phases (CSPs) and as chiral mobile phase additives (CMAs) but their low solubility in hydro-organic solvents has limited their applicability as CMA in the reversed-phase (RP) mode. The solubility of β-CD in pure water is 1.67 x 10^{-2} M at 25° C. Chiral resolution was attained only when the

concentration of β-CD was increased to levels surpassing its solubility in neat water. Two approaches have been used to overcome the solubility problem. One approach takes advantage of the fact that it is possible to increase the solubility of the native CD by the addition of urea or sodium hydroxide to the mobile phase (30). Aqueous solutions saturated with urea were more effective in attaining chiral separations than aqueous solutions containing sodium hydroxide. The proposed separation mechanism for the native CDs in the reversed-phase mode involved the formation of an inclusion complex, driven by hydrophobic interactions, in which each enantiomer is complexed somewhat differently in the CD cavity. Disparate retention times are noted for the optical isomers. The relative size and geometry of the guest molecule in relation to the dimensions of the host CD cavity are important parameters in determining if an inclusion complex can be formed (31). Other parameters include: the concentration of the CMA, the concentration and the type of organic modifier in the mobile phase, and the retention behavior of enantiomers.

Another approach benefits from the enhanced solubility of derivatized cyclodextrins in hydro-organic solvents relative to that of the native CD. The derivatization process involves the formation of ethers due to the addition of propylene oxide to the hydroxy groups on the mouth and base of the CD cavity. Armstrong, Faulkner, and Han separated a variety of racemic compounds using hydroxypropyl- and hydroxyethyl- derivatized β-CD as CMAs (32).

The majority of reports of CD as a CMA are for TLC whereas most applications of CD-CSPs are for work in HPLC or GC. Although many compounds separate well with both methods, it is interesting to note that sometimes there is a poor correlation between the enantiomeric separations

obtained with CD-CMA versus CD-CSP. Reasons for this discrepancy may be: (1) β-CD is linked to the silica gel via an 6-10 atom spacer and these spacer arms may provide additional interaction sites for the complexed analyte and/or these arms may restrict motion of the CD; (2) if the CD is in the mobile phase, it is available for multiple complexation. The most notable difference that has been observed between the use of CD as CMA and CSP in reversed-phase media is the reversal of elution order of enantiomers. For example, with DL amino acid derivatives, the D enantiomer elutes ahead of the L enantiomer when the chiral agent is in the mobile phase but a reversal of the retention behavior is noted if the chiral selector is bonded to the stationary phase, exclusively (30).

Cyclodextrin bonded phases have been used for the reversed phase separation of a variety of enantiomers (33), diastereoisomers (34), structural isomers (34), enzymes (35), and routine compounds (36). Of these, the enantiomeric separations probably have received the greatest attention. In order to evaluate the mechanism of enantioselective chromatography, a number of empirical and theoretical studies have been done (37-41). In specific cases involving cyclodextrins, the formation of an inclusion complex seems to be a fundamental part of the chiral recognition and separation process (33, 34, 36, 41). As yet, there have been no reports of normal phase, enantiomeric separations on native cyclodextrin bonded phase columns. Indeed, the fact that facile enantiomeric resolution obtained in the reversed phase-mode could not be duplicated in the normal phase-mode has been used as indirect evidence to support the premise that inclusion complexation is necessary for enantioselectivity (41). However, native cyclodextrin bonded phases have been used successfully in normal phase LC for a number of achiral

separations (42-44). The retention behavior was somewhat like that of a diol column. It is believed that the nonpolar portion of the mobile phase (e.g., hexane, heptane, etc.) occupies the cavity of cyclodextrin and that solute retention was due mainly to interaction with the external hydroxyl groups that line the top and bottom of the cyclodextrin torus (42-45).

Among the available CSPs for LC are the polymeric chiral phases (46), the crown ether phases (47), the protein CSPs (48, 49), the π-complex, hydrogen bond CSPs (Pirkle-type) (50), and the cyclodextrin (CD) bonded CSPs (33). The number of commercially available HPLC-CSP has risen to over fifty and has continued to grow annually (51). There have been several reviews of HPLC-CSPs which were designed to help determine which CSP to use for a particular problem (52-56). It should be noted that research has concentrated on HPLC while the progress in TLC has advanced much more slowly.

There have been relatively few reports of CSP for TLC and only for a limited number of compounds. Yuasa announced the partial separation of D, L-tryptophan on a crystalline cellulose-coated plate (57). Wainer, *et al.* published the separation of racemic 2, 2, 2-trifluoro-1-(9-anthryl)ethanol on a chiral dinitrobenzoylphenylglycine bonded phase (58). Brunner, *et al.* reported the use of a naphthylethylurea TLC-CSP for the separation of enantiomeric amides (59). Alak and Armstrong published the separation of several racemic amino acids and ferrocene derivatives on a β-cyclodextrin bonded phase (60). While β-CD was used successively as a bonded TLC phase, attempts to coat a TLC plate with β-CD were unsuccessful (61). The problem with coating the plates was due to the low solubility of β-CD in organic solvents. Weinstein (62), Grinberg and Weinstein (63), and Gunther, et al. (64) reported the separation of several racemic dansyl

amino acids on reversed-phase (RP) plates impregnated with copper (II) complexes of chiral alkyl α-amino acid derivatives. Results obtained with TLC-CSPs are comparable to those obtained using analogous HPLC-CSPs. At this time, the only CSPs which are commercially available for TLC are the ternary complex-ligand exchange plates. Some of the problems in developing chiral TLC plates were discussed by Wilson (61).

Several important differences exist between HPLC and TLC. The major advantage that HPLC has over TLC is better reproducibility, greater sensitivity and more theoretical plates. The fact that the HPLC column is equilibrated with the mobile phase before use whereas the TLC stationary phase is initially dry has resulted in several important differences. The quantity of mobile phase in the bed varies according to location on the TLC plate, such that the phase ratio varies during development. Second, heat is given off when a solvent wets a dry bed and this results in a small temperature differential between the solvent front and the rest of the bed. Third, in TLC, the mobile phase may change composition during development of a binary or more complex solvent due to differential adsorption on the stationary phase. In addition, the velocity of the solvent front is inversely proportional to the distance that the solvent front has traveled, so the flow rate is not constant with time or distance. Also, the amount of chiral agent in the mobile phase may change with TLC due to preferential adsorption of the chiral selector as the solvent front migrates along the chromatographic bed. In contrast, the concentration of the CMA remains constant during high performance liquid chromatographic analysis due to pre-equilibration of the LC column.

An advantage of HPLC is that the analyst can choose between gradient or isocratic elution. It is also possible to change the flow rate in HPLC to

optimize separations. Precise control of pH and ionic strength during the separation process and ease of temperature control are additional advantages offered by high performance LC. Despite these differences, it is sometimes possible to attain similar results with both chromatographic methods.

TLC has achieved popularity due to its simplicity, low cost and wide versatility and applicability. In his review, Stahl stated that TLC was the easiest and most economical chromatographic technique for fast separation and visual evaluation (65). The main advantage of TLC over HPLC has been the ability to screen a large number of compounds concurrently thereby saving time and operating expenses. One problem that has been noted is the elongation of spot size and/or irregular spot size for polar compounds using this method. The introduction of high performance TLC has helped this problem somewhat. The spot diameter is controlled by molecular diffusion in high performance plates (66). In some cases, TLC could be used to determine what mobile phase to use in HPLC if the same CSP or CMA was utilized. In the case of enantioselective synthesis, it could provide useful information as to the progress of a reaction.

Theoretically, it is possible to choose the mode which best suits the needs of the analyst. The main obstacle to this idea has been the limited commercial availability of CSPs for TLC. Although there are a greater number of commercially available CSPs for HPLC, these columns can be expensive and may be easily damaged. A viable alternative to CSP is the use of achiral stationary phases with the appropriate CMA. Some disadvantages of using CMAs are: (1) the isolation of a pure chiral analyte may be difficult, and (2) the method of detection must be compatible with the CMA used. For example, if a compound which absorbs ultraviolet

light is used as a CMA, one can use an ultraviolet detector in HPLC and adjust the baseline accordingly but in TLC it would not be possible to detect the samples using an ultraviolet detector. Advantages of using CMA are: (1) it is easy to change the chiral selector being used, and (2) the lifetime of conventional achiral columns is typically longer than that of chiral columns.

In this dissertation, the use of various CMAs and CSPs in TLC and HPLC for the resolution of chiral analytes will be discussed. A method for the TLC separation of enantiomers and diastereomers involving the use of chiral ion interaction agents will be described. Several aromatic amino alcohols were resolved by TLC using a mobile phase containing (1R)-(-)-ammonium-10-camphorsulfonate or N-carbobenzoxy(CBZ)-glycyl-L-proline (ZGP). Many of these chiral aromatic amino alcohols are of pharmacological importance as α- and β-adrenergic blockers, adrenergic compounds, and anti-glaucoma agents. A comparison was made between various N-CBZ-amino acid derivatives as chiral counter ions/chiral mobile phase additives. Various mobile phase compositions and numerous commercially available achiral TLC stationary phases were evaluated.

Amino acid derivatives, nicotine derivatives and cinchona alkaloids were separated by thin layer chromatography on different types of reversed phase plates using a mobile phase containing maltosyl-β-cyclodextrin. A comparison is made between octadecyl, diphenyl and ethyl reversed-phase TLC plates. Several derivatized amino acids are separated by thin layer chromatography utilizing hydroxypropyl-β-cyclodextrin (HP-β-CD). Studies were conducted to determine the effect that the degree of substitution of HP-β-CD would have on: the resolving abilities of the CMA, the time of development and the viscosity of the hydroxypropyl-β-

CD mobile phase solutions. Comparisons will be made between the native and the derivatized CD used as CMAs in RP-TLC.

The separation of some drug stereoisomers using native CD CSPs and hydroorganic mobile phases will be presented. The principal features of the CD chiral recognition mechanism will be reiterated. In addition, several different derivatized β-cyclodextrins were synthesized and used as chiral stationary phases in normal phase liquid chromatography. The multiply substituted derivatives were made with acetic anhydride, (R)- and (S)-1-(1-naphthyl)ethylisocyanate, 2,6-dimethylphenylisocyanate and p-toluoyl chloride. The first successful cyclodextrin-based, normal phase separation of enantiomers was accomplished on these derivative phases. In contrast to chiral separations on the native β-cyclodextrin stationary phase, the enantiomeric separation mechanism on these new phases in the normal phase mode was not thought to be dependent on inclusion complexation. The similarities and differences between the derivatized cyclodextrin stationary phases and the derivatized cellulosic stationary phases will be discussed.

Analysis of free form amino acids in commercial samples and various physiological fluids was conducted. Detectable levels of free D-amino acids were found in human urine and blood serum but not in human amniotic fluid. This was accomplished using a liquid chromatographic coupled column (*i.e.*, achiral-chiral) method that was developed for the rapid and sensitive analysis of these compounds. The technique was tested on a series of commercial L-amino acids. Care was taken to minimize racemization. Trace to percent levels of contaminating D-enantiomers were found in all commercial samples. In urine the D-amino acids ranged from several hundredths of a percent up to percent levels of the

corresponding excreted natural L-amino acids. There were no apparent correlations between the amount of amino acids excreted and the relative amount of D-enantiomers present. In blood serum samples, the D-amino acids ranged from less than 0.05% to 0.50% levels of corresponding natural L-amino acids. The limit of detection for the amino acids in the analyzed physiological fluids was 0.05%.

It was possible to analyze four amino acids (phenylalanine, tyrosine, tryptophan and leucine) in urine. In blood serum, three out of four of the above-mentioned amino acids were analyzed. The majority of the tryptophan in the blood sample was lost during the solid phase extraction step. Four amino acids (phenylalanine, tyrosine, leucine and methionine) were analyzed in the human amniotic fluid samples.

II. BACKGROUND

A. History of Chiral Separations

Stereochemistry is that part of science which deals with structure in three dimensions. One aspect of stereochemistry is stereoisomerism: isomers which differ only in the way that the atoms are oriented in space. Stereoisomers which rotate plane polarized light are called optical isomers.

In 1815 the French physicist, Jean-Baptiste Biot, found that α quartz rotated plane polarized light (67). This was the first report of optical activity. However, it was not until 1848 that the first deliberate separation of enantiomers from a racemic mixture was reported (68). In 1848 at the Ecole normale in Paris, a young chemist made a set of observations which led him to make a proposal that now forms the foundation of stereochemistry. This young man, Louis Pasteur, has just won his doctorate in science and he was attempting to gain some experience in crystallography. He was duplicating earlier work on tartaric salts when he noticed something no one had perceived before: optically inactive sodium ammonium tartrate consisted of a mixture of two distinctive types of crystals. Figure 2 depicts the two types of sodium ammonium tartrate crystals. Pasteur was able to separate the morphologically different crystals by hand using a hand lens and a pair of tweezers. While the original mixture was optically inactive, each set of crystals was found to be optically active in aqueous solution. Furthermore, all physical and chemical properties of the individual solutions were identical except the specific rotations of the two solutions which were equal but of opposite sign. Pasteur's method of mechanical separation demonstrated the

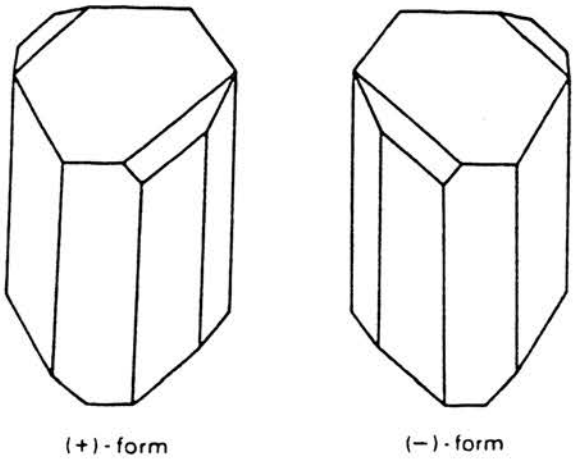
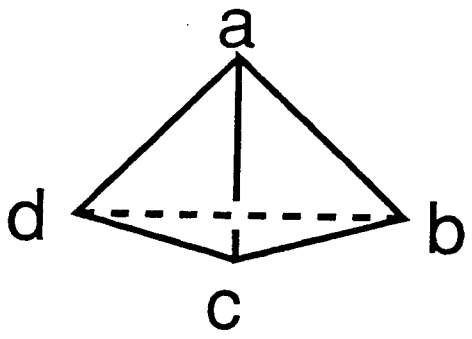


Figure 2. Configuration of sodium ammonium tartrate crystals. Racemic sodium ammonium tartrate forms two sets of crystals, distinguished by the non-superimposable mirror-image relation of their crystal facets and the opposite sign of their optical activity.

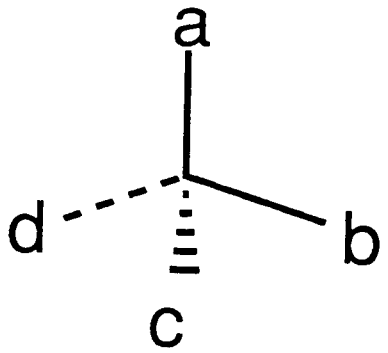
human ability to distinguish left from right, by explicit or implicit reference to a chiral object, such as a hand. Thus was coined the phrase "right- or left-handedness". Pasteur concluded that molecular disymmetry was necessary for optical activity in small molecules or crystals since the difference in optical rotation was observed in solution. He proposed that the molecules composing the crystals were mirror images of each other but he was unable to determine why a molecule would be disymmetric or chiral.

In 1874, van't Hoff and Le Bel individually reported that the "asymmetric carbon atom" was the reason for chirality (69, 70). Van't Hoff elucidated the correct proposed structure, tetrahedral rather than square pyramidal, so he was given the lion's share of the credit for this discovery (See Figure 3). It is now known that other types of chirality exist. It is possible to have "chiral centers" as well as "chiral axes" and/or "chiral planes" of symmetry. Some examples of chiral molecules which do not contain "chiral centers" are shown in Figure 4. Also, organic molecules can contain "chiral heteroatoms" such as nitrogen or phosphorous. Recently it has been proposed to use the term stereogenic center rather than "chiral center" or "chiral atom" since, technically, an atom or point cannot be chiral.

There are two different conventions for the designation of the three dimensional structure of a chiral entity. The first system was devised by Emil Fisher in his attempt to interrelate the absolute stereochemistry of optical isomers (71). In the late 1880's, Fisher arbitrarily assigned the absolute configuration of (+)-glucose and the Fisher projection scheme forms the backbone of present day carbohydrate chemistry. A Fisher projection of D, L -glyceraldehyde is shown in Figure 5. In 1902, Fisher

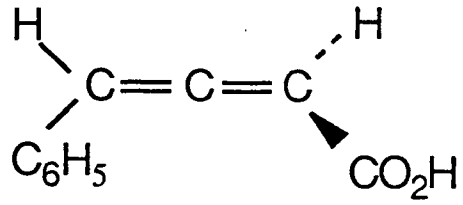


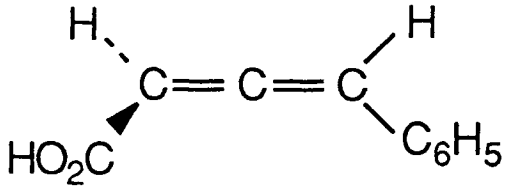
Square pyramidal

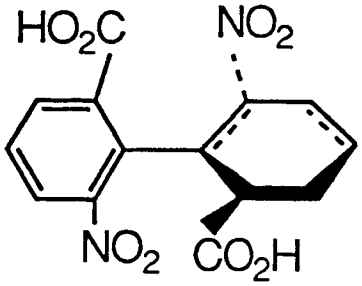


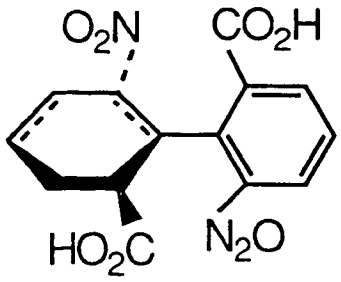
Tetrahedral

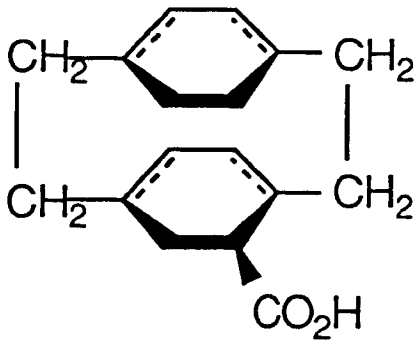
Figure 3. Square pyramidal and tetrahedral structure











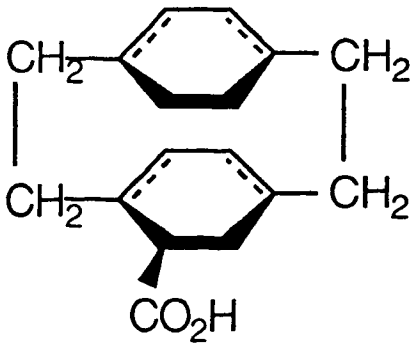


Figure 4. Examples of Chiral Molecules which do not contain "chiral centers".

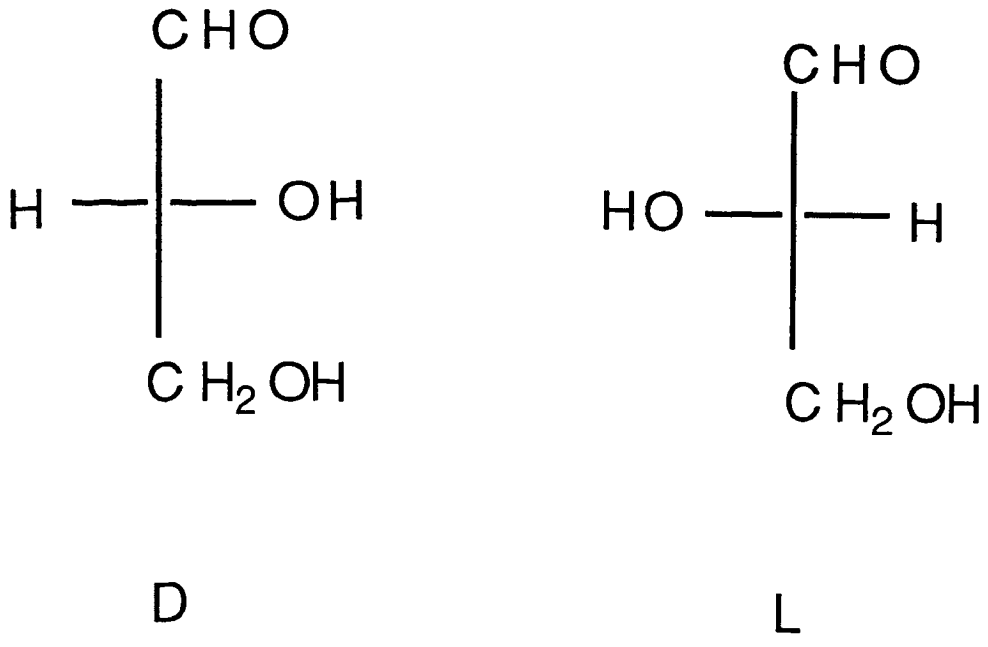


Figure 5. Fisher projection of D, L-glyceraldehyde

was honored for his groundbreaking work by becoming a Nobel Prize recipient. The D, L system devised by Fisher is still used to describe amino acids, sugars and their derivatives.

Another useful procedure for designating the spatial structure of molecules involved the use of the prefixes R and S. These prefixes were assigned according to a procedure devised by R. S. Cahn, Sir Christopher Ingold and V. Prelog. There is no essential connection relating the D, L and the R, S conventions. The D, L convention was based on Fisher projections whereas the R, S convention was based on Cahn-Ingold-Prelog sequence rules.

The designation (+) and (-) indicated the direction of specific rotation of plane-polarized light. If the rotation of the plane of light was to the right, the substance was dextrorotatory (d) or (+); and if the direction of rotation was to the left, the substance was levrorotatory (l) or (-). The symbols d and l could also be used to designate the direction of specific rotation.

When Fisher reported his Nobel Prize work, there was no method for the determination of absolute configuration so he arbitrarily assigned his D, L symbols. It was not possible to answer the question of absolute configuration until 1951 (72). J. M. Bijvoet reported the determination of the actual spatial arrangement of atoms of an optically active compound, d(+)-Na Rb tartrate, using x-ray crystal analysis. Luck was with Fisher for he had arbitrarily assigned the correct configuration for L-glyceraldehyde.

Ever since Pasteur's pioneering work, investigators have searched for improved methods to resolve and purify enantiomers. There are several methods for resolution of enantiomers. These are: (a) conversion to diastereomers, (b) biochemical processes, (c) mechanical separation, (d)

differential reactivity, (e) selective crystallization, and (f) differential adsorption. The dominant approach has been conversion of enantiomers to diastereomers followed by crystallization. Most of these methods were characterized by multiple steps and low efficiency. The introduction of chromatography as a separation tool allowed for efficient enantiomeric separations. This work focuses on chiral separations that use differential adsorption properties of enantiomers and/or diastereomeric ion-pairs in a process known as chromatography.

In 1903, the first application of differential adsorption in a flowing system with a mobile phase and a stationary phase to separate analytes was reported by Tswett (73). He passed an organic solvent solution of plant pigments through a glass column packed with finely divided inulin (a polysaccharide) and observed the formation of several sharply defined colored bands or zones. The pigments were separated due to differing abilities to adsorb upon the inulin thereby creating the colored bands noted by Tswett. This process was referred to as chromatography.

Chromatography means the production of a color scheme or to write in colors. This technique, introduced by Tswett, is known as adsorption chromatography. It operates on a principle which depends upon the differential adsorption characteristics of analytes in a system composed of a solid stationary phase and a liquid mobile phase. The behavior of the analytes is affected primarily by the selection of solid adsorbent and solvent.

In the beginning, there was only one type of chromatographic technique available for the scientist, this involved a polar stationary phase and a nonpolar mobile phase. Also, it was only possible to analyze colored substances. Many methods were utilized to adapt adsorption

chromatography to the analysis of colorless compounds. The formation of colored derivatives (74) and the use of dyes (75) proved successful.

As technological advances were made in the twentieth century, instrumentation was developed to enhance the capabilities of chromatography. There are now a wide variety of commercially available detectors which allow detection of unlimited numbers of compounds. Derivatization of analytes is now used as often to enhance separation as for detection. Tswett used a glass column and the flow through the column resulted from gravitational force. Modern liquid chromatography uses microbore columns capable of withstanding pressures up to several atmospheres and mechanical pumps are used to maintain a designated flow rate.

In addition to changes in technology, scientists were discovering new applications and methodology based on Tswett's early findings. Research by Martin marked the beginning of modern paper chromatography (76). The first gas chromatography paper appeared in 1951 (77). Stahl reported the first use of thin-layer chromatography five years later (78). Liquid chromatography emerged on a comparable basis to gas chromatography with the introduction of work by Huber and Hulsman (79). Halasz and Sebastian reported the first chemically bonded stationary phases for liquid chromatography (80). With the introduction of bonded stationary phases, it became necessary to denote the original polar stationary phase/nonpolar mobile phase method as the normal-phase mode and the newer nonpolar stationary phase/polar mobile phase approach as the reverse-phase mode.

The literature contains references dating as far back as 1904 which catalog attempts to resolve racemic compounds using adsorption methods (81). A review of early chromatographic literature indicates that the first

complete resolution of enantiomers was achieved on paper chromatography. In 1939, Henderson and Rule reported the chiral separation of d, l-p-phenylenediiminocamphor on d-lactose (82). Kotake, et. al., researched the influence of a chiral mobile phase on the resolution of several amino acids in paper chromatography in 1951 and he ascribed the separation to the chirality of the support, cellulose (83). In 1952, Dalgliesh proposed that a three-point simultaneous attachment was necessary to resolve amino acids using a cellulosic surface, this model was later modified to include additional interactions, such as repulsion and/or steric hindrance (84). Figure 6 shows a simplified model of a three-point interaction between chiral molecules.

Another polysaccharide, starch, was used by Prelog et. al. to separate Troger's base (85) and by Krebs et. al. (86) to resolve various acids. The first use of a modified chromatographic support for chiral separation was reported in 1960 (87). Klenn and Reed impregnated silica with a nitroaromatic entity because such compounds were known to complex other aromatic hydrocarbons via $\pi-\pi$ interactions.

In the 1960s compounds were derivatized with chiral reagents to form diastereomers and then chromatographically analyzed. Development of chiral separation techniques was dominated by gas chromatography during the 1960's. Gil-Av, *et. al.* reported the first successful direct enantiomeric separation in gas chromatography in 1966 (88). This approach employed the differential association of enantiomers with a chiral chromatographic media composed of derivatized amino acids. Short-lived diastereomeric adsorbates which differ in stability were formed. The enantiomer which formed the more stable complex was preferentially retained. This idea forms the foundation for modern chiral stationary phases and would

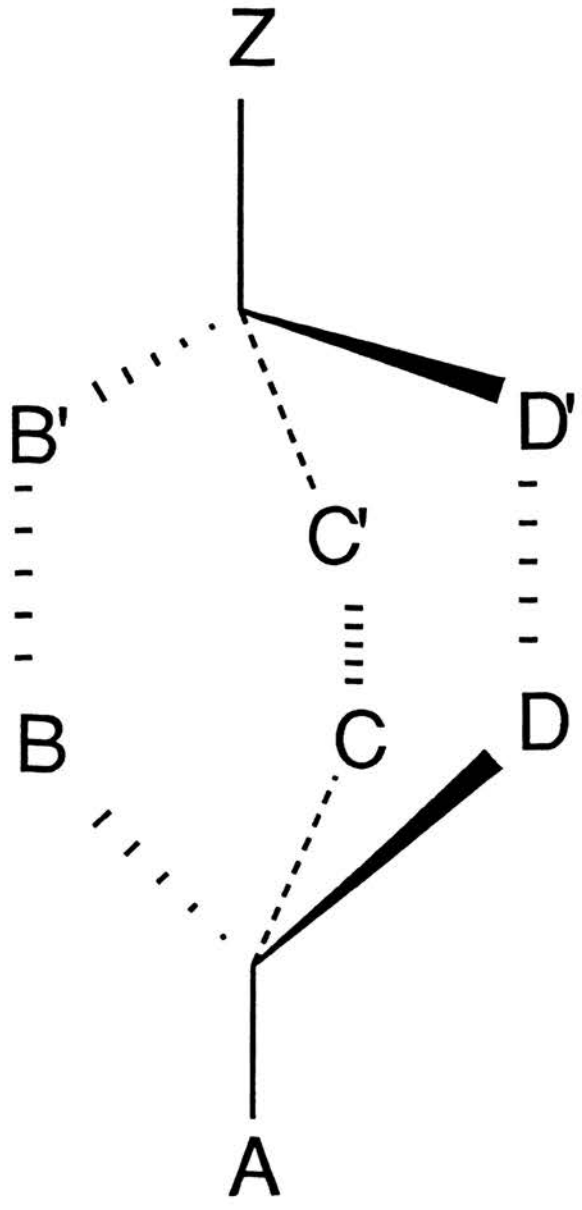


Figure 6. Simplified model of three point interaction between chiral molecules

eventually lead to the development of a wide variety of chiral LC and GC chromatographic supports.

In 1968 Rogozhin and Davankov (89) reported that ligand exchange chromatography (LEC) could be utilized for liquid chromatographic chiral separations. If a metal was introduced into a system which was capable of reversibly coordinating with an asymmetric stationary phase and the individual enantiomers, a complex diastereomeric specie could be dynamically formed (See Figure 7). Rogozhin and Davankov covalently bonded amino acids, such as L-proline, to a polystyrene-p-divinylbenzene cross-linked copolymer support and used copper (II) as the chelating metal to resolve free amino acid racemates. This method proved to be an effective technique for separating underivatized amino acids.

A different type of complexation, inclusion complexation, was reported by Cram and his group (90, 91). Cram synthesized various chiral binaphthyl based crown ethers and discovered that the 18-crown-6 proved to be a successful chiral stationary phase (47). The chiral crown ether used as a CSP in this study is shown in Figure 8. It was possible to resolve chiral compounds which contain primary amines because the 18-crown-6 ether moiety complexes ammonium ions (See Figure 9).

Up to this point, progress in the field of chiral separations was moving at a slow pace. The 1980s marked a rapid evolutionary period for chiral stationary phase development and commercialization, followed by global public acceptance and use. The turning point was marked by the introduction of the 'Pirkle' bonded phase which was heralded for its wide applicability and became the first commercialized CSP (50). Pirkle's rationale in designing a chiral stationary phase was based on the theories of Dalgleish and Gil-Av. Pirkle tried to create a three point simultaneous

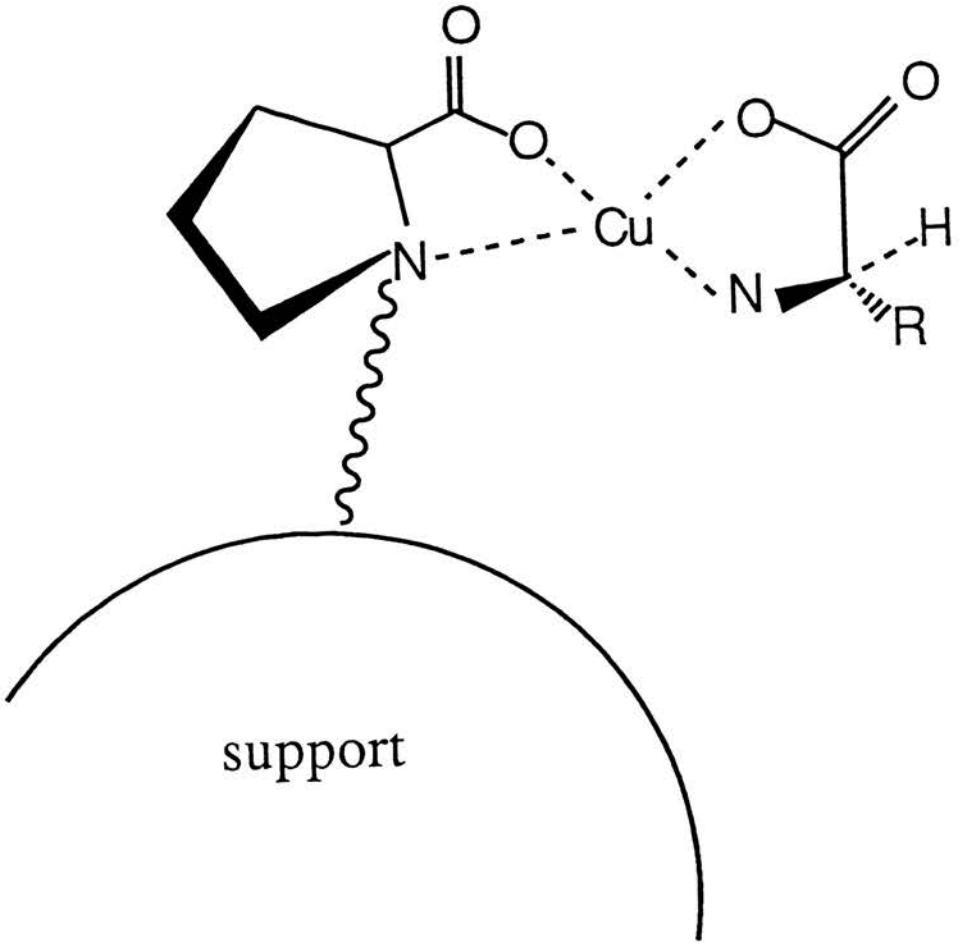


Figure 7. Simplified schematic of ternary complex formed in chiral LEC

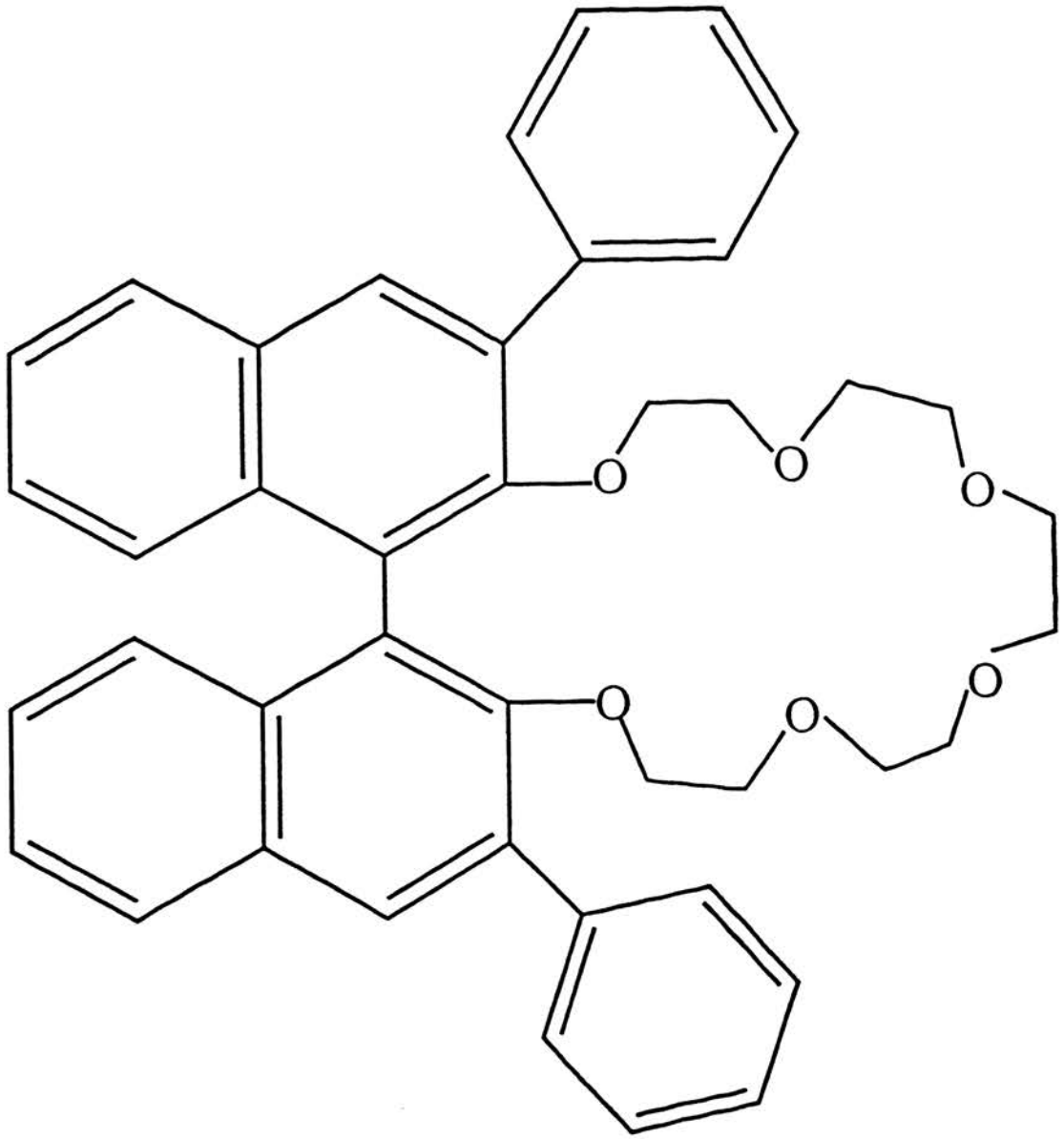


Figure 8. Crown ether used in amino acid studies

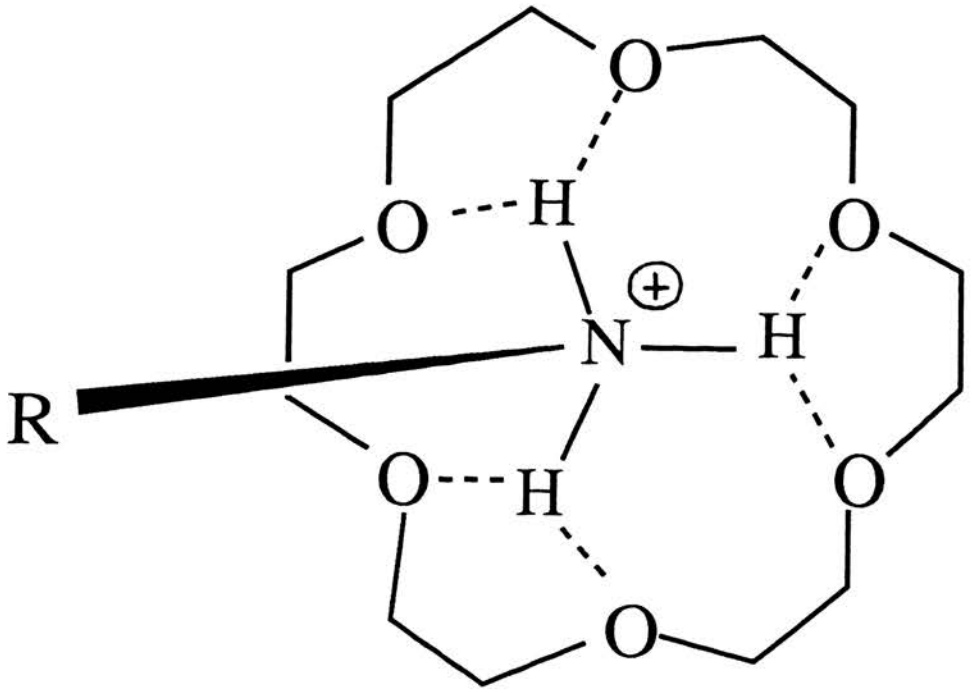
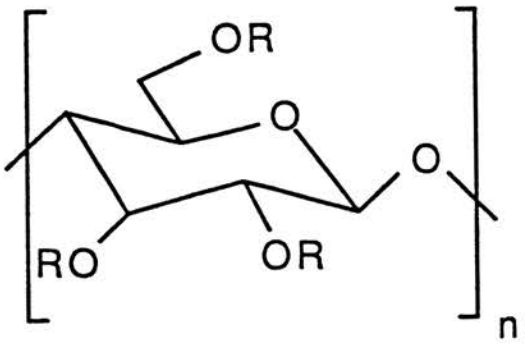


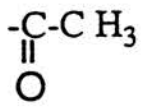
Figure 9. Complexation of primary amine with chiral crown ether in acidic media

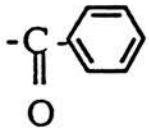
interaction between the analyte and a chiral bonded molecule via π–π interactions, hydrogen bonding, and dipolar interactions. The initial commercial 'Pirkle' CSP consisted of (R)-N-3,5-(dinitrobenzoyl)-phenylglycine bonded to γ-aminopropylsilanized silica gel. These CSPs can be used with relatively polar mobile phases, but enantioselectivity and stability are enhanced in the normal-phase mode. A wide variety of π-complex-hydrogen bond CSPs have been synthesized by Pirkle and by other scientists, but the general applicability remains the same (92, 93). The second generation 'Pirkle' phases have improved enantioselectivity (37) in comparison to that of the first generation.

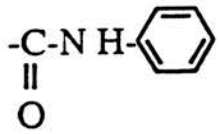
In the ongoing search for new and improved CSPs, it is not surprising to note the continuing adaptation of saccharides as potential CSPs. Linder and Mannschreck reported the use of triacetyl cellulose as a CSP in 1980 (94). Various derivatized cellulosic phases (See Figure 10) have been commercialized and have proven successful in separating racemic thioamides, sulfoxides, organophosphorus compounds, pharmaceuticals and amino acid derivatives. Another commercially available polysaccharide stationary phase was developed by Armstrong in 1984 (95). This was the cyclodextrin bonded phase. A simplified schematic depicting inclusion complexation between a solute and a native cyclodextrin stationary phase is shown in Figure 11. Cyclodextrin stationary phases have been utilized primarily in the reverse-phase mode for the resolution of pharmaceuticals, nicotine analogues, derivatized amino acids, steroids, metallocenes, binaphthyl crown ethers and various aromatic compounds. The structural similarities between cellulose and cyclodextrin are shown in Figure 12.

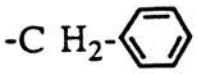
Another type of commercially available CSP involved natural and synthetic polymers. In 1981, Okamoto reported the use of a helical



R=  triacetate

 tribenzoate

 trisphenylcarbamate

 tribenzyl ether

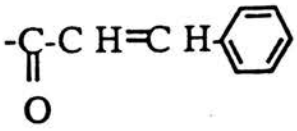
 tricinnamate

Figure 10. Cellulose derivatives utilized as chiral stationary phases

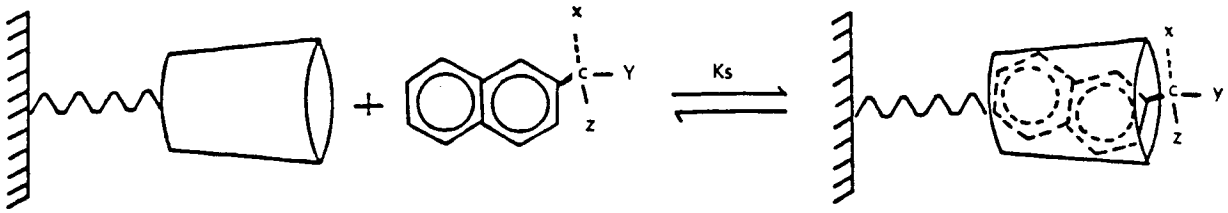


Figure 11. Simplified schematic of inclusion complexation with a native cyclodextrin

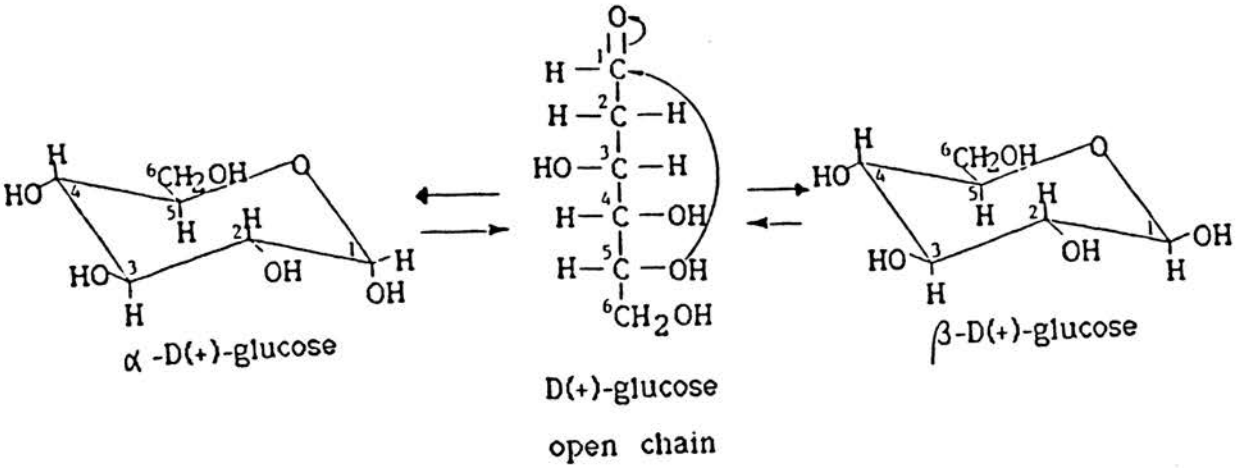


Figure 12. Open chain and cyclic structures and stereochemistry of D-(+)-glucose. Upon ring closure, α andβ anomers are formed. The α anomer forms cyclic molecules with a specific molecular weight; cyclodextrin. The β anomer forms linear molecules with a wide range of molecular weights.

synthetic polymer, (+)-poly(triphenylmethyl methacrylate) as a chiral stationary phase (46). This polymer can be used either as solid high molecular weight polymeric particles or as soluble low molecular weight fractions which are adsorbed onto diphenyl silanized silica gel. The state of the polymer can affect the enantioselectivity and the adsorbed version appears to have a wider applicability. In 1983, two protein bonded LC phases were reported. Allenmark developed the bovine serum albumin (BSA) phase (48) and Hermansson produced the α_1-acid glycoprotein (AGP) phase (49). Both of these natural polymeric phases were shown to be very useful. The BSA column can be utilized to separate derivatized amino acids, aromatic sulfoxides and certain pharmaceuticals. The AGP column can resolve a wide variety of cyclic and aromatic drugs. The major complaint for the protein bonded phases has been the frailty of these columns and the inability to do preparative scale separations. The second generation columns are proving somewhat hardier.

It should be noted that amino acid and chiral crown ether bonded chiral stationary phases have been commercialized for use with ligand exchange chromatography and the high performance liquid chromatography, respectively.

It is important to note that despite the changing face of technology, the basic principles of adsorption chromatography are still the same. In liquid chromatography (LC), the adsorption of an analyte is determined by several parameters: the nature of the solvent, the nature of the adsorbent, the nature of the analyte, temperature, and pH.

III. EXPERIMENTAL

A. MATERIALS

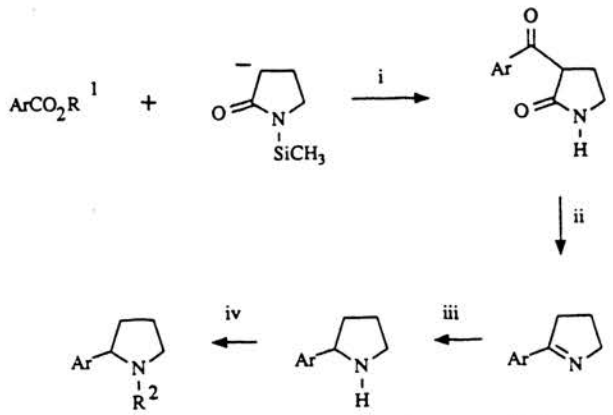
1. Thin layer chromatography

Silica gel (K5F, 250 micrometer [μm] layer thickness, 5 x 20 centimeter [cm]), high performance silica gel (HP-KF, 200 μm layer thickness, 5 x 5 cm), cellulose (K2F, 250 μm layer thickness, 5 x 20 cm), chemically bonded octadecyl (KC18F, 200 μm layer thickness, 5 x 20 cm), chemically bonded ethyl (KC2F, 200 μm layer thickness, 5 x 20 cm) and chemically bonded diphenyl (Diphenyl-F, 250 μm layer thickness, 5 x 20 cm) TLC plates were obtained from Whatman Chemical Separation Division, Inc. (Clifton, NJ). Aluminum oxide (250 μm layer thickness, 5 x 20 cm) and diol (HPTLC Diol $F_{254}s$, 200 μm layer thickness, 10 x 10 cm) TLC plates were obtained through Alltech Associates, Inc. (Deerfield, IL).

Phenylpropanolamine, timolol, isoproterenol, metoprolol, propranolol, octopamine, pindolol, N-carbobenzoxy-alanyl-L-proline, N-carbobenzoxy-isoleucyl-L-proline, N-carbobenzoxy-L-proline, N-carbobenzoxy-glycyl-L-proline, dansyl-DL-leucine, dansyl-DL-methionine, dansyl-DL-norleucine, dansyl-DL-phenylalanine, dansyl-DL-valine, cinchonidine, cinchonine, quinine, quinidine, heptakis (2,6-di-O-methyl)-β-cyclodextrin, and sodium chloride were obtained from Sigma (St. Louis, MO). Norphenylephrine, 1-pentanol, molecular sieves (5 Angstrom, 4-8 mesh), (1R)-(-)-ammonium-10-camphorsulfonate, DL-alanine-β-naphthylamide, DL-methionine-β-naphthylamide and RS-5-(4-methylphenyl)-5-phenylhydantoin were obtained from Aldrich (Milwaukee, WI). Methylene chloride (CH_2Cl_2), ethylene glycol, acetonitrile, ethanol, 2-propanol, methanol (MeOH), and 1-butanol were

obtained from Fisher Scientific (Fair Lawn, NJ). N'-(2-naphthylmethyl)-nornicotine, a nicotine analogue, was synthesized according to the following scheme (31).

Scheme I[a]



[a] i, Lithium diisopropylamide; ii, hydrochloric acid, heat, then hydroxide; iii, sodium cyanoborohydride; iv, *n*-butyllithium, then R^2Br.

Maltosyl-β-CD was supplied by P.C. Hermann. Two different companies, Pharmatec and Consortium Fur Elektrochemische Industrie GMBH (Wacker), supplied the HP-β-CD for the study regarding the effect of the degree of substitution. Pharmatec indicated the degree of substitution (DS) as 2, 7 and 9 for their product. The average molar substitution (MS) of the HP-β-CD supplied by the Wacker company was 0.6 and 0.9. The degree of substitution indicates the number of substitutents per CD molecule whereas the molar substitution is the number of substitutents per anhydroglucose unit. The β-CD molecule consists of seven linked glucose units so if the MS was 0.6 for the derivatized CD; the DS would be 4.2.

2. High Performance Liquid Chromatography

Huperzine A, a drug under investigation for Alzheimer's dementia (44), was kindly provided by Professor A. P. Kozikowski and Dr. Yan Xia of the University of Pittsburgh. SQ 30840 is a drug under investigation at the Squibb Institute for Medical Research, Princeton (NJ). It was supplied by Dr. Kimball. Efaroxan, Idazoxan and derivatives are drugs under investigation at Reckitt & Coleman. They were supplied by N. A. Hyde (Danson Lane, Kingston-upon-Hull, UK). Flouxetine is a drug under investigation at Eli Lilly Laboratories (Indianapolis, IN). It was provided by Thomas L. Jeatran. Ibuprofen was supplied by Eli Lilly. The other drugs were obtained as previously described (41). Etomidate and Norgestrel were supplied by Advanced Separations Technologies (Whippany, NJ). Methanol and acetonitrile were from Fisher Scientific (Fair Lawn, NJ).

The structures of the solutes, which were obtained from various sources, are presented in the tables. Hexane and isopropanol were obtained from Fisher Scientific (St. Louis, MO).

Dinitrobenzoyl chloride, 2,6-dimethylphenylisocyanate, (R) and (S)-1-(1-naphthyl)ethylisocyanate and p-toluoyl chloride were obtained from Aldrich Chemical Company (Milwaukee, WI). The acetylated β-CD column was 250 x 4.6 mm column obtained from Advanced Separation Technologies, Inc. (Whippany, NJ). The chromatographic columns used in the normal phase HPLC study were synthesized according to the procedure listed under preparation of bonded phase. The columns were packed by Tom Beesley at Advanced Separations Technologies, Inc. (Whippany, NJ).

The perchloric acid, mercaptoethanol, boric acid, o-phthalaldehyde, and DL-amino acids were obtained from Aldrich (Milwaukee, WI). Methanol, ethanol and potassium hydroxide were supplied by Fisher (St. Louis, MO). The L-amino acids were provided by the following companies: Kodak (Rochester, NY), Lancaster Synthesis (Windham, NJ), Fluka (Ronkonkoma, NY), Sigma (St. Louis, MO), Spectrum (Gardena, CA) and Research Plus, Inc. (Bayonne, NJ). All of the samples and mobile phase solutions were filtered using 0.2 μ filters supplied by Alltech Associates, Inc. (Deerfield, IL).

The chromatographic system was a Shimadzu Gradient System, with two LC-6A pumps, a SCL-6A gradient controller, and a UV detector, model SPD-6A. The recorder was the Shimadzu model C-R2AX integrator. The column dimensions were 25 cm x 4.6 mm, i.d. Cyclobond I bonded with β-cyclodextrin (seven glucopyranoside units) from Astec. A Cyclobond II column bonded with γ-cyclodextrin (eight glucopyranoside units) was also used.

The chromatographic columns used in the urine, blood and amniotic fluid studies included the Crownpak CR(+) and CR(-) columns supplied by Michael Henry at J.T. Baker (Phillipsburg, NJ) and 5 μ octadecylsilane (C_{18}) columns obtained from Advanced Separation Technology (Whippany, NJ).

The LC system used for the analysis of commercial sources of L-amino acids consisted of a Shimadzu LC-6A pump and CR3A chromatopac recorder, an ESA (Bedford, MA) Coulochem model 5100A detector and analytical cell model 5011, and a Rheodyne (Cotati, CA) model 7125 injector. Two different chromatographic systems were used for the urine study. The electrochemical (LCEC) system consisted of two Rheodyne

injectors (models 7125 and 7010), two Shimadzu LC-6A pumps, a Perkin Elmer (Norwalk, CT) LC-85B spectrophotometric detector, the above-mentioned ESA electrochemical detection system, a Perkin Elmer R100A recorder and a Shimadzu CR3A Chromatopac recorder. The LC system which utilized fluorescence detection was made up of the following Shimadzu devices: two LC-6A pumps, a SCL-6B system controller, a CR601 chromatopac recorder, SPD-6AV UV/VIS spectrophotometric detector, and a RF-535 fluorescence HPLC monitor. The "fluorescence" chromatographic system also included a Rainin (San Carlos, CA) model A-30-S pump, a Scientific Systems, Inc. (State College, PA) pulse dampener model LP-21 and two Rheodyne injectors (models 7125 and 7010) for post column derivatization.

A Labconco (Kansas City, MO) Freeze Dryer model 75018 was used to concentrate some samples and a Precision Clinical Centricone manufactured by Precision Scientific Company (Chicago, IL) was used for centrifugation.

B. METHODS

1. Normal phase TLC

The molecular sieves (Type 5A) were dried for twenty four hours at three hundred and fifty degrees centigrade and stored in a drying oven at one hundred and twenty five degrees centigrade. Fifty grams of molecular sieves were used to dry two hundred and fifty milliliters of solvent. The solvents were dryed for twenty four hours before use. The TLC plates were placed in a drying oven at one hundred and twenty five degrees centigrade for three to four hours and then stored in a dessicator until use.

It took approximately thirty to forty-five minutes to completely develop a 5 x 5 cm TLC plate. All developments were done at room temperature (20°c) in 6 cm internal diameter (i.d.) x 23 cm cylindrical glass chambers. Spot visualization was performed by use of a fixed wavelength, 254 nm, ultraviolet (UV) lamp. A Schimadzu dual wavelength TLC scanner (CS-910) was used to measure resolution. Various wavelengths were scanned to provide supporting evidence as to the validity of the enantiomeric separation.

2. Reverse phase TLC

Sodium chloride was used to stabilize the plate binder. 0.6M sodium chloride was added to the mobile phases containing greater than 50% water by volume. Acetonitrile and methanol were used as organic modifiers.

The plates were developed at room temperature (20°c) in 6 (i.d.) x 23 cm cylindrical glass chambers. Spot visualization was performed by use of a fixed wavelength (254 nm) ultraviolet lamp. A Shimadzu dual wavelength TLC scanner (CS-910) was used to measure resolution in the HP-β-CD study.

It took approximately thirty-six to forty-eight hours to completely develop a 5 x 20 cm TLC plate when maltosyl-β-CD was the CMA. It took approximately thirty minutes to completely develop a 5 x 20 cm TLC plate when the CMA was dimethyl-β-CD. The approximate development times are discussed in the results section for the data obtained when HP-β-CD was the CMA.

3. Amino Acid Study

Prior to using the LCEC systems, voltage scans were run on several essential amino acids. The following amino acids were chosen for the LCEC commercial study: cysteine, methionine, ornithine, tryptophan and tyrosine. The applied voltage for the analysis of cysteine, methionine, tyrosine, and tryptophan was +0.5 volts. A somewhat better response was noted for these compounds if the voltage was greater than +0.5 and less than +0.8 volts but the background current was considered to be too high (greater than 1 μ amp). A high background current can have detrimental effects on the electrodes. Ornithine was analyzed at -0.10 volts.

Before beginning this study, it was necessary to optimize the separation of enantiomers by modifying the mobile phase conditions, the flow rate and the column temperature. Cysteine and ornithine elute rather quickly so the optimum temperature was determined to be 0°C and 10°C, respectively. The optimum flow rate for these two amino acids was 0.1 ml/min. The mobile phase composition was aqueous perchloric acid (pH = 1.5 or 2.0). Under the stated conditions, it was possible to quantitate the amount of D-amino acid present without interference from the void volume peak. Methionine, tryptophan, phenylalanine, and tyrosine had longer retention times so it was possible to analyze these amino acids at room temperature. The flow rate for methionine and tyrosine was 0.3 ml/min; for phenylalanine, 0.5 ml/min; and the flow rate for tryptophan was 0.8 ml/min. The mobile phase composition was 97.5/2.5 aqueous perchloric acid/methanol (v/v) or 100% pH=2 $HClO_4$ solution for these three compounds.

A C_{18} column was used to isolate the individual amino acids. The mobile phase consisted either of 95:5 water:methanol (v:v) or 95:5, $5x10^{-3}$ M $HClO_4$:methanol (v:v). The flow rate was 1.0 or 1.5 ml/min. The column was at room temperature and the UV detector setting was at 200 nm.

A column switching or coupled column method was used for the analysis of tyrosine, phenylalanine, tryptophan and leucine in physiological fluids (Figure 26). Urine specimens taken in the early morning were preferred as they contained higher overall amino acid concentrations. The optimum separation conditions for the chiral separation are as listed above and in the individual figures.

Two different methods of detection were used in the urine study: electrochemical and fluorescence. The applied voltages for the LCEC experiments were the same as those listed above. Post column OPA derivatization of the amino acids was required for fluorescence detection. The excitation wavelength was 340 nm and the emission wavelength was 450 nm. The Rainin pump was the post column pump for this system. The OPA solution was prepared as follows. The OPA reagent was prepared by dissolving 700 mg of o-phthalaldehyde in 15 ml of ethanol. Three hundred milliliters of mercaptoethanol was added to this solution. The solution was then added to 1 liter of 3% boric acid solution which had been adjusted to pH=10.0 with KOH. Teflon tubing (0.5 millimeters i.d.) was used with the post column apparatus and the post column reactor was 3 meters long.

C. PREPARATION OF DERIVATIZED CD BONDED PHASE

β-Cyclodextrin was attached to 5 μ spherical silica as previously reported (96). Four grams of the β-CD bonded phase were dried overnight under vacuum in a drying gun using MeOH and P_2O_5. The dried sorbent was placed in a 3-necked round bottom flask. Anhydrous solvent (Aldrich) (100 mL) was added. Pyridine was used as the solvent for the isocyanate derivatized phases and toluene (+ 3 mL pyridine) was used for the acid chloride derivatized phase. The mixture was refluxed until all the water was removed (as an azeotrope into a Dean-Stark trap). The derivatizing agent was added (neat) and the mixture was refluxed for about four hours (three hours for the acid chloride derivatized phase). The isocyanate derivatized β-CD bonded phases were collected on a fritted glass filter and washed with approximately 100 mL of pyridine followed by 200 mL of MeOH and then air dried. The acid chloride derivatized β-CD phase was also collected on a fritted glass filter and washed with approximately 100 mL each of pyridine and MeOH, about 10 mL of H_2O followed by MeOH and then air dried.

The bonded sorbents were submitted for carbon analysis. The surface concentration was calculated according to the equation (97).

$$(\mu moles/m^2) = \frac{\%C \times 10^6}{S[1200N_C - \%C(M - 1)]}$$

where N_C is the number of carbons in the ligand, M is the molecular weight of the ligand and S is the surface area of the substrate, which, according to the manufacturer, is 170 m^2/g. For β-CD (N_C = 42, M =

1135), the coverage was calculated to be 0.20 μmoles/m^2. To determine the degree of substitution on the β-CD of the derivatized β-CD phases, the %C from the CD + linkage chain was subtracted from the total %C and M - 1 was substituted by M in the denominator. The degree of substitution for each of the phases were calculated and are reported in Table I. The sorbents were all packed into 250 x 4.6 mm stainless steel columns.

D. SAMPLE PREPARATION

1. Urinalysis

All samples were dissolved in the mobile phase solution immediately prior to analysis. The results of this study were reproducible if the column and the detector current were allowed to stabilize before beginning the analysis. The average concentration of the L-amino acids was 10 mM. Despite the large amount of sample injected, it was still possible to attain baseline resolution with a single column. When using fluorescence detection the average concentration of the L-amino acid standard solution was 0.2 mM.

Urine samples were collected from young healthy adults. The mobile phase solution and the urine samples were filtered with 0.2 μ filters. If the urine appeared cloudy, the sample was centrifuged at 1550 rpm for 5 minutes. The supernatant was filtered and analyzed. All remaining urine samples were refrigerated at 4°C.

In one set of experiments the samples were concentrated by freeze drying. The freeze-dried samples were dissolved in the mobile phase solution immediately prior to analysis.

2. Blood serum analysis

Venous blood was collected from young healthy adults. The blood samples were placed in a refrigerator for twenty-four hours to allow coagulation. The samples were centrifuged at 1550 rpm for fifteen minutes. The supernatent was collected and filtered with a 0.2μ filter. Solid phase extraction (SPE) was performed to remove proteins. A C18

SPE cartidge was utilized. The cartridge was washed with 2 x 2 milliliters (mL) of MeOH and H_2O, respectively. Two to three mL of sample was introduced onto the cartridge. The sample was eluted using one mL of aqueous perchloric acid (pH=2.0). This sample was immediately analyzed using a coupled column system.

In one set of experiments the samples were concentrated by freeze drying. The freeze-dried samples were dissolved in the mobile phase solution immediately prior to analysis.

3. Amniotic fluid analysis

Frozen amniotic fluid samples were graciously donated by Dr. Sau W. Cheung, Ph. D at Washington University School of Medicine. These samples were collected from pregnant women and stored in a freezer at 4° C until analysis. The age of the samples ranged from several days to several years.

The amniotic fluid samples were thawed for five to ten minutes at room temperature. The samples were filtered with a 0.2μ filter and analyzed using a coupled column system.

In one set of experiments the samples were concentrated by freeze drying. The freeze-dried samples were dissolved in the mobile phase solution immediately prior to analysis.

IV. RESULTS AND DISCUSSION

A. CHIRAL COUNTER IONS IN NORMAL PHASE TLC

A method was developed for the thin layer chromatographic (TLC) separation of enantiomers and diastereomers involving the use of chiral ion interaction agents based on prior normal-phase HPLC studies (26-28). Several aromatic amino alcohols were resolved by TLC on diol and/or high performance silica gel plates using a mobile phase containing (1R)-(-)-ammonium-10-camphorsulfonate (CSA) or N-carbobenzoxy-glycyl-L-proline (ZGP). Results which were attained using ZGP or CSA as chiral mobile phase additives in TLC are similar to those obtained using the same CMAs in HPLC. One major difference was that separations of aromatic amino alcohols in HPLC involved the (+) antipode of 10-camphorsulfonate whereas the same work performed in TLC used the (-) antipode. The chiral separation mechanism for each chromatographic method (HPLC and TLC) was the formation of diastereomeric ion-pairs.

For the TLC studies as well as the HPLC studies, no enantiomeric separations could be achieved with any of the ion interaction agents unless the solvents and the chromatographic bed were thoroughly dried. Presumably, this was because an intimate, multi-point interaction must occur between the chiral ion interaction agent and the racemic substrate. Hydrogen bonding is particularly important when these associations occur (π–π interactions, dipole-dipole interactions and steric interactions are also pertinent). If too much water is present, the water may preferentially associate via hydrogen bonding with the chiral selector and/or racemate thereby negating interactions between these species that are important for chiral recognition.

Dichloromethane was the major component of the mobile phase. However, it was difficult to dissolve ammonium camphorsulfonate (CSA) in dry dichloromethane. Several organic co-solvents were tested to evaluate their ability to solubilize CSA. These solvents included: 1-pentanol, 1-butanol, 2-propanol, ethanol, methanol, acetonitrile, and ethylene glycol. CSA was most soluble if ethylene glycol was used but the solution appeared to form two layers and thus was impractical. CSA was soluble in solutions which contained the lower molecular weight alcohols. As was expected, the higher the percent of alcohol in the mobile phase, the greater the solubility of the chiral ion interaction agent. It was possible to attain resolution of metoprolol at 95/5 (v/v) dichloromethane/methanol although CSA displayed limited solubility in mobile phases which contained less than 10% alcohol by volume. The best results were obtained when dry methanol or 2-propanol were present in the mobile phase.

No solubility problem was encountered with these mixed mobile phases when using ZGP as a chiral mobile phase additive, so it was possible to study the effects of different CMA concentrations. In this study, the concentration of ZGP was varied from 6.5-16.8 mM without any significant change in the retention factor (R_f) or the separation factor (α) (Table I). The capacity factors (k') for the enantiomers usually decrease with an increase in the concentration of the counter ion. This is probably due to increased competition between the counter ion and the diastereomeric ion-pair for adsorption sites on the stationary phase.

Separations obtained on silica gel (K5F) and high performance silica gel (HP-KF) gave approximately the same R_f values but the K5F did not

Table I. Effect of Varying Concentration of ZGP on Enantioselectivity for Propranolol in TLC Study

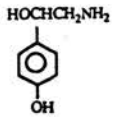
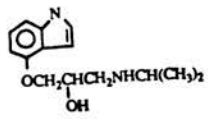
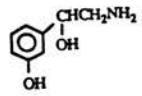
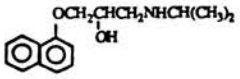
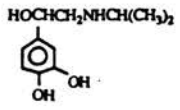
Concentration of ZGP (mM)	R_{f1}	R_{f2}	α
6.5	0.07	0.23	1.7
6.8	0.07	0.24	1.9
13.6	0.06	0.21	1.8
16.8	0.08	0.20	1.6

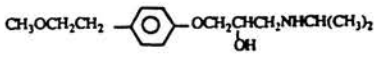
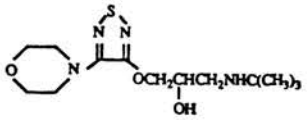
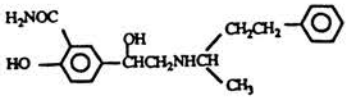
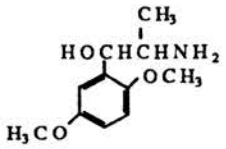
*Experimental conditions: The mobile phase consisted of 90/10, methylene chloride/methanol. The stationary phase was HP-KF silica gel. The method of detection was a Schimadzu dual wavelength TLC scanner (CS-910).

offer the same selectivity as HP-KF. Separations on the ethyl (KC2F) plates gave higher R_f values than the HP-KF plates but no enantiomers were resolved on the KC2F plates. The ethyl and diol plates gave similar R_f values except there was much greater streaking on the ethyl plates. There was very little spot movement on either the aluminum oxide plates or the cellulosic plates. The only TLC plates on which effective, reproducible enantiomeric separations could be obtained were the diol and the high performance silica gel plates (HP-KF).

The structures of the resolved compounds as well as pertinent separation data are given in Table II. Note that all of the compounds have three functionalities in common: an aromatic ring, an α-hydroxyl group and a β-amine. Table II shows that it was possible to achieve very efficient separations with the use of CSA and ZGP as chiral mobile phase additives (CMAs). Several compounds were separated with both CMAs. In four out of five of these compounds (Table II) a higher selectivity was obtained by adding 5 mM triethylamine (TEA) when ZGP was the CMA. A majority of the racemates were resolved on the HP-KF plates. Retention on the stationary phases was due to the OH-groups present on both the HP silica gel and the diol plates. These types of plates displayed a certain similarity in their chromatographic behavior, however the silica gel plates were more strongly adsorbing than the diol plates (98). Note that the mobile phase which was used in conjunction with the diol plates had a lower percentage of alcohol and used a less polar alcohol in comparison with the mobile phases used with the HP silica gel plates. There were two similarities among the compounds resolved with the diol plates: none of these compounds contained a primary amine and each racemate had a terminal branched hydrocarbon group. The separations achieved on the diol plates

Table II. Compounds Separated Using ZGP or CSA as Chiral Mobile Phase Additives

Enantiomers	R_{f1}	R_{f2}	α	Conditions
Octopamine	0.15	0.33	2.2	7.9 mM CSA[a]
				
Pindolol	0.07	0.12	1.7	6.7 mM ZGP[b]
				
Norphenylephrine	0.05	0.26	5.2	5.9 mM ZGP[a]
	0.03	0.14	4.7	6.8 mM CSA[c]
Propranolol	0.08	0.20	2.5	6.7 mM ZGP[c]
	0.05	0.20	4.0	9.3 mM CSA[c]
Isoproterenol	0.14	0.38	2.7	5.9 mM ZGP[a]
	0.12	0.30	2.5	6.8 mM CSA[a]

Metoprolol 	0.11	0.17	1.5	10.7 mM CSA[b]
Timolol 	0.26	0.51	2.0	5.8 mM ZGP[a]
	0.39	0.55	1.4	13.9 mM CSA[b]
Diastereomers				
Labetalol 	0.01	0.05	5.0	6.7 mM ZGP[b]
	0.02	0.05	2.5	9.3 mM CSA[b]
Methoxamine 	0.15	0.32	2.1	6.8 mM CSA[c]
Phenylpropanol-amine 	0.06	0.26	4.3	6.9 mM ZGP[a]

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[a] HP Silica gel plates with 75/25 (v/v) methylene chloride/methanol

[b] DIOL plates with 95/5 (v/v) methylene chloride/2-propanol

[c] HP Silica gel plates with 90/10 (v/v) methylene chloride/methanol

NOTE: All separations achieved with ZGP contained 5 mM TEA.

gave lower α values than those attained with the HP-KF plates. None of the adrenergic compounds were resolved on the diol plates. Separations were optimized for most of the adrenergic compounds by using a higher alcohol content in the mobile phase whereas most of the separations for the β-adrenergic blockers were optimized with a lower percentage alcohol content and/or a less polar alcohol in the mobile phase.

Figure 13 shows two TLC chromatograms. These chromatograms illustrate the high degree of stereoselectivity obtainable with this method. Both chromatograms were developed on HP-KF plates using 75:25 (v/v) dichloromethane/methanol but each used a different CMA. The plate on the left illustrates the separation of isoproterenol and propranolol using CSA as a chiral ion interaction agent. The plate on the rightt shows the resolution of methoxamine, norphenylephrine, phenylpropranolamine and octopamine employing ZGP and triethylamine (TEA). Methoxamine and phenylpropranolamine have two stereogenic centers so there are four stereoisomers for each compound. The number of stereoisomers is determined by application of the 2^n rule were n = the number of stereogenic centers for the compound. The TLC densitometric scans for the above-mentioned plate showed three peaks for methoxamine and two peaks for phenylpropranolamine. The resolution of methoxamine was not reported under these conditions because it was not possible to determine which stereoisomers were resolved. The separation of phenylpropanolamine was presumed to be between diastereomers rather than between enantiomers.

Figures 14 and 15 show three TLC densitometric profiles of TLC plates scanned at 254 nm, 275 nm and 300 nm, respectively. Notice that

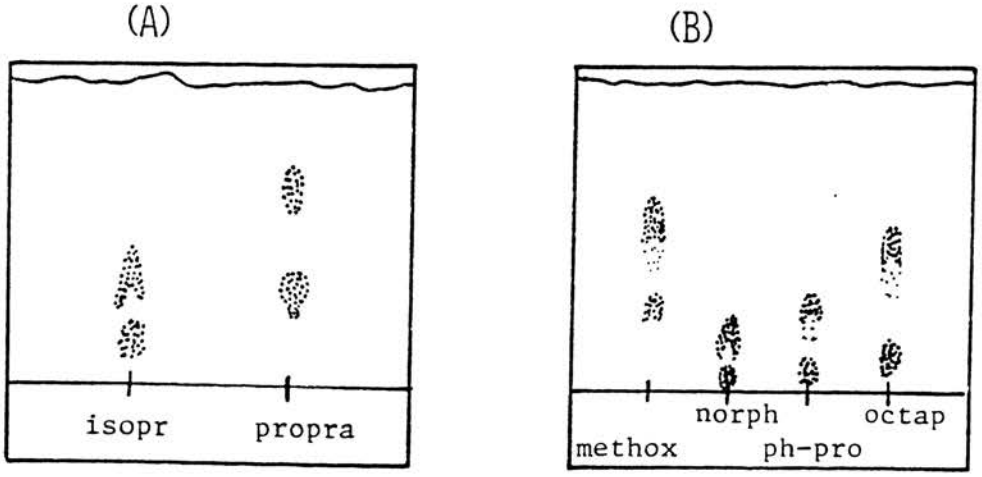


Figure 13: Thin layer chromatograms on 5 x 5 cm HPTLC silica gel. (A) Mobile phase 75:25 CH_2Cl_2: MeOH containing 6.8 mM camphorsulfonic acid (CSA). isopr (isoproterenol); propra (propranolol). (B) Mobile phase 75:25 CH_2Cl_2: MeOH containing 6.9 mM N-carbobenzoxy-glycyl-L-proline (ZGP) and 5 mM triethylamine. methox (methoxamine); norph (norphenylephrine); ph-pro (phenylpropanolamine); octop (octapamine).

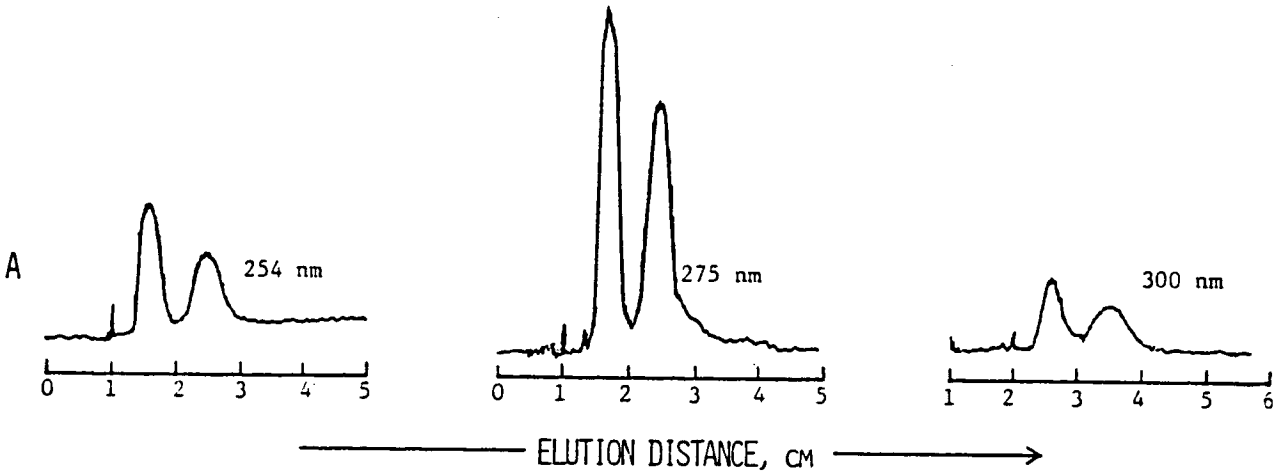


Figure 14: TLC densitometric scans (using a Shimadzu CS-910 at three different wavelengths) showing the resolution of racemic norphenylephrine. The stationary phase was a HPTLC silica gel plate (5x5 cm). The mobile phase consisted of 6.6 mM N-CBZ-gly-L-proline + 5 mM triethylamine dissolved in 75:25 (v/v) CH_2Cl_2: MeOH.

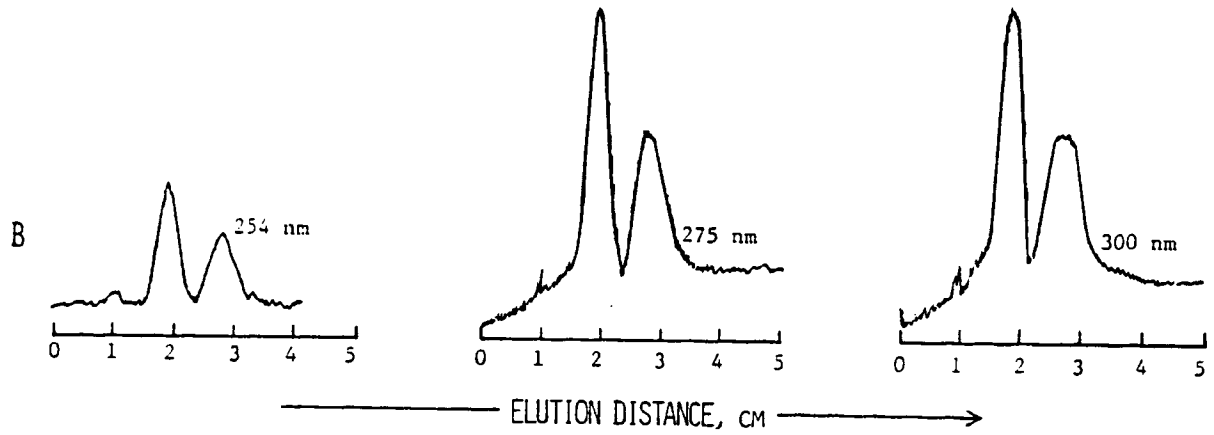


Figure 15: TLC densitometric scans (using a Shimadzu CS-910 scanner) showing the resolution of racemic isoproterenol (a bronchodilator). The stationary phase was a HPTLC silica gel plate (5x5 cm). The mobile phase consisted of 6.8 mM camphorsulfonic acid dissolved in 75:25 (v/v) CH_2Cl_2: MeOH.

the peak areas maintain the same relative proportions at different wavelengths thereby making it possible that these were enantiomeric separations. The stationary phase was HP silica gel and the mobile phase was 75:25 (v/v) dichloromethane/methanol for both separations. In Figure 14, norphenylephrine was resolved using ZGP and TEA. Figure 15 illustrates the resolution of racemic isoproterenol; the chiral ion interaction agent was CSA.

A comparison was made between various N-CBZ-amino acid derivatives as CMAs: N-CBZ-alanyl-L-proline (ZAP), N-CBZ-isoleucyl-L-proline (ZIP), N-CBZ-L-proline (ZP) and N-CBZ-glycyl-L-proline (ZGP). Table III shows that it was possible to attain some separations with all of the N-CBZ-amino acid derivatives. However, ZGP separated the largest number of compounds and gave higher α values than the other N-CBZ-amino acid derivatives. Note that the only compound resolved using diol plates and 2- propanol with an N-CBZ derivative was metoprolol. Also, metoprolol was the only compound resolved using ZAP or ZP as a CMA. It was possible to separate two adrenergic compounds and one β-adrenergic blocker using ZIP as a CMA (Table III). From these results it was clear that ZGP was the best chiral ion interaction agent of the N-CBZ-amino acid derivatives in resolving the racemates of interest in this study.

In thin layer chromatography, ZGP and CSA demonstrated unique abilities in separating enantiomeric amino alcohols. Of the ten pairs of enantiomers resolved in this TLC study; two compounds could only be separated using ZGP, three compounds could only be resolved using CSA and five of the compounds analyzed could be separated using either CMA. Of the enantiomers which could be resolved with either CMA, a higher stereoselectivity was obtained in four out of five of these compounds

Table III. Compounds Separated Using Other N-CBZ Amino Acid Derivatives as CMAs.

Compounds	R_{f1}	R_{f2}	α	Conditions
Norphenylephrine	0.20	0.48	2.4	7.5 mM ZIP[a]
Octopamine	0.20	0.52	2.6	7.5 mM ZIP[a]
Metoprolol	0.17	0.28	1.6	6.9 mM ZIP[b]
	0.17	0.28	1.6	7.0 mM ZAP[b]
	0.17	0.28	1.6	7.8 mM ZP[b]
Propranolol	0.10	0.30	3.0	6.3 mM ZGP[a]
Isoproterenol	0.04	0.10	2.5	6.3 mM ZGP[a]
Phenylpropanolamine	0.08	0.32	4.0	6.3 mM ZGP[a]
Timolol	0.20	0.40	2.0	5.8 mM ZGP[a]

[a]HP Silica Gel Plates with 75/25 CH_2Cl_2/MeOH
[b]Diol plates with 95/5 CH_2Cl_2/2-propanol
ZP: N-CBZ-L-proline
ZIP: N-CBZ-isoleucyl-L-proline
ZAP: N-CBZ-alanyl-L-proline
ZGP:N-CBZ-glycyl-L-proline

NOTE: The TEA was not used as an additive for these separations.

(Table II) when ZGP was the CMA. It was possible to obtain better stereoselectivity in HPLC with ZGP than with CSA for the amino alcohols analyzed (99). In HPLC, the preferred stationary phase was the DIOL. In TLC, the largest number of compounds were separated with the high performance silica gel plates although it was necessary to use the DIOL plates to resolve some of the analytes. The only compound which could be separated on either type of TLC plate was timolol.

There were several mobile phase dissimilarities which could be noted in a comparison between HPLC and TLC when ZGP and CSA are used as CMAs. The addition of triethylamine (TEA) to the mobile phase in HPLC decreased retention times but did not effect stereoselectivity. The use of TEA in the mobile phase in TLC gave different results with different compounds (Table IV) and no general trend was noted. Acetonitrile, tetrahydrofuran, 1-pentanol and ethyl acetate could be used as polar modifiers in HPLC but 1-pentanol was the only modifier which did not contribute to peak asymmetry. Solute movement in TLC was restricted to mobile phases which contained an alcohol as co-solvent. The R_f values in TLC increased with an increase in the amount of alcohol in the mobile phase. This is expected because the solutes travel farther on the plate if interactions with the mobile phase are enhanced. Conversely, an increase in the alcohol content resulted in decreased retention times in HPLC. If mobile phase interactions are enhanced in HPLC, the solute elutes from the column faster. The α values changed, in TLC, if the amount of alcohol in the mobile phase was varied.

This approach was highly successful in separating chiral aromatic amino alcohols. Once the separation was optimized, it was possible to attain enantiomeric separations with good α values in approximately 30

Table IV. The Effect on Enantioselectivity when TEA is added to Mobile Phases Containing ZGP in TLC Studies

Compound	with TEA			without TEA		
	R_{f1}	R_{f2}	α	R_{f1}	R_{f2}	α
Isoproterenol	0.14	0.38	2.7	0.04	0.10	2.5
Phenylpropranol-amine	0.06	0.26	4.3	0.08	0.32	4.0
Timolol	0.26	0.51	2.0	0.20	0.40	2.0
Norphenylephrine	0.05	0.26	5.2	0.05	0.26	5.2
Propranolol	0.08	0.20	2.5	0.10	0.30	3.0

*Experimental conditions: The mobile phase consisted of 75/25, methylene chloride/methanol. The stationary phase was HP-KF silica gel. A Schimadzu dual wavelength TLC scanner(CS-910) was used for detection.

minutes. This appeared to be a good method for screening specific types of racemic compounds and different solvent systems for HPLC in a relatively short time. Many of these chiral ion interaction agents were relatively inexpensive and provided an easily accessible pathway for enantiomeric separations, but the supply of CSPs for TLC remains limited.

B. CHIRAL MOBILE PHASE ADDITIVES IN REVERSE PHASE TLC

Recent investigations into the use of CDs as chiral separation agents involved the utilization of different types of cyclodextrin derivatives as CMA in reverse-phase media. Derivatization of some of the CD hydroxyls resulted in enhanced aqueous solubility relative to the native CD (32). Similar mobile phases were used for the native and derivatized cyclodextrins. Some compounds, such as dansyl amino acids, could be separated using either the native or the derivatized CD.

For the maltosyl-β-CD study, three types of reversed-phase TLC plates were used: octadecyl, diphenyl, and ethyl. Table V contains data and conditions for compounds separated using maltosyl-β-CD as a CMA. Separations were achieved for the dansyl amino acids on all three types of plates. The best separation for dansyl leucine was obtained using the octadecyl plates whereas the best results for dansyl valine were obtained with the ethyl plates. The separation factors (α) obtained with the dansyl amino acids showed little variance between the plate types indicating that the plates affected efficiency more than selectivity. It was possible to separate the diastereomers, quinine and quinidine, with all plate types as well. The separation factor (α) was the same for the ethyl and diphenyl plates but the α value achieved with the octadecyl plates was much lower. The preferred TLC plate was the ethyl (KC2F) because a greater number of compounds were separated using this plate type. Note that in all cases the mobile phase consisted of an acetonitrile-water mixture with a derivatized β-cyclodextrin as the CMA. The best results were obtained with 30/70 (v/v) acetonitrile-water mixtures and 0.4 M maltosyl-β-CD.

Table V. Compounds Separated Using Maltosyl β-Cyclodextrin as CMA

Enantiomers	Rf1	Reversed Phase Rf2	α	Plate Type
(1) DL-Alanine-β-naphthylamide	0.71	0.66	1.08	ethyl
(2) Dansyl DL-leucine	0.22	0.16	1.38	octadecyl
(3) Dansyl DL-valine	0.40	0.31	1.29	diphenyl
(4) DL-Methionine-β-naphthylamide	0.39	0.34	1.15	ethyl
(5) N'-(2-Naphthylmethyl)nornicotine	0.50	0.30	1.67	ethyl
Diastereomers				
(1) Cinchonidine/Cinchonine	0.23	0.12	1.92	ethyl
(2) Quinidine/Quinine	0.09	0.03	3.00	ethyl*

*Analysis using diphenyl plate produced the same value for selectivity (α).

The mobile phase consists of: 30/70, acetonitrile/water which contains 0.4 M maltosyl-β-CD and 0.6 M NaCl.

When greater than 30% acetonitrile was added, some streaking occurred and most of the solutes traveled with the solvent front. There was more streaking and no separations were achieved when methanol was used as an organic modifier. The minimum concentration of maltosyl-β-CD required for a chiral separation was 0.4 M.

The retention behavior of the the amino acid derivatives was the same as observed in prior studies (30). In Figure 16, the D enantiomer elutes ahead of the L enantiomer because the chiral agent is in the mobile phase. A reversal of the retention behavior is seen if the chiral agent is bonded to the stationary phase exclusively.

All of the compounds in Table V contain a naphthyl group or two fused aromatic rings. The size of the internal diameter of β-CD is approximately the size of a naphthyl moiety. There is a relatively "tight fit" for molecules with two or more rings which are complexed by β-CD (41). It is believed that chiral recognition is enhanced if the conformation and movement of the complexed solute is restricted inside the CD cavity (100). The largest separation factors indicated in Table V were for compounds which had the chiral center located between two aromatic ring systems, e.g.-N'-(2-naphthylmethyl)nornicotine, quinidine/quinine and cinchonidine/cinchonine. This was in agreement with prior work which indicated that enantioselectivity was enhanced when a chiral center was "sandwiched" between two π systems (101). This enhancement was believed to be due to the rigidity of the chiral molecule and the fact that the chiral center appeared to be the main point at which rotation can occur between the two π systems. In addition, diastereomers have different chemical properties and one would expect to see larger separation values for the cinchona alkaloids.

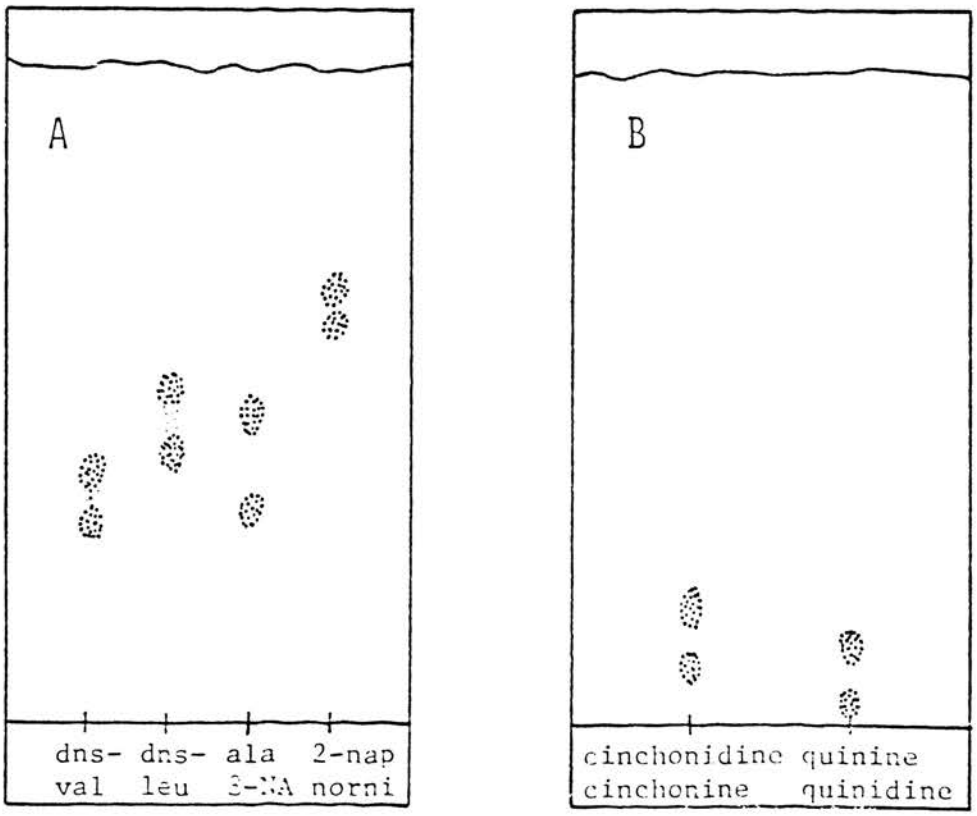


Figure 16. Reversed phase TLC chromatograms showing the separation of enantiomers (A) dansyl D,L-valine; dansyl D,L leucine; D,L-alanine-β-naphthylamide and racemic N'-(2-naphthylmethyl)nornicotine. Chromatogram (B) shows a typical separation of the indicated diastereomers. The mobile phase consists of: 30/70 acetonitrile/water which contains 0.4 M maltosyl-β-CD and 0.6 M NaCl.

No separations were obtained using dimethyl-β-CD as a CMA. The range of CMA concentration was 0.4 - 1 x 10^{-3} M. The organic modifier used was acetonitrile. Due to the scarcity of available planar chromatographic techniques for resolving enantiomers, the use of substituted CDs as CMAs in reverse-phase media remains a viable alternative to CSPs.

C. TLC STUDY OF THE EFFECT OF THE DEGREE OF SUBSTITUTION OF HYDROXYPROPYL-β-CYCLODEXTRIN

Several derivatized amino acids were separated by thin layer chromatography (TLC) utilizing hydroxypropyl-β-cyclodextrin (HP-β-CD). Studies were conducted to determine the effect that the degree of substitution of HP-β-CD would have on: the resolving abilities of the CMA, the time of development and the viscosity of the hydroxypropyl-β-CD mobile phase solutions. Unfortunately, maltosyl derivatized β-CD having different degrees of substitution was unavailable.

Table VI contains viscosity data (in centiposes) and development times (in hours) for the HP-β-CD solutions which were used in this study. Two mobile phase compositions were used in this study. As seen in Table VI, the viscosity of the solutions increased and the % plate traversed decreased as the number of substitutents per CD molecule increased. The % plate traversed indicated how far the solvent front traveled during development relative to the length of the plate. The experiment (i.e., plate development) was stopped when the solvent front ceased movement. The % plate traversed increased and the development time decreased if the top of the development chamber was covered in parafilm rather than a small glass beaker. The parafilm formed a better seal on the container thereby preventing the loss of vapor.

Table VII contains separation data for several enantiomeric and diastereomeric compounds. Figures 17 and 18 show the separation of stereoisomers using hydroxypropyl-β-cyclodextrin supplied by Wacker and Pharmatec, respectively. It was important to note that the best separations for all of the analytes were obtained using the CMA with the lowest

Table VI. Viscosity Data (μ) and Development Times (T_d) for Hydroxypropyl-β-CD Derivatives Study

CD Derivative	μ_1(cp)	T_{d1}(hrs)	% plate developed
DS = 2	28.5	48	98
7	35.7	40	95
9	64.6	45	75
MS =0.6	33.8	40	75
0.9	62.8	45	41

	μ_2(cp)	T_{d2}(hrs)	% plate developed
DS =2	8.2	17	88
7	14.2	16	84
9	17.8	17	66
MS =0.6	11.0	25	84
0.9	16.3	27	77

μ_1 and T_{d1} are values obtained using solution 1. The mobile phase in solution 1 consisted of: 30/70 (v/v) acetonitrile/water which contained 0.4 M HP-β-CD and 0.6 M NaCl.

μ_2 and T_{d2} are values for solution 2. The mobile phase in solution 2 consists of: 35/65 (v/v) acetonitrile/water which contained 0.3 M HP-β-CD and 0.6 M NaCl.

The top of the TLC development chamber was covered in parafilm.

Pharmatec indicated the degree of substitution (DS) of the CD molecule and Wacker indicated the molar substitution (MS) of their product.

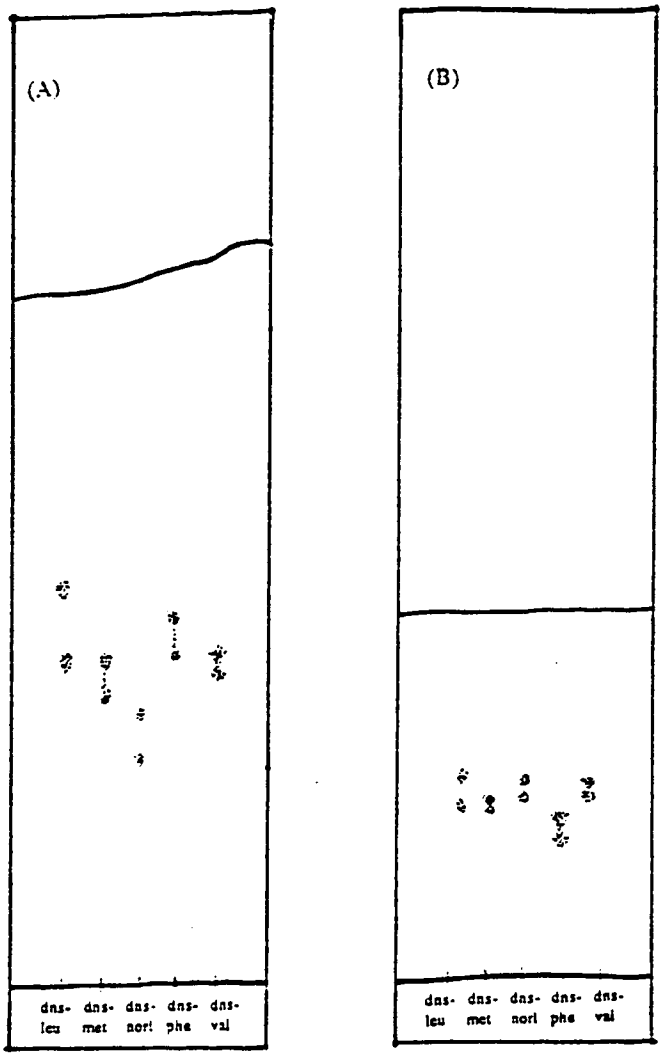


Figure 17. Reversed-phase TLC chromatograms showing the separation of enantiomers (A) and (B) dansyl DL-leucine; dansyl DL-methionine; dansyl DL-norleucine; dansyl DL-phenylalanine and dansyl DL-valine. The mobile phase consists of: 30/70 acetonitrile/water which contains 0.4 M HP-β-CD (Wacker) and 0.6 M NaCl. The stationary phase was octadecylsilane.

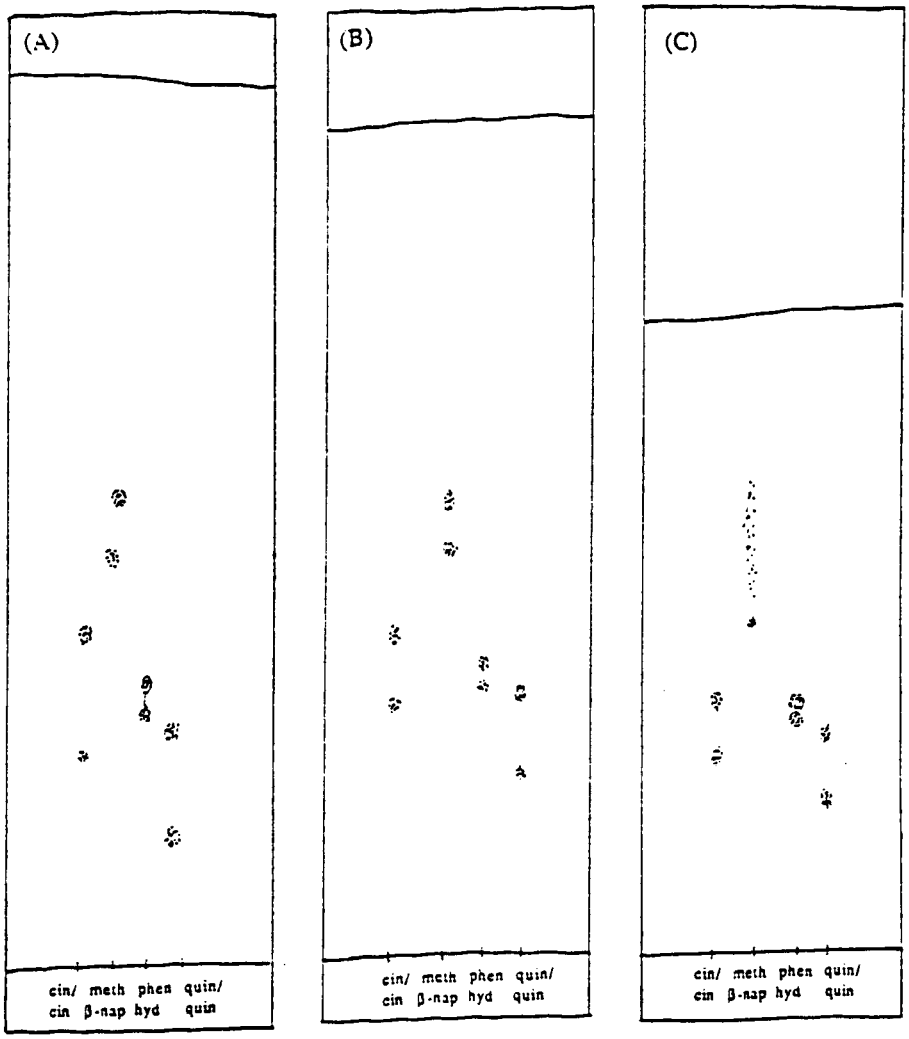


Figure 18. Reversed-phase TLC chromatograms showing the separation of enantiomers and diastereomers(A),(B), and (C) cinchonine/cinchonidine; methionine-β-naphthylamide; 5-(4-methylphenyl)-5-phenylhydantoin and quinine/quinidine. The conditions for each chromatogram was the same except the degree of substitution of the CMA. The mobile phase consists of: 35/65 acetonitrile/water which contains 0.3 M HP-β–CD (Pharmatec) and 0.6 M NaCl. The stationary phase was octadecylsilane.

number of substitutents. The general trend was that the separation factor (α) decreased as the degree of substitution (or the molar substitution) increased. This held true for all enantiomeric separations. The only exceptions noted were with the diastereomeric separations where the α value was larger if the DS of the CMA was nine rather than seven. Since diastereomers have different chemical properties, this different behavior may be due to factors other than the enantioselective properties of the CMA.

The changes in chiral selectivity between the native and the derivatized CD may be due to the loss of hydrogen bonding groups alpha to the chiral centers of the CD molecule and/or the gain of additional interaction sites about the mouth and the base of the derivatized CD cavity. If there was a small number of substituents on the cyclodextrin ring, it appeared that these substituents offered additional interaction sites for the analyte without prohibiting the formation of an inclusion complex. A large number of substituents about the mouth and base of the CD cavity may interfere with the formation of an inclusion complex, which is thought to be important for chiral recognition.

This study indicated that the degree of substitution on functionalized CDs was an important factor which may affect the chiral separation mechanism. The aqueous solubility of hydroxypropyl β-CD appeared to increase as the degree of substitution increased. Increases in solubility allowed larger concentrations of the CMA which may lead to enhanced enantioselectivity. Therefore, in using derivatized CDs as CMAs, it appeared that there was a tradeoff between the enhancement of the solubility of the CMA in aqueous media and the stereoselectivity of the derivatized CD. The degree of CD substitution should be high enough to

Table VII. Compounds Separated Using Hydroxypropyl-β-CD Derivatives as CMAs in RP-TLC

Enantiomers	DS	α	MS	α
Dansyl DL-leucine	2	1.26	0.6	1.22
	7	1.16	0.9	1.17
	9	1.09		
Dansyl DL-methionine	2	1.15	0.6	1.10
	7	1.04	0.9	-
	9	-		
Dansyl DL-norleucine	2	1.21	0.6	1.19
	7	1.10	0.9	1.08
	9	1.06		
Dansyl DL-phenyl-alanine	2	1.15	0.6	1.10
	7	1.06	0.9	-
	9	-		
Dansyl DL-valine	2	1.07	0.6	1.05
	7	-	0.9	-
	9	-		

DL-Methionine-β-naphthylamide	2	1.20	0.6	1.10
	7	1.12	0.9	1.12
	9	1.10		
(RS)-5-(4-methyl-phenyl)-5-phenyl-hydantoin	2	1.10	0.6	1.10
	7	1.09	0.9	1.08
	9	1.10		

Diastereomers

Quinine/Quinidine	2	1.90	0.6	1.90
	7	1.40	0.9	1.40
	9	1.50		
Cinchonine/Cinchonidine	2	1.50	0.6	1.70
	7	1.27	0.9	1.35
	9	1.30		

increase the solubility to approximately 0.3-0.4 M, however the cyclodextrin should not be exhaustively derivatized since this appears to inhibit the formation of an inclusion complex and reduce the enantioselectivity for many racemates. A delicate balance must be attained so that the degree of derivatization is adequate to increase the selectivity and solubility of the CMA but not large enough to obstruct the formation of an inclusion complex or to unnecessarily lengthen development times. The development times were longer for the derivatized CD than for the native CD (in saturated urea solution) because a greater concentration of CMA was used and the viscosity of the solution correspondingly increased.

D. NATIVE β-CYCLODEXTRIN IN REVERSE PHASE HPLC

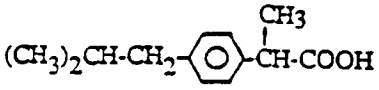
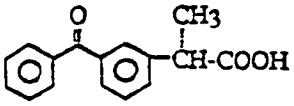
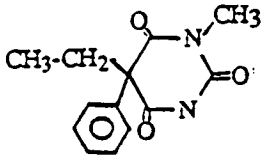
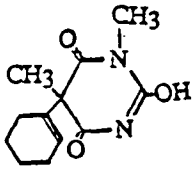
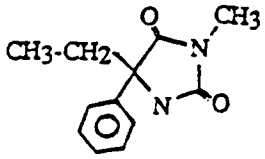
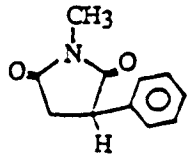
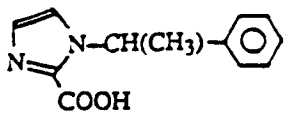
The majority of reports of CD-CMA were performed in TLC whereas most applications of CD-CSP were in HPLC and gas chromatography (GC). Cyclodextrin bonded silica gel can be used as a CSP in LC to resolve racemic mixtures of pharmaceutical compounds. The enantiomers of 25 different racemic drugs were resolved on CD-CSPs in the reverse-phase mode and studies were conducted regarding the chiral recognition mechanism applicable in these separations.

Table VIII lists the names, formulae and chromatographic conditions of the racemic drugs whose enantiomers were separated using native CD-bonded CSPs. The k' values listed correspond to the first eluting enantiomer; α is the ratio of the first eluting k' value to the last eluting k' value, and R_S is the resolution factor.

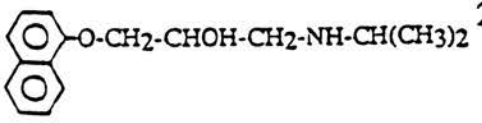
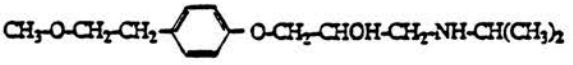
All separated drugs, except norgestrel, contain at least one aromatic ring. As shown previously (41, 95, 102), the internal toroidal β-cyclodextrin (β-CD) cavity has a diameter varying from 0.6 nm at the bottom to 0.8 nm at the top of the cavity and is relatively non-polar. It could easily accommodate molecules or parts of molecules having the size of a five, six, or seven-atom aromatic ring. The secondary 2- and 3-hydroxyl groups of each glucopyranoside ring stand axially at the mouth of the CD cavity, forming a polar crown that is most important for chiral recognition.

It was thought that there should be a difference in chiral CD recognition between compounds with the chiral center in an acyclic portion of the molecule (101). While this seemed to be a factor, it also appeared that the hybridization of the groups attached to the chiral center

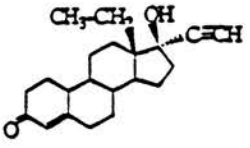
Table VIII. Chromatographic Parameters of the Separation of Enantiomeric Drugs

Therapeutic category & drug name	Formula	Mobile phase % v/v	k'	α	Rs	rem.
Anti-inflammatory agent						
Ibuprofen		MeCH-Buffer[a] 70 - 30	8.04	1.1	0.7	
Ketoprofen		MeOH-Buffer[a] 27 - 73	7.67	1.06	1.2	g,h
Sedative, anticonvulsivant						
Hexobarbital		MeOH-Buffer[a] 15 - 85	9.39	1.14	1.5	g
Mephobarbital		MeOH-Buffer[a] 20 - 80	14.8	1.14	1.6	g
Mephenytoin		MeOH-Buffer[a] 40 - 60	0.48	1.33	1.8	g
Phensuximide		MeCN-Buffer[a] 10 - 90	1.97	1.15	1.5	g
Etomidate		MeCN-Buffer[b] 10 - 90	2.7	1.46	0.9	
		MeCN-Water[c] 5 - 95	1.8	1.70	1.1	

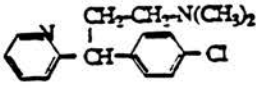
β-adrenergic blocker

Propranolol 	MeOH-Buffer[a] 25 - 75	2.78	1.04	1.4	g,h
Metoprolol 	MeOH-Buffer[a] 32 - 68	3.51	1.03	0.9	g,h

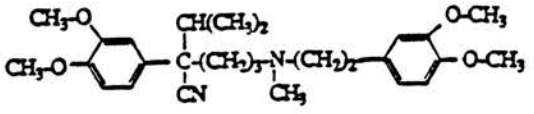
Progestrin

Norgestrel 	MeCN-Water 30 - 70	0.48	1.24	1.1	i

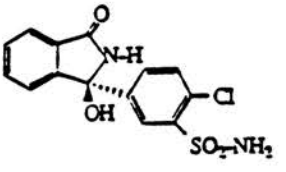
Antihistamine

Chlorpheniramine 	MeCN-Buffer[a] 15 - 85	5.86	1.07	1.5	g

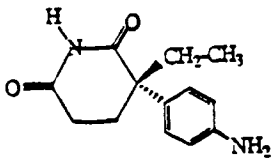
Vasodilatator

Verapamil 	MeCN-Buffer[a] 15 - 85	2.94	1.03	0.7	g,i

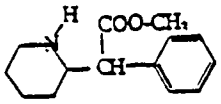
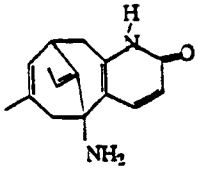
Diuretic

Chlorthalidone 	MeOH-Buffer[a] 30 - 70	0.50	1.44	1.9	g

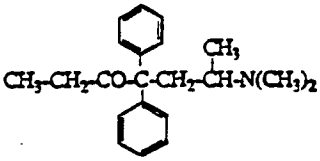
Anticorticosteroid

Aminoglutethimide		MeCN-Buffer[a] 15 - 85	7.49	1.03	0.9	g,j

Central nervous system stimulant

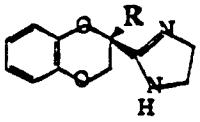
Methylphenidate		MeCN-Buffer[a] 10 - 90	1.17	1.14	1.6	g,h
Huperzine A		MeCN-Buffer[f] 10 - 90	1.83	1.05	0.8	

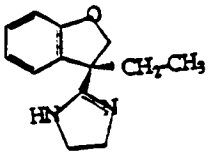
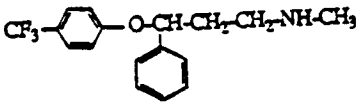
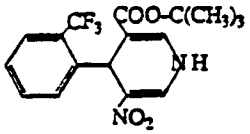
Narcotic, analgesic

Methadone		MeCN-Buffer[a] 15 - 85	2.38	1.04	0.8	g,j

Compounds under pharmacological investigation

Idazoxan derivatives

R = ethyl		Buffer[d]	5.74	1.19	1.3
R = isopropenyl		MeOH-Buffer[e] 10 - 90	3.20	1.30	2.3
R = phenyl		MeOH-Buffer[e] 10 - 90	6.12	1.19	1.3
R = methoxy		MeOH-Buffer[e] 10 - 90	1.21	1.40	2.5
R = ethoxy		MeOH-Buffer[a] 10 - 90	1.62	1.40	2.5

Efaroxan		MeOH-Buffer[a] 2.55 5 - 95	1.2	1.1
Fluoxetine		MeOH-Buffer[a] 5.02 40 - 60	1.1	0.7
SQ 30840		MeCN-Buffer[a] 2.33 20 - 80	1.39	3.7

Column 25 cm x 4.6 mm i.d. Cyclobond I (β-cyclodextrin). Mobile phase composition indicated, MeOH = methanol, MeCN = acetonitrile, flow rate 1 mL/min. Room temperature.

a) 1% (w/v) triethylammonium acetate in water (TEAA), pH 4.1.
b) 5% (w/v) triethylammonium acetate in water (TEAA), pH 7.
c) 0.01% (w/v) diethylamine in water.
d) 1% (w/v) triethylammonium acetate in water (TEAA), pH 5.98.
e) 1% (w/v) triethylammonium acetate in water (TEAA), pH 4.2.
f) 1% (w/v) triethylammonium acetate in water (TEAA), pH 7.
g) Adapted from ref. [13].
h) Two 25 cm Cyclobond I columns were used in series.
i) A Cyclobond II column was used (γ-cyclodextrin).
j) A gradient from 10% MeCN to 20% MeCN in 20 min. was used; mean composition MeCN 15% v/v.

have a significant effect. The highest resolution factor was obtained for compounds (i) whose chiral center was part of a ring and (ii) whose chiral center was linked to at least two sp^2 hybridized carbon atoms (101). The highest resolution factor, $R_S = 3.7$ was obtained for compound SQ 30840 whose chiral center was part of a ring and is linked to three sp^2 hybridized carbon atoms. The lowest resolution factor, $R_S = 0.7$, was obtained for flouxetine whose chiral center is not part of a ring and is linked to only one sp^2 hybridized carbon atom.

The effects of substituents were demonstrated with the Idazoxan derivatives. Figure 19 shows the chromatogram of the methoxy, isopropenyl, and phenyl Idazoxan derivatives. The resolution was maximal with the two ether substituents (methoxy and ethoxy, $R_S = 2.5$) and with the isopropenyl substituent (sp^2 hybridized carbon atom, $R_S = 2.3$). The phenyl substituent, also contains a sp^2 hybridized carbon atom; however, it produced a slightly lower resolution factor ($R_S = 1.3$; Table VIII). In this case, steric effects must be taken into account and it is likely that the bulky phenyl groups interfered somewhat with chiral recognition thereby negating the advantage of having an additional sp^2 hybridized carbon linked to the chiral center.

The comparison of mephobarbital and mephenytoin was interesting: these two compounds are structurally identical, but the carbonyl group attached to the chiral center of mephobarbital was missing in mephenytoin. Although the CD chiral recognition of the two compounds was similar (R_S= 1.6 and 1.8, α = 1.14 and 1.33, respectively); the small carbonyl structural change made a tremendous difference in the global retention of the two molecules (k' = 14.8 and 0.48, respectively). It must be noted

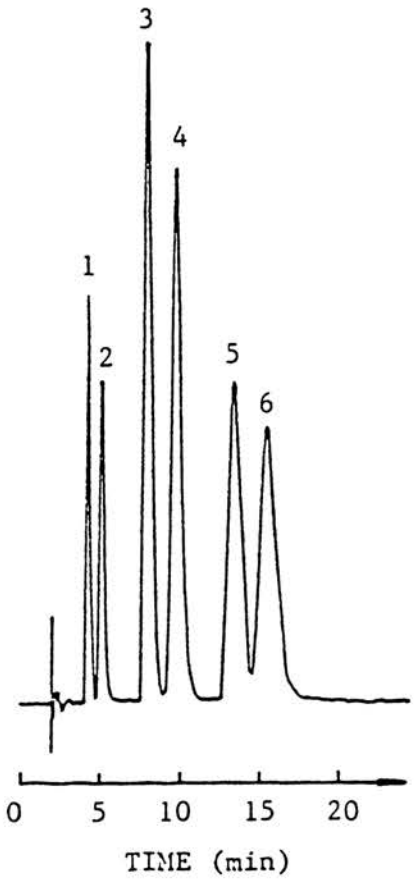


Figure 19. Separation of Idazoxan derivatives. Column β-cyclodextrin 250 x 4.6 mm i.d.; mobile phase: 95% buffer (1% TEAA w/v, pH=4.2)/ 5% acetonitrile v/v, 1.5 ml/min; detection: UV 270 nm, 0.01 a.u.f.s. 1,2: methoxy derivatives, 3,4: isopropenyl derivatives, 5,6: phenyl derivatives

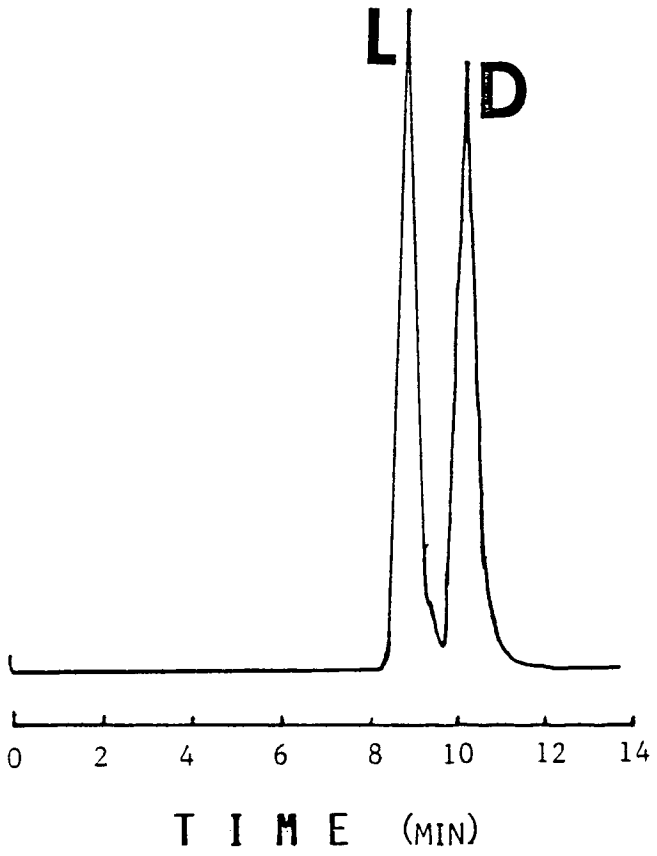


Figure 20. Separation of D and L norgestrel on a 25 cm γ-cyclodextrin column; mobile phase: acetonitrile-buffer (TEAA 1% w/v) 30/70 v/v, 1 ml/min. The L enantiomer eluted first.

that part of the k' difference was due to the mobile phase composition (Table VIII).

The size of the molecule was also an important parameter in chiral recognition by CD. If the molecule was too big to fit inside the CD cavity, chiral recognition was hindered. This was the case for norgestrel. The two isomers of this steroid were not separated by the β-CD bonded column. The chiral center of norgestrel was part of a ring and has an sp hybridized carbon atom. Although there is no aromatic ring in this molecule, it should be possible to obtain a chiral separation on CD-bonded CSPs. Figure 20 shows the separation obtained on a γ-CD-bonded column. γ-CD has eight glucose units and a larger cavity that can accommodate steroids.

Figure 21 is the circular dichroism spectrum of the two peaks obtained with huperzine A. Huperzine A was injected five times and its first and second peaks were collected in two different vials. The circular dichroism of each collected peak was obtained using a Jasco J600 polarimeter. The two circular dichroism spectra were superimposed in Figure 21. For any wavelength, the circular dichroism of the first peak was opposite to the one of the second peak. This is absolute proof of enantiomeric separations. However, this experiment did not allow the determination of the absolute chemical configuration of the enantiomers. Indeed, the D and L structures are not systematically linked to a positive or negative light polarization rotation.

Triethylammonium acetate (TEAA) was added at relatively high concentration (0.062 M or 1% w/v) in the aqueous fraction of the mobile phases to decrease the retention and to improve the efficiency of amino compounds. Basic amino groups have a high affinity for the hydroxyls on

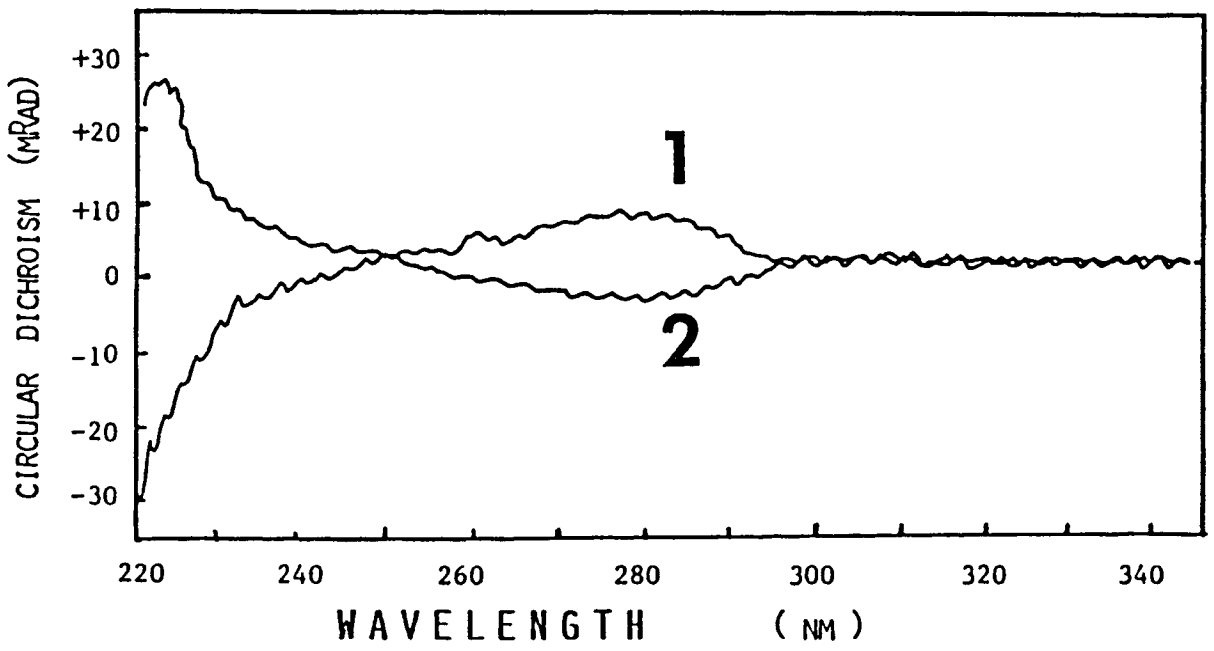


Figure 21. Circular dichroism superimposed spectra of peak 1 and peak 2 obtained with huperzine A.

the CDs. This may produce high retention times and peak tailing. The addition of 1% , (w/v), of TEAA to the mobile phase can solve this problem; the TEA+ ions bind to the silanol groups thereby producing sharper peaks for amino compounds (41). Figure 22 shows the evolution of the separation of the etomidate enantiomers. The use of a diethylamine (DEA, 0.01%, v/v) solution (95% and 5% acetonitrile, v/v) produced an even better separation. With two columns in series using the TEAA buffer, the separation and resolution factors were 1.46 and 0.90, respectively (Fig. 22A). With only one column and the DEA mobile phase, the separation and resolution factors were 1.70 and 1.30 respectively (Fig. 22B). It must be noted that the pH of the DEA mobile phase was 9.5. This high pH value could damage the column irreversibly by dissolving the silica, cleaving some Si-C bonds and releasing the bonded CDs. However, for short periods of time (as in this separation), it is sometimes advantageous to use mobile phases at higher pH values.

These phases are able to resolve enantiomers that cannot be separated by the traditional native CD stationary phases. The use of CSPs in LC is a new technology that can dramatically improve the quality of chiral drugs (104). A full clinical study of the pharmacological and toxicological effects on both enantiomers of any new chiral drug may soon be required to obtain an authorization for marketing the drug as a racemic mixture.

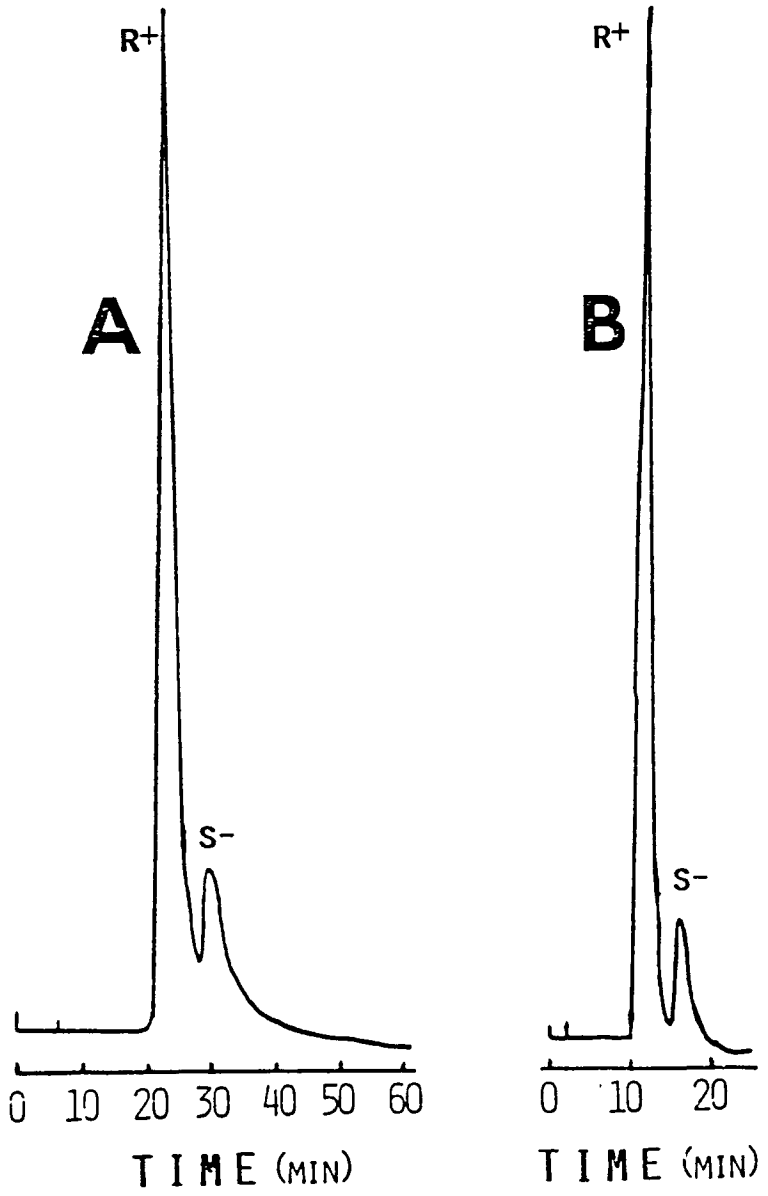
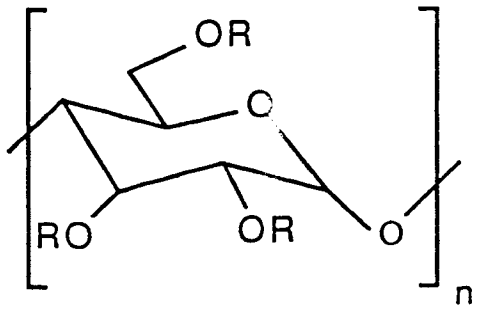


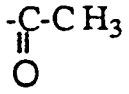
Figure 22. Separation of R and S etomidate. A: two 25 cm Cyclobond I columns connected in series; mobile phase: buffer (1% TEAA, pH=7)/acetonitrile (90/10 v/v), 0.9 ml/min. B: one 25 cm Cyclobond I column; mobile phase: water (0.01% v/v diethylamine, pH=9.5)/ acetonitrile (95/5 v/v), 0.8 ml/min.

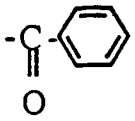
E. DERIVATIZED CYCLODEXTRIN IN NORMAL PHASE HPLC

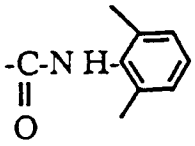
Several different derivatized β-cyclodextrins were synthesized and used as chiral stationary phases in normal phase liquid chromatography (See Figure 23). The multiply substituted derivatives were made with acetic anhydride, (R)- and (S)-1-(1-naphthyl)ethylisocyanate, 2,6-dimethylphenylisocyanate and p-toluoyl chloride. The first successful cyclodextrin-based, normal phase separation of enantiomers was accomplished on these derivative phases. In contrast to chiral separations on the native β-cyclodextrin stationary phase, the enantiomeric separation mechanism on these new phases is not thought to be dependent on inclusion complexation. The similarities and differences between the derivatized cyclodextrin stationary phases and the cellulosic stationary phases will be discussed.

β-Cyclodextrin has 21 hydroxyl groups available for modification. The seven primary 6-hydroxyls are at the "base" or narrow end of the CD torus while the fourteen secondary 2- and 3- hydroxyl groups are at the "mouth" or wide end of the CD torus. Also, the CD was attached to silica gel via its hydroxyl groups through an average of two linkage chains. In order to obtain the maximum degree of substitution per cyclodextrin, a large molar excess of derivatizing agent was used under vigorous conditions. The average degree of substitution for naphthylethylisocyanate was ~ 6; 10, for 2,6-dimethylphenylisocyanate and 13.for p-toluoyl chloride. Acetyl modified β-CD was thought to be about 90% substituted. Figure 24 is an idealized model of toluoyl derivatized β-CD. With the exception of the acetyl modified cyclodextrins, the new derivatized CD phases were not exhaustively derivatized and therefore have residual



R=  peracetyl

 toluoyl

 2,6-dimethylphenyl isocyanate

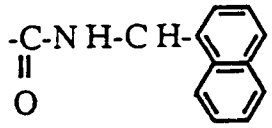
 S-naphthylethyl isocyanate

Figure 23. Derivatized cyclodextrin utilized as chiral stationary phases.

Table IX. List of Bonded Sorbents

Derivatizing Agent	Amount of Reagent	%C	Units/CD
dimethylphenylisocyanate	5g	7.87	10
p-toluoyl chloride	5g	8.33	13
(R)-(-)-1-(1-naphthyl)ethylisocyanate[a]	3g	7.58	6.6
(S)-(+)-1-(1-naphthyl)ethylisocyanate[a]	3g	8.89	6.3

[a] The (R)- and (S)-naphthyl derivatized phases were prepared from different lots of CD bonded sorbent. The difference in %C between the two phases may reflect the lot to lot substrate variation in surface area, etc. However, the degree of substitution is calculated from

$$\text{degree of substitution} = (\mu\text{moles/m}^2 \text{ of naphthyl})/(\mu\text{moles/m}^2 \text{ of CD})$$

which eliminates the variation due to surface area.

chirally selective hydrogen bonding sites as well as additional hydrogen bonding sites and aromatic groups for π–π interactions. It appeared that cyclodextrins modified with the larger, bulkier substituents consistently had a lower degree of substitution. Presumably, this was because of steric reasons. Lower degrees of substitution could be obtained by using less derivatizing agent and shorter reaction times. An (S)-naphthylethyl isocyanate derivatized β-CD stationary phase, with degree of substitution of 3, (DS-3) was made in this manner. In comparing the DS-3 and DS-7 (S)-naphthylethyl isocyanate derivatized-β-CD phases, it was noted that under identical conditions, a solute's retention time increased as the degree of substitution increased. However, the selectivity (α) did not change appreciably for most compounds, provided that low levels of analyte were used.

Most of the prior work done with CD has been in the reversed-phase mode but recent developments with derivatized CD columns utilized the normal-phase (NP) mode. In the presence of nonpolar solvents (such as hexane-isopropanol mixtures) it may be difficult for trace organic solutes to form traditional inclusion complexes with cyclodextrins because the hydrophobic cavity is presumably filled with the nonpolar solvent and the probability of the formation of an inclusion complex is much lower than in reversed-phase. However, the presence of aromatic and carbonyl groups in modified CDs provide opportunities for π–π interactions that do not exist with native cyclodextrins. This, combined with the hydrogen bonding sites of the residual hydroxyl groups, provide the type of interactions commonly associated with Pirkle-type chiral stationary phases (105). Furthermore, the nonpolar solvents do not compete with the solute for the residual hydrogen bonding sites on the cyclodextrin.

The first successful CD-based, normal phase separation of enantiomers was accomplished using derivatized CD-CSP. Figure 24 shows a simplified model of toluoyl derivatized β-CD chiral stationary phase. Table X lists twenty different enantiomeric compounds that were resolved on one or more of the derivatized cyclodextrin stationary phases. Seven of these racemic compounds (Nos. 13,14 and 16-20) also could be resolved on native β-cyclodextrin columns in the reversed-phase mode (31,106), but the rest of the compounds could not. For the compounds that were resolved on both types of columns, the selectivity (α) obtained on the derivatized β-cyclodextrin columns was comparable or better (and with smaller k'-values) than that obtained on the native β-cyclodextrin columns. Also, it should be noted that for compounds 18, 19 and 20, two conventional β-cyclodextrin columns (a 25 cm and 15 cm column) used in series were required to obtain similar selectivity.

Occasionally, two different modified CD columns could resolve the same racemate (see Nos 3-6; Table X). Figure 25 shows the normal phase separation of compound No. 4 on two different derivatized β-CD columns. Note the retention and resolution characteristics were significantly different. All racemates were tested under a variety of conditions on all of the modified CD columns. Each derivatized-CD seemed to have a distinct enantioselectivity. Several of the compounds were highly selective for one particular stationary phase (Table XI). The seemingly random selectivities, column efficiencies and mobile phase requirements of the derivatized CD column were analogous to those reported for the modified cellulosic chiral stationary phases (107). In general, the isocyanate-derivatized phases seemed to have the best selectivity. The resultant carbamate linkage of the isocyanate-derivatized phases may provide additional sites for hydrogen

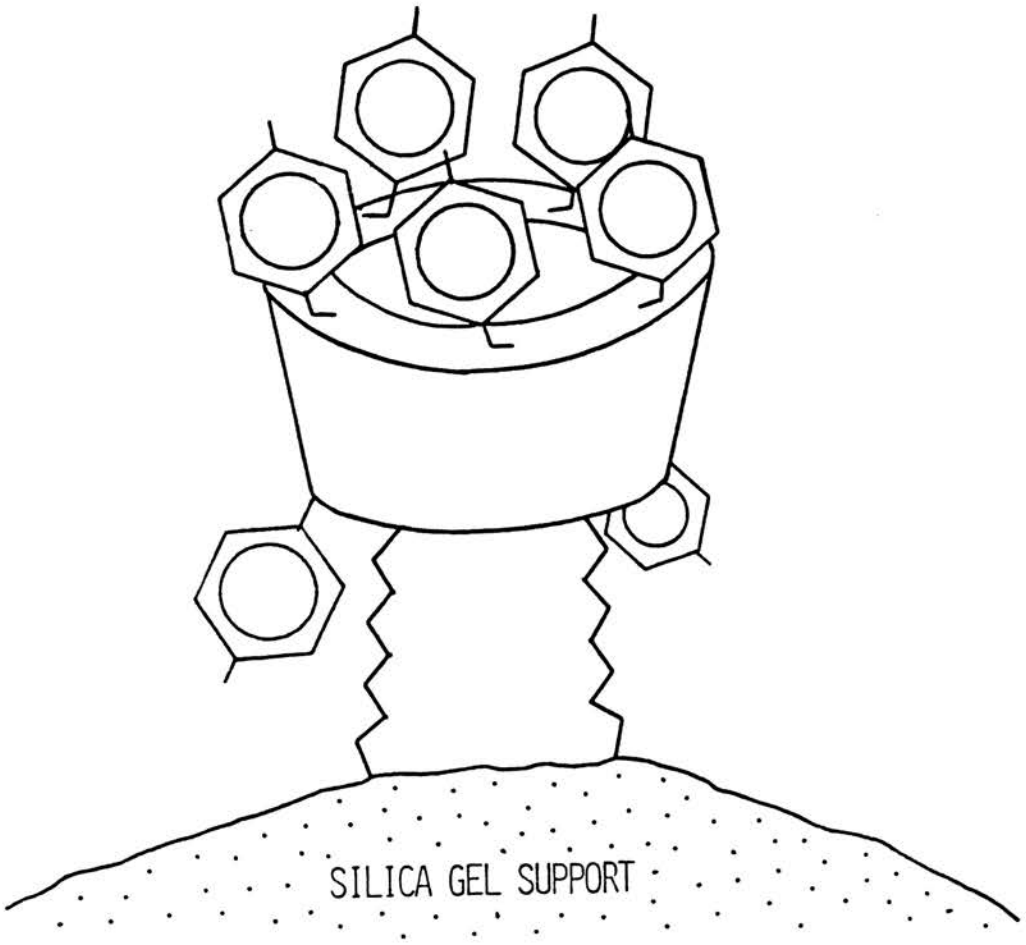


Figure 24. Simplified model of toluoyl derivatized β-cyclodextrin.

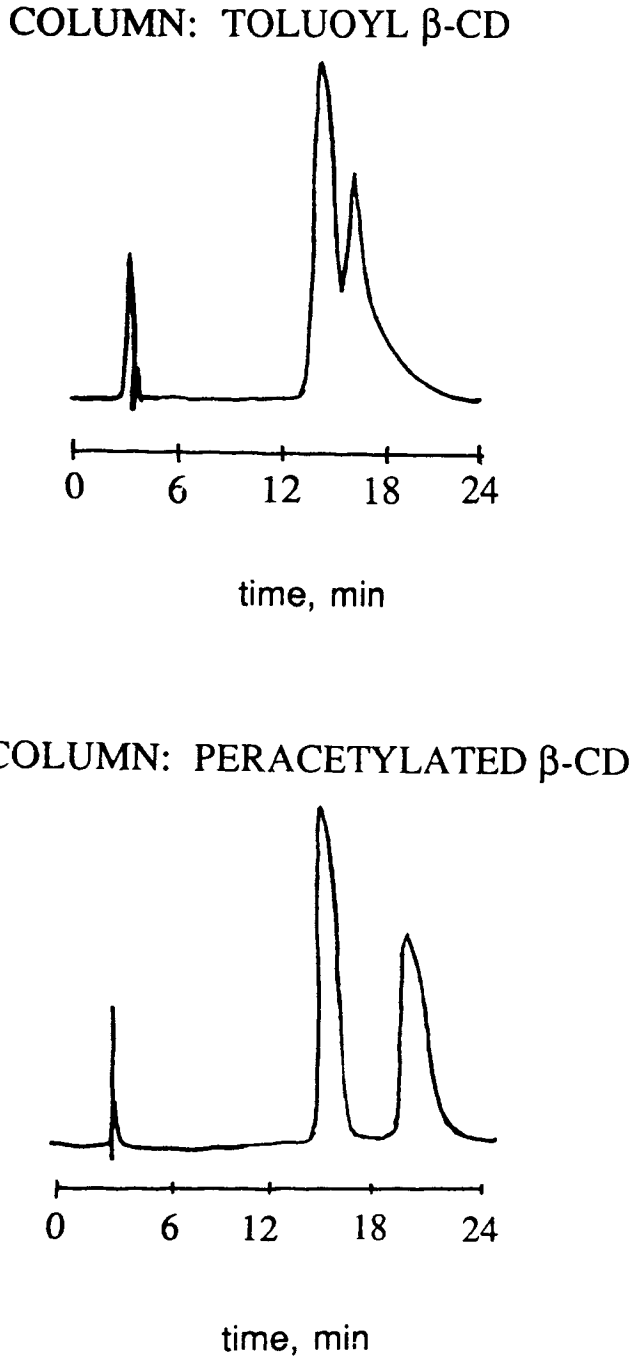
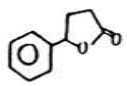
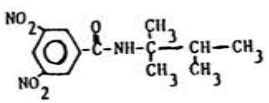
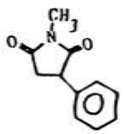
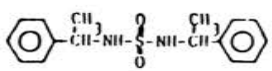
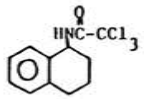
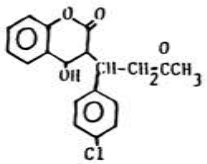
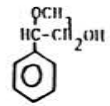
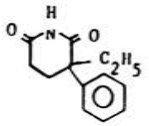
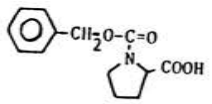
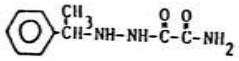
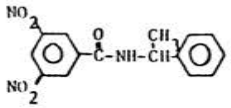
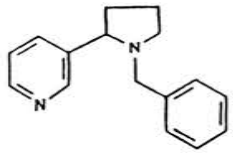
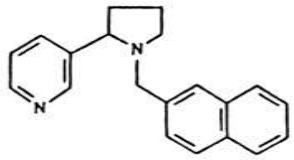


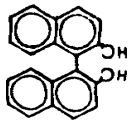
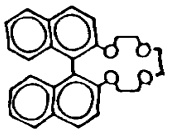
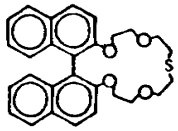
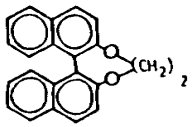
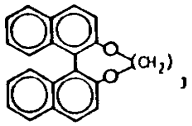
Figure 25. Comparison of toluoyl and peracetylated β-CD CSP for the normal phase separation of (R,R)-(S,S)-N,N'-bis-(α-methylbenzyl)-sulfamide. The mobile phase was 90/10 (v/v) hexane/isopropanol and the flow rate was 1.0 ml/min. The conditions were the same for the two chromatograms, the only difference was the stationary phase used.

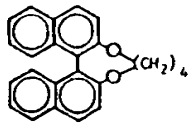
Table X Normal Phase LC separation data for a variety of Enantiomeric solutes on Bonded Derivatized β-Cyclodextrin columns

Compound	Structure	k'_1	α	Mobile Phase[a]	Stationary Phase
1. (±)-γ-phenyl-γ-butyrolactone		14.5	1.10	98:2, hex:ipa	2,6-dimethylphenyl-isocyanate
2. N-(dinitrobenzoyl)-2-amino-2,3-dimethyl butane		5.87	1.20	90:10, hex:ipa	R-naphthylethylisocyanate derivatized β-CD
3. *(±)-phensuximide*		5.60	1.15	90:10, hex:ipa	2,6-dimethylphenyl-isocyanate derivatized β-CD
		3.38	1.07	90:10, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD
4. (R,R)-(S,S)-(±)-N,N'-bis-(α-methylbenzyl)-sulfamide		4.33	1.21	90:10, hex:ipa	peracetyl derivatized-β-CD

		2.13	1.08	90:10, hex:ipa	toluoyl derivatized-β-CD
5. (R,S)-N-trichloroacetyl-1,2,3,4-tetrahydro-1-naphthylamine		7.70	1.3	95:5, hex:ipa	2,6-dimethylphenylisocyanate derivatized-β-CD
		1.28	1.06	90:10, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD
6. D,L-3-(α-acetonyl-4-chlorobenzyl)-4-hydroxycoumarin		23.6	1.12	70:30, AcN:ipa	R-naphthylethylisocyanate derivatized-β-CD
		21.5	1.13	60:40, hex:ipa	peracetyl derivatized-β-CD
7. (R,S)-α-methoxyphenyl-acetic acid			1.27	99.5:0.5, EtOH:HoAC	toluoyl derivatized-β-CD
8. (R,S)-2-methoxy-2-phenylethanol		4.5	1.1	95:5, hex:ipa	2,6-dimethylphenylisocyanate derivatized-β-CD

9. (±) glutethimide		14.3	1.1	90:10, hex:ipa	2,6-dimethylphenlisocyanate derivatized-β-CD
		3.1	1.08	90:10, hex:ipa	toluoyl derivatized-β-CD
10. N-CBZ-DL-proline		13.3	1.16	99.9:0.1, EtOH:HOAc	R-naphthylethylisocyanate derivatized-β-CD
11. (±)-5-(α-phenethyl)-semioxamazide		3.31	1.03	90:10, hex:ipa	peracetyl derivatized-β-CD
12. (R,S)-N-(3,5-dinitrobenzoyl)-α-methyl benzylamine		6.2	1.6	70:30, hex:ipa	2,6-dimethylphenylisocyanate derivatized-β-CD
13. N'-benzyl nornicotine		2.61	1.18	98:2, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD
14. N'-(2-naphthylmethyl) nornicotine		4.4	1.13	98:2, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD

15. (R,S)-2,2'-bi-2-naphthol		23.4	1.10	98:2, hex:ipa	2,6-dimethylphenylioscyanate derivatized-β-CD
16. (R,S)-2,2'-binaphthyldiyl--crown 4		2.6	1.2	95:5, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD
17. (R,S)-2,2'-binaphthyldiyl--17-thiacrown 5		5.2	1.08	95:5, hex:ipa	toluoyl derivatized-β-CD
18.		2.9	1.06	98:2, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD
19.		4.20	1.07	98:2, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD

20.		3.14	1.07	98:2, hex:ipa	R-naphthylethylisocyanate derivatized-β-CD

a hex is the abbreviation for hexane and ipa is the abbreviation for isopropanol.

bonding and/or stronger dipole-dipole interactions relative to the ester linkage of the acid chloride-derivatized phases. The naphthylethyl-isocyanate derivatized β-CD columns had a definite logic for the separation of at least one group of compounds. Solutes that contain an aromatic π-acidic group and an amide, urethane or urea group usually were resolved. Derivatization of analytes (such as chiral amines, alcohols, carboxylic acids, etc.) by the addition of a 3,5-dinitrobenzyl group expanded the range of compounds which could be separated by providing additional sites for interaction with the chiral agent. In this respect, the naphthylethylisocyanate derivatized-CD phase was somewhat analogous to the naphthylvaline reciprocal CSPs developed by Pirkle and Pochapsky (108).

As has been noted, there are a number of analogies that can be drawn between the derivatized cyclodextrin bonded phases and modified cellulosic phases. For example, both are modified carbohydrates containing glucose monomer units. Many of the substituents attached to the 2-, 3- and 6-hydroxyl groups of cellulose and cyclodextrins are identical (i.e., acetyl and toluoyl) or analogous (i.e., the aromatic isocyanates). However, there are a number of important differences in these two classes of chiral stationary phases which can significantly affect the chromatographic approach and results. A comparison of these two classes of CSP is given in Table XI. One of the obvious differences is that the derivatized cyclodextrins are discrete molecules covalently bonded to silica gel while the cellulosic CSPs consist of a distribution of polymers that are adsorbed onto silica gel. As a result, the cellulosic phases are limited to fairly large pore-size silica gels (e.g., $\geq$ 300Å), so that the cellulose polymer can go into the pores and coat the internal surface) while the

Table XI. Comparison of Derivatized Cyclodextrin and Derivatized Cellulosic Chiral Stationary Phases for Normal Phase LC Separation

Derivatized Cyclodextrin	Derivatized Cellulose
1. Individual molecules covalently bonded to silica gel.	1. Distribution of polymers adsorbed on silica gel.
2. No solvent restrictions except for strong acids and bases.	2. Must avoid halogenated solvent or any other mobile phases that will dissolve and remove the substituted cellulose.
3. Can be stored at room temperature indefinitely in typical normal phase solvents. No irreversible conformational changes that affect chiral recognition are possible.	3. Storage recommended hexane at ~4°C. Upon standing at room temperature, polar organic modifiers (isopropanol, etc.) sometimes can cause an alteration in the secondary structure of adsorbed cellulose polymers resulting in a loss of chiral recognition.
4. Can be bonded to a variety of different pore-size silica gels (60-300Å).	4. Must be adsorbed on a large enough pore size silica gel (~300Å) that the polymer can enter the pore.
5. Highly purified derivatives can be crystallized thereby allowing computer modeling and theoretical evaluation of enantioselective properties.	5. Cannot be crystallized at the present time. Secondary structure that contributes to enantio-selectivity is unknown.

derivatized-CD CSPs are not. At least two precautions must be observed with the coated chiral stationary phases that are unnecessary with bonded varieties. For example, mobile phase solvents that dissolve the polymer (such as halocarbons, THF, etc.) must be strictly avoided for the cellulosic CSPs as they quickly strip the derivatized cellulose from the silica gel. Also, there seems to be a definite secondary structure to the adsorbed cellulose polymer which leads to chiral recognition. If this secondary structure is altered or changed, enantioselectivity could be lost even though the chiral polymer remains on the silica gel. Storing these columns in mobile phases containing a more polar solvent (such as isopropanol) at room temperature was sometimes sufficient to cause a change in the secondary structure of the polymer.

Despite the empirical similarities between the derivatized cyclodextrin and derivatized cellulosic stationary phases, they do not have identical enantioselectivities. There were racemates that have been resolved on the derivatized-CD columns that have not been resolved on the analogous cellulosic phases (e.g., compounds 5 and 19 of Table X) and vice versa. Other racemates could be resolved on both phases thereby giving one a choice. Having such a choice frequently is useful since the efficiency, selectivity, loading capacity, retention time, optimal solvent composition, cost, etc. usually differ for different CSPs.

As the number of available chiral stationary phases increases, there is bound to be overlap in the types of compounds separated. Clearly, more studies need to be done comparing many of the existing columns. This is likely to occur in the near future.

F. AMINO ACID STUDY

The column switching set-up for this study is shown in Figure 26. The general racemization profile versus pH for many free amino acids is illustrated in Figure 27. Typically there are minima between pHs of 0 to 3 and a plateau region between pHs 3 to 7 (109). At very high (> 12) and low (< 0) pHs, the rate of racemization increases substantially. In addition to pH, racemization rates also are influenced by temperature, ionic strength, presences of metal ions and so forth. Each amino acid has an individual racemization profile and rate. Aspartic acid, for example, has a small maximum at pH ~ 3.8 and another minima at pH ~ 5.0 (109). One goal of the analytical methodologies developed for this work was to minimize the effect of racemization on the measured enantiomeric purities. Hence most samples were analyzed immediately after they were obtained. The analyses were complete within 45 minutes from sample introduction. No pH or temperature extremes that could lead to high racemization rates were used at any time. No precolumn reaction or derivatization of the amino acids were done (see Materials and Methods).

Each analytical system was "tested" with commercial L-amino acid preparations. Typical results for five amino acids are shown in Table XII. D-amino acids were found in all commercial samples although some were at very low levels. Clearly, analytical methodologies for determining enantiomeric purities are reaching high levels of sophistication and sensitivity. In many cases the sensitivity is such that an enantiomeric impurity is almost always found (Table XII). Of the L-amino acids evaluated, tryptophan usually had the lowest levels of the "unnatural" D-amino acid. It was not unusual to see tenths of a percent and occasionally

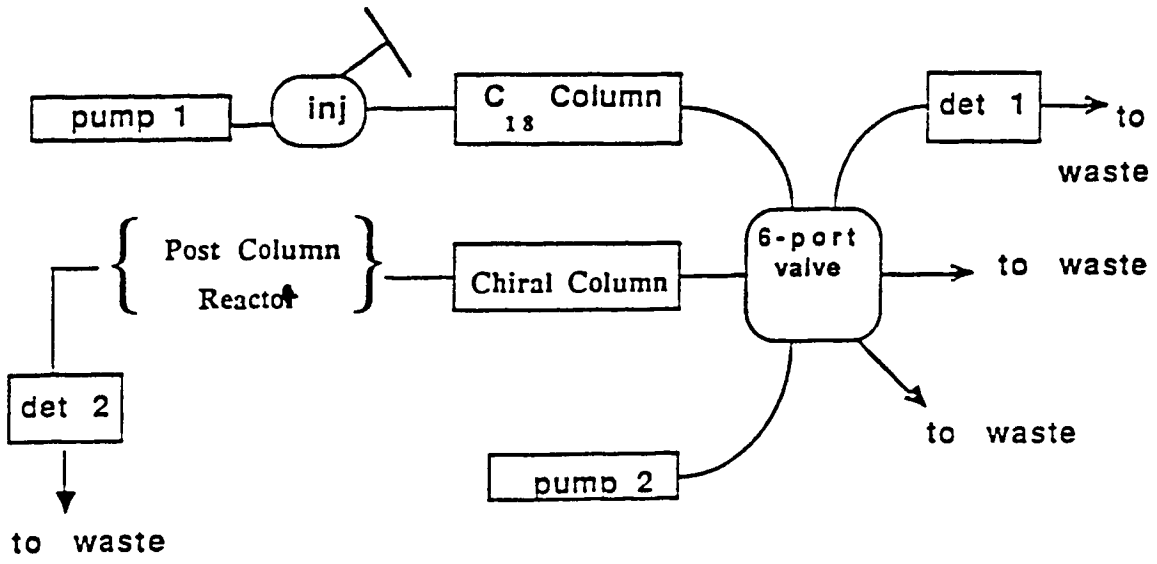


Figure 26. Schematic showing the coupled column (i.e., achiral-chiral) system used for the determination of the enantiomeric purity of amino acids in urine. Note, when electrochemical detection was used (detector 2) there was no post-column derivatization. When fluorescence detection was used (detector 2), post-column OPA derivatives of the amino acids were made.

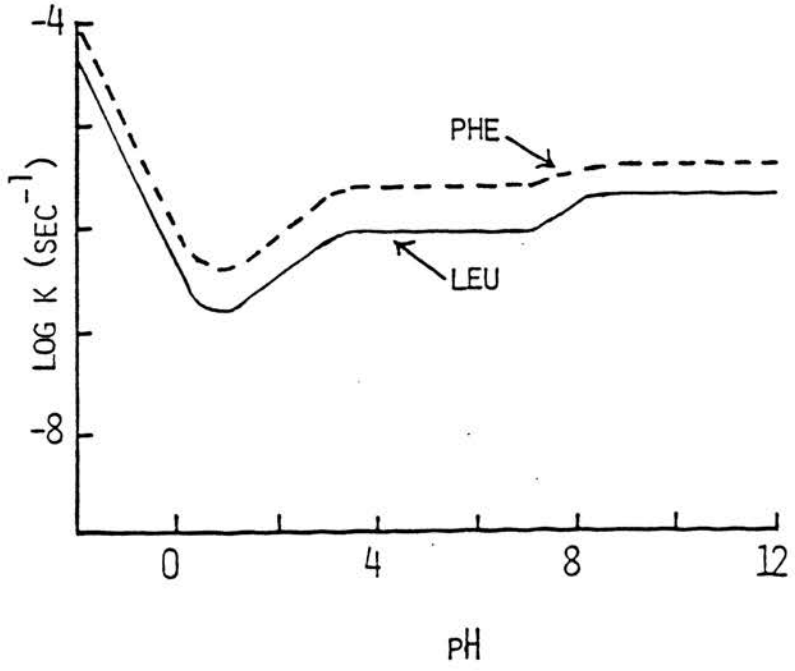


Figure 27. Plot showing the rate of L:D interconversion versus pH for phenylalanine and leucine at 142°C. Data redrawn from Bada (1985).

Table XII. Data Showing the Amount of Contaminating D-Enantiomers in Commercial Samples of L-Amino Acids

Sample Source	Cysteine % D[a]	Methionine % D[a]	Ornithine % D[a]	Tyrosine % D[a]	Tryptophan % D[a]
A. Fluka	2.9×10^{-1}	4.2×10^{-3}	1.7×10^{-3}	3.6×10^{-2}	1.9×10^{-3}
B. Kodak	8.0×10^{-1}	6.0×10^{-3}	7.6×10^{-2}	2.1×10^{-2}	5.7×10^{-3}
C. Lancaster Synthesis	3.9×10^{-1}	5.0×10^{-3}	3.9×10^{-1}	6.5×10^{-3}	8.9×10^{-4}
D. Sigma	4.1×10^{-1}	3.3	3.5×10^{-2}	2.9×10^{-2}	7.3×10^{-3}
E. Spectrum	6.5×10^{-1}	3.8×10^{-1}	3.0×10^{-2}	1.5×10^{-3}	1.0×10^{-3}

[a]% D denotes the % D-amino acid enantiomer present in the commercially available L-amino acid samples. All samples were prepared immediately prior to analysis. Conditions for separation are indicated in the experimental section.

percent levels of the D-enantiomers. There were significant batch to batch differences in the enantiomeric purity of individual amino acids from the same company. These variations often were as great as the variations between companies (Table XIII). It might be interesting to consider the effect that the varying levels of enantiomeric impurities could have on some of the numerous biological, biochemical and medicinal experiments that utilize these amino acids. Figure 29 shows the chromatographic separation of D-cysteine and D-tryptophan from the dominant L-enantiomer in two commercial samples.

Several essential amino acids were analyzed in human urine, blood serum and amniotic fluid. Because of the large number of contaminating compounds and high ionic strength, it was not advisable to directly inject biological samples onto a chromatographic chiral stationary phase (CSP). A column switching procedure was essential for accurate results and for increasing the longevity of the CSP. The amniotic fluid samples were much cleaner than the urine and the blood serum samples but the same precautions were taken. The fresh sample was directly injected onto a reversed phase C_{18} column which separated the amino acids from one other as well as from the other compounds in the sample. Figures 29, 30 and 31 show typical C_{18} chromatograms of urine, blood serum and amniotic fluid, respectively. Upon elution, the amino acid of interest was then switched onto the CSP for enantiomeric resolution and quantitation. Figure 32 shows the enantiomeric separation of phenylalanine from human urine on a chiral crown ether column (110). Figure 33 shows the enantiomeric separation of tyrosine from human blood serum utilizing the same column. Some amino acids can be detected directly by

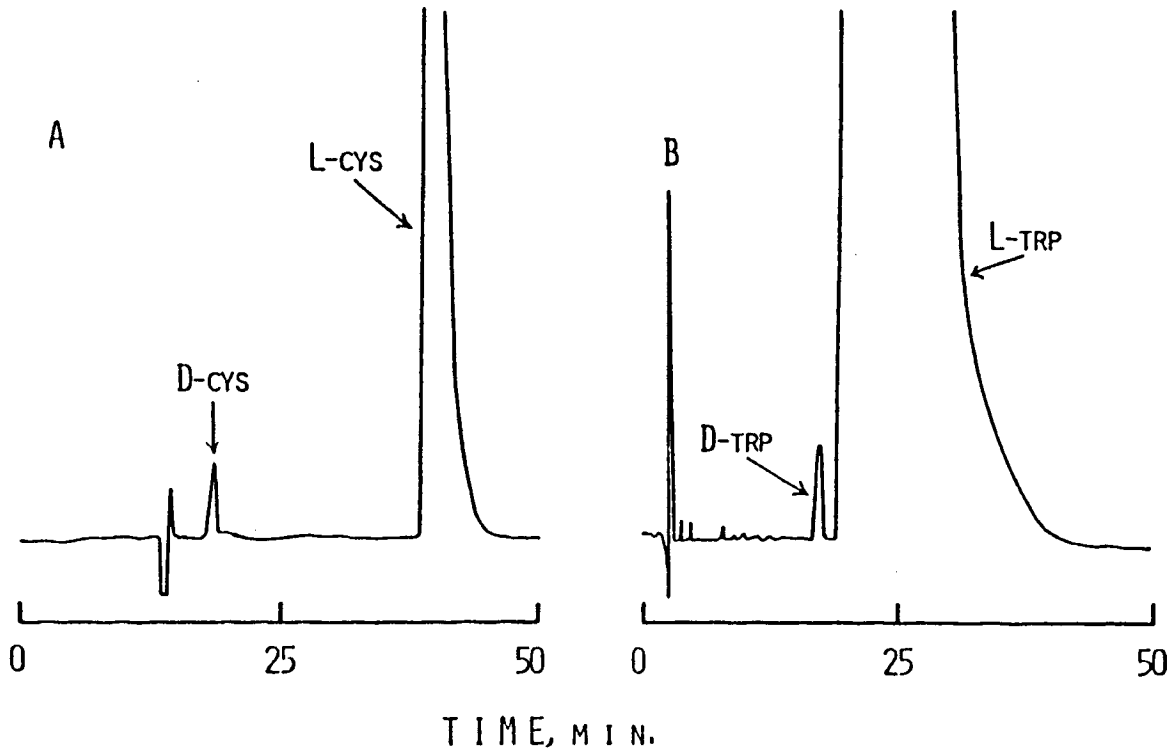


Figure 28. LC chromatograms used to evaluate the enantiomeric purity of L-cysteine from Fluka and L-tryptophan from Sigma. Electrochemical detection was used in both cases. For chromatogram A, the mobile phase was pH 1.5 aqueous $HClO_4$; the flow rate was 0.1 ml/min; the voltage was +0.50v and the gain was 1x50. For chromatogram B, the mobile phase was pH 1.5 aqueous $HClO_4$; the flow rate was 0.8 ml/min; the voltage was +0.50v and the gain was 10x5.

Table XIII. Batch Comparison for L-amino acids from company D

Amino Acid	% D in Batch 1	% D in Batch 2
Cysteine	0.405	1.109
Methionine	3.35	2.32
Tyrosine	0.029	0.029
Tryptophan	0.073	0.038

Note: Batch 2 material was ordered in preparation for the commercial study. Batch 1 material was on the lab shelf prior to preparation for the commercial study.

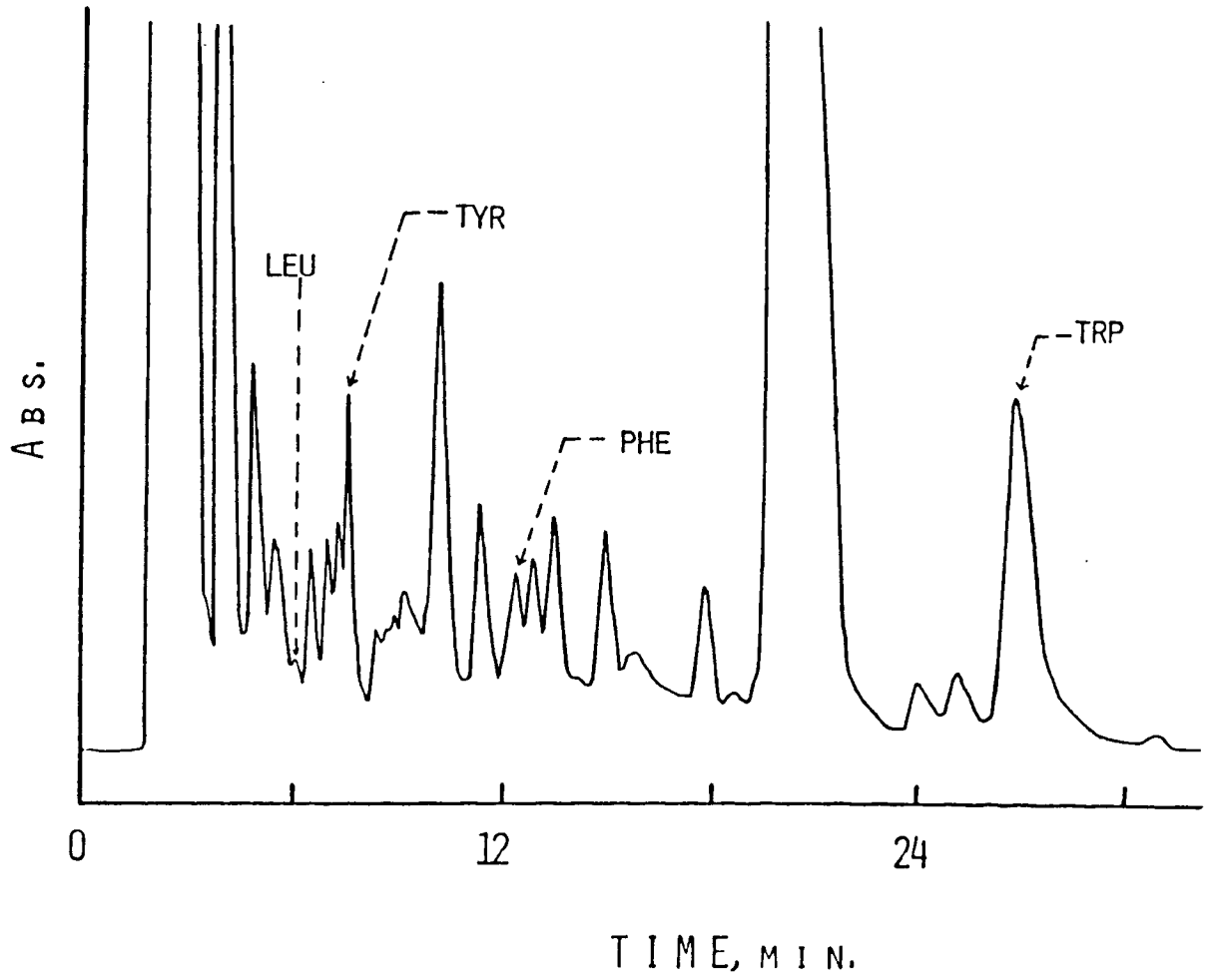


Figure 29. A C_{18} chromatogram for the direct injection of urine. Peaks for four amino acids (Leucine, tyrosine, phenylalanine and tryptophan) are indicated. UV detection at 200 nm was used. The injection volume was 20 ml, the flow rate was 1.5 ml/min and the mobile phase consisted of 5:95 (v:v) methanol:$5x10^{-3}$M $HClO_4$. Note: in this chromatogram the peak labeled tryptophan actually consists of tryptophan plus another impurity. However, these two components are resolved when using a mobile phase consisting of 5:95 (v:v) methanol:water.

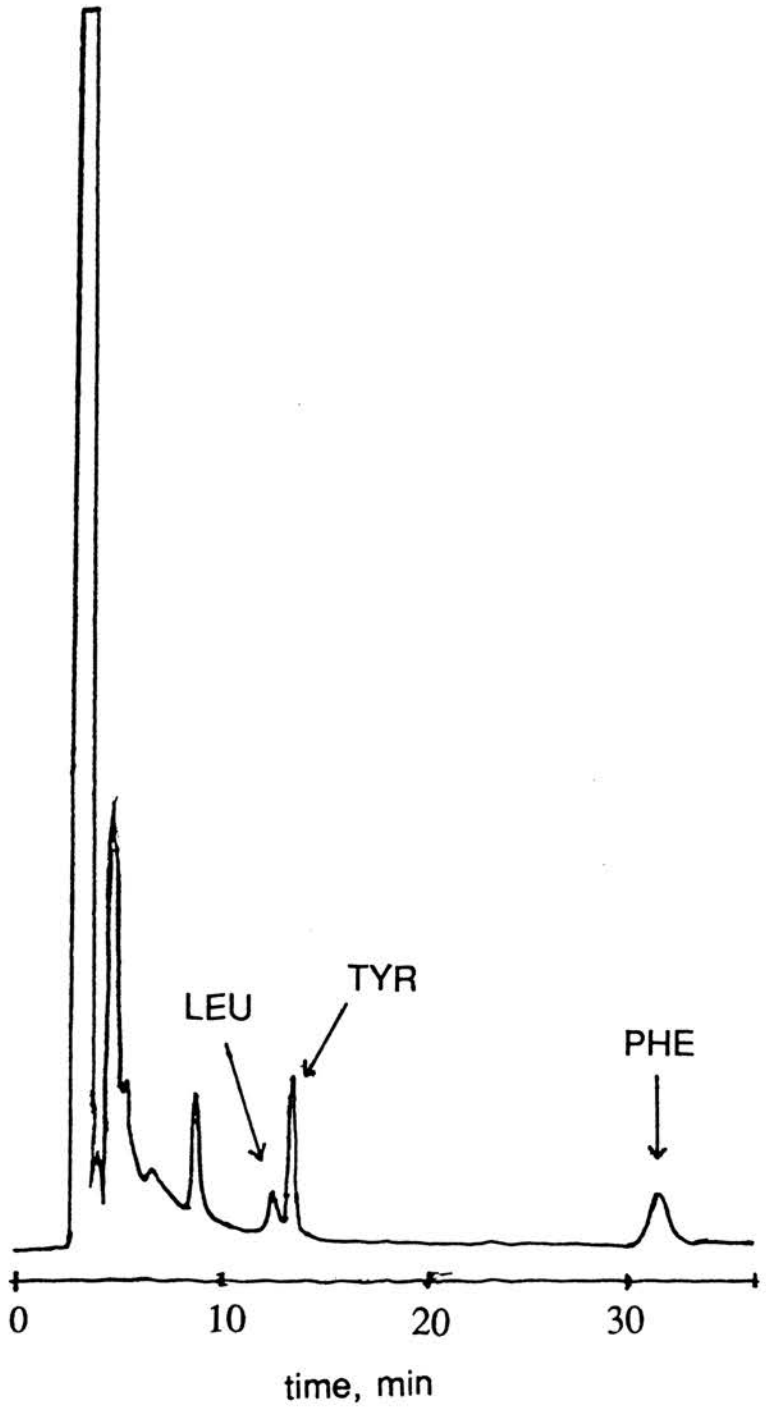


Figure 30. A C_{18} chromatogram for the direct injection of blood serum. UV detection at 200 nm was used. The injection volume was 20 µl, the flow rate was 1.0 ml/min and the mobile phase 5:95 (v:v) MeOH:H_2O.

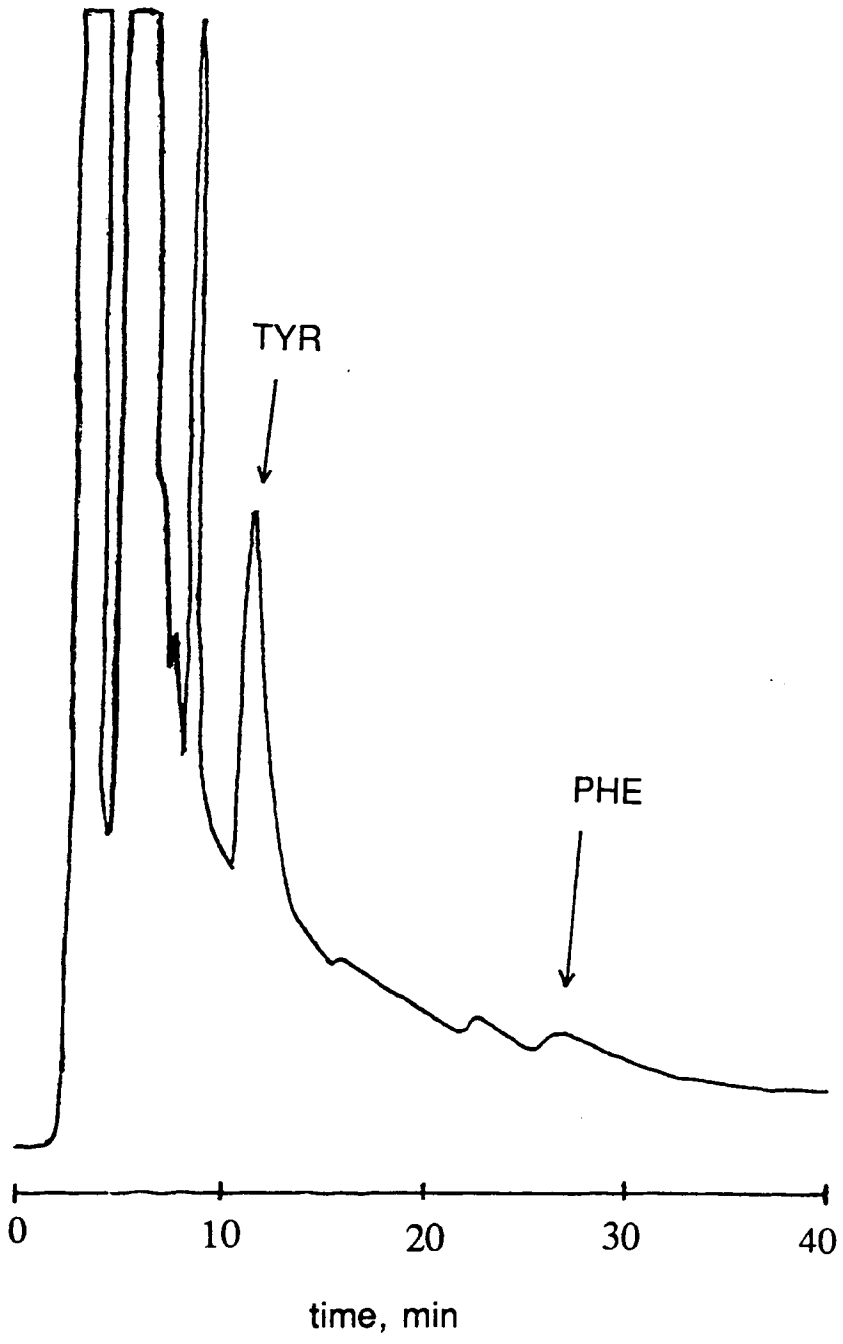


Figure 31. A C_{18} chromatogram for the direct injection of amniotic fluid. UV detection at 200 nm was used. The injection volume was 20 µl, the flow rate was 1.0 ml/min and the mobile phase 5:95 (v:v) MeOH:H_2O.

electrochemical detection, while others must undergo postcolumn derivatization to form a fluorescent analogue (see Experimental Section). Table XIV summarizes the analytical data for four of the amino acids found in human urine. The total levels of these amino acids were within ranges previously reported for human urine (111). Trace levels of the "unnatural" D-amino acids were found in all cases. The relative amounts of D-amino acids present in these samples ranged from several hundredths of a percent to percent levels (Table XIV). In general, there were greater amounts of D-amino acids found in human urine than were found in commercial standards for L-amino acids (Table XII). The range of total amino acid concentrations as well as the percent D-enantiomer content varied not only from person to person but also for one person from day to day. For example, in the case of the phenylalanine data in Table XIV, both the high (5.1%) and low (0.3%) values came from two different specimens provided by the same individual two weeks apart. In general, the relative amount of D-phenylalanine was greater than that found for D-tyrosine and D-tryptophan. There did not seem to be a correlation between the total amino acid concentration in urine and the relative amount of the D-enantiomer.

In order to quantitate the low levels of D-amino acids, relatively large samples (20 - 200 μl) had to be injected. If amino acid levels were too low, a preconcentration step was necessary. This was the case for some of the amino acids not reported in this work. Although preconcentration increased the level of the trace analyte in a given volume of sample, it also increased the level of interfering contaminants. This could lead to overlapping peaks or column overloading and poor resolution. Lypholization seemed to be the most effective way to

Table XIV. Summary of Free Amino Acid Data from Human Urine Specimens

Amino Acid	Number of Urine Samples Analyzed	Total Range of AA Concentration mM/L	% D-Amino Acid, Range	Average % D-Amino Acid
Phenylalanine	11	35 - 130	0.3 - 5.1	1.9
Tyrosine	9	45 - 210	0.1 - 0.7[a]	0.3[a]
Tryptophan	8	46 - 280	0.06 - 0.4	0.2
Leucine	3	b	b	b

[a]One result (i.e., 6.6% D-tyrosine) was excluded from this data as it was approximately an order of magnitude higher than the next closest value. Four different individuals provided all samples for this study. However, the high tyrosine value (vide supra) was the sole sample provided by one individual. Currently, it is not known if this high value is legitimate or is due to an impurity or some other error.

[b]Peaks were obtained for both the D- and L-leucine. However, neither peak was baseline resolved from other contaminate peaks. This made accurate quantitation difficult. Although we are reasonably confident that some D-leucine is present, we would prefer to improve the analytical methodology somewhat before reporting any numbers.

concentrate excreted amino acids. Solid phase extraction of amino acids from urine proved to be ineffective. Solid phase extraction of amino acids was performed with the blood serum samples to remove proteins. Table XV compares the measured levels of D-tryptophan and D-tyrosine in lypholized urine samples versus untreated urine samples that were analyzed directly. The lypholization step did not affect the measurement of D-tryptophan in urine. However,the apparent amount of D-tyrosine in lypholized urine was anomalously high (Table XV). This was thought to be the result of overlapping impurity peaks rather than to racemization during lypholization. However, further investigations must be completed before racemization can be completely disregarded.

Table XVI summarizes the analytical data for three of the essential amino acids found in human blood serum. The total levels of these amino acids were within ranges previously reported for human blood serum. Trace levels of D-amino acids were found in some cases. The relative amounts of D-amino acids were lower than that reported for human urine. Each person donated a single sample, so individual variations were not studied. The lowest concentration of D-amino acid was found for phenylalanine and the highest concentration was for tyrosine. No "unnatural" D-amino acids were detected in the amniotic fluid samples for the four amino acids analyzed. The limit of detection was 0.05%.

This study provides evidence for the presence of free D-amino acids in human urine and blood serum. In some cases the "unnatural" D-enantiomer was present at percent levels relative to the corresponding L-amino acid concentration. However, somewhat lower levels were more common. Care must be taken to resolve the amino acids of interest from other similarly retained components so as not to overestimate the amount

Table XV. Data Showing the Effect of a Freeze-Drying Preconcentration Step on the Measurement of D-Amino Acid Levels.

Amino Acid	Range of D-Amino Acids		Average % D-Amino Acid Concentration	
	Direct Injection	Perconcentration by lypholization	Direct Injection	Preconcentration by lypholization
Tryptophan	0.1 - 0.36	0.06 - 0.6	0.18	0.21
Tyrosine	0.1 to 0.70	3.0 to 9.2	0.3	4.8

of the D-enantiomer. Any biochemical, physiological or clinical significance of these enantiomers has yet to be established. Certainly there are a number of interesting possibilities. Many past studies on the efficiency of free amino acid reabsorption by the kidney have not taken into account possible differences in the rates or mechanism of D- versus L-amino acid absorption, for example.

Table XVI. Summary of free amino acids data from human blood serum samples

Amino Acid	Number of Urine Samples Analyzed	Total Range of AA Concentration mM/L	% D-Amino Acid, Range	Average % D-Amino Acid
Phenylalanine	13	35 - 130	<0.05-1.00	0.10
Tyrosine	8	45 - 210	<0.05-0.51	0.31
Leucine	4	a	<0.05	<0.05

[a] Same as Table XIV.

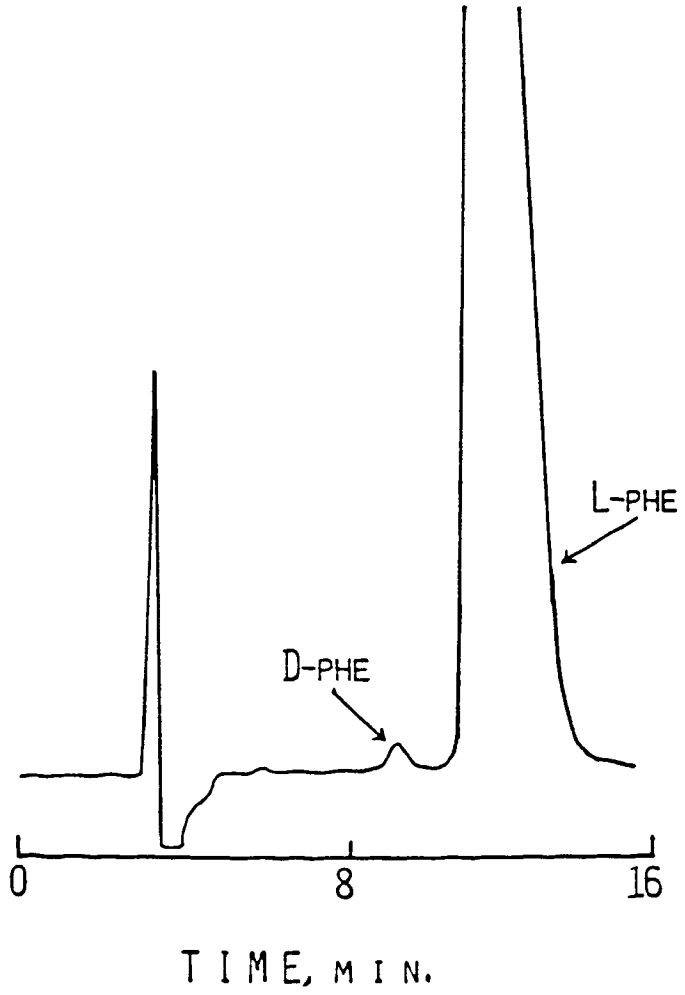


Figure 32. Chromatogram showing the enantiomeric purity of phenylalanine from the direct injection of a urine sample onto the coupled column system (Figure 26). The conditions for the C_{18} separation were the same as in figure 29. Note the absence of interfering peaks. A chiral crown ether column was used at room temperature. The mobile phase consisted of 0.02 M $HClO_4$, and the flow rate was 0.5 ml/min. Fluorescence detection was used after post column OPA derivatization (see experimental).

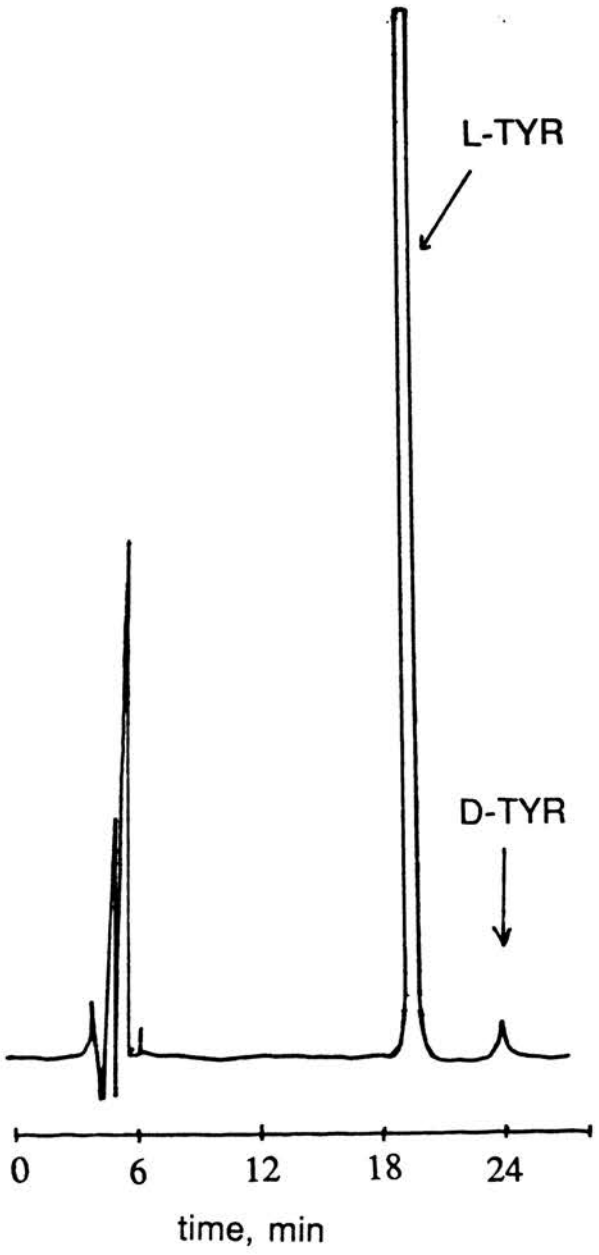


Figure 33. Chromatogram showing the enantiomeric purity of tyrosine from the direct injection of a human blood serum sample onto the coupled column system (Figure 26). The conditions for the C_{18} separation were the same as in figure 29. Note the absence of interfering peaks. A chiral crown ether column was used at room temperature. The mobile phase consisted of 0.02 M $HClO_4$, and the flow rate was 0.5 ml/min. Electrochemical detection was used: v=$^{+}$0.5volts.

CONCLUSIONS

Chiral mobile phase additives were studied in both normal and reverse phase thin layer chromatography. N-carbobenzoxy-glycyl-L-proline and (1R)-ammonium-10-camphorsulfonate were effective CMAs for various aromatic amino alcohols, but it was not possible to obtain chiral separations unless the solvents were properly dried. Derivatized β-cyclodextrins proved more soluble in hydro-organic solvents than the native β-CD. Maltosyl- and hydroxypropyl-β-CD were useful as CMAs but dimethyl-β-CD was not. It was determined that the degree of substitution of the derivatized CD can effect the viscosity of the mobile phase, the time of development and the enantioselectivity. In enantiomeric separations involving CMAs two parameters had to be optimized to develop a plausible TLC technique. The first parameter was the type of TLC plate utilized and the second parameter was the mobile phase composition.

Native and derivatized β-CD chiral stationary phases were successful in resolving a variety of chiral analytes in reverse and normal phase high performance liquid chromatography, respectively. Results indicated that the enantioselectivity can be different for some compounds when comparing the different types of derivatized CD columns. The same can be said of a comparison of the derivatized cyclodextrin and the derivatized cellulosic stationary phases.

The potential of CSPs based on CDs is tremendous, and recently CD derivatives that can be used as CSPs in gas chromatography have been developed (103). Derivatized CDs can be used in LC to improve chiral separations or to extend the applicability of such CSPs. Currently this research group is completing experiments on the use of derivatized CDs as stationary phases for both reversed and normal-phase LC.

In a comparison between the L-amino acid product of six prominent companies, the range of D-amino acid impurity was from 1.0×10^{-1} to 8.9×10^{-4}%. Although the human body is known to be composed of L-amino acids, experimental results indicated that there are trace to percent levels of D-amino acids in blood serum and urine. There were no detectable levels of D-amino acids found in human amniotic fluid.

BIBLIOGRAPHY

1. A. Ariens, W. Soudijn, and P. Timmermans, *Stereochemistry and Biological Activity of Drugs*, Blackwell Scientific Publication, Oxford, 1983.
2. I. W. Wainer and T. D. Doyle, *LC-GC*, 2 (1984) 88.
3. E. J. Ariens in *Chiral Separations by HPLC* ; A. M. Krstulovic, Ed., Wiley, New York (1989).
4. E. O. Field, J. E. Gibbs, D. F. Tucker and K. Hellmann, *Nature*, 211 (1966) 1308.
5. W. H. DeCamp, *Chirality*, 1 (1989) 2.
6. W. Linder, M. Rath, K. Stoschitzky and H. J. Semmelrock,*Chirality*, 1 (1989) 10.
7. B. Testa, *TIPS*, 7 (1986) 155.
8. Guideline for Submitting Supporting Documentation in Drug Applications for the Manufacture of Drug Substances. Office of Drug Evaluation and Research (HFD-100), Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, 1987.
9. P. M. Hardy in *Chemistry and Biochemistry of Amino Acids* ; G. C. Barrett, Ed., Chapman and Hall, New York (1985).
10. F. Kogl and H. Erxleben, *Z. Physiol. Chem.*, 258 (1939) 57.
11. J. A. Miller, *Cancer Research*, 10 (1950) 65.
12. F. Kogl, *Experientia*, 5 (1949) 173.
13. A. Meister, *Biochemistry of the Amino Acids*, Academic Press, New York, 1957.
14. S. Moore and W. H. Stein, *J. Biol. Chem.*, 192 (1951) 663.
15. S. Moore and W. H. Stein, *J. Biol. Chem.*, 211 (1954) 893.

16. D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
17. P. B. Hamilton, *Anal. Chem.*, 30 (1958) 914.
18. P. B. Hamilton, *Anal. Chem.*, 32 (1962) 1779.
19. P. B. Hamilton, D. C. Bogue and R. A. Anderson, *Anal. Chem.*, 32 (1960) 1782.
20. P. Furst, L. Pollack, T. A. Graser, H. Gudel and P. Stehle, *J. Chromatogr.*, 499 (1990) 557.
21. J. F. Davey and R. S. Ersser, *J. Chromatogr.*, 528 (1990) 9.
22. W. S. Hancock, C. A. Bishop and M. T. W. Hearn, *Anal. Biochem.*, 92 (1979) 170.
23. P. E. Hare and D. Gil-Av, *Science*, 204 (1979) 1226.
24. J. Lepage, W. Lindner, G. Davies and B. Karger, *Anal. Chem.*, 51 (1979) 433.
25. J. Debowski, D. Sybilska and J. Jurczak, *J. Chromatogr.*, 237 (1982) 303.
26. C. Pettersson and G. Schill, *J. Chromatogr.*, 204 (1981) 179.
27. C. Pettersson and G. Schill, *J. Liq. Chromatogr.*, 9 (1986) 269.
28. C. Pettersson and M. Josefsson, *Chromatographia*, 21 (1986) 321.
29. J. H. Knox and J. Jurand, *J. Chromatogr.*, 234 (1982) 222.
30. D. W. Armstrong, F. He and S. M. Han, *J. Chromatogr.*, 448 (1988) 345.
31. J. I. Seeman, H. Secor, D. W. Armstrong, K. D. Timmons, and T. J. Ward, *Anal. Chem.*, 60 (1988) 2120.
32. D. W. Armstrong, J. R. Faulkner, Jr., and S. M. Han, *J. Chromatogr.*, 452 (1988) 323.

33. S. M. Han and D. W. Armstrong in *Chiral Separations by HPLC*; A. M. Krstulovic, Ed., John Wiley and Sons, New York (1989).
34. D. W. Armstrong, W. DeMond, A. Alak, W. L. Hinze, T. E. Reihl and K. H. Bui, *Anal. Chem.*, 57 (1985) 234.
35. C. A. Henson and J. M. Stone, *J. Chromatogr.*, 469 (1989) 361.
36. D. W. Armstrong, A. Alak, W. DeMond, W. L. Hinze and T. E. Reihl, *J. Liq. Chromatogr.*, 8 (1985) 261.
37. W. H. Pirkle and T. C. Pochapsky, *J. Chromatogr.*, 369 (1986) 175.
38. K. Lipkowitz, J. M. Landwer and T. Darden, *Anal. Chem.*, 58 (1986) 1611.
39. S. Topiol, M. Sabio, J. Moroz and W. B. Caldwell, *J. Am. Chem. Soc.*, 110 (1988) 8367.
40. R. E. Boehm, D. E. Martire and D. W. Armstrong, *Anal. Chem.*, 60 (1988) 522.
41. D. W. Armstrong, T. J. Ward, R. D. Armstrong and T. E. Beesley, *Science*, 232 (1986) 1132.
42. C. A. Chang, Q. Wu and D. W. Armstrong, *J. Chromatogr.*, 354 (1986) 454.
43. C. A. Chang, Q. Wu and L. Tan, *J. Chromatogr.*, 361 (1986) 199.
44. D. W. Armstrong and H. L. Jin, *J. Chromatogr.*, 462 (1989) 219.
45. K. R. Lindner and A. Mannschrek, *J. Chromatogr.*, 193 (1980) 308.
46. Y. Okamoto, S. Honda, I. Okamoto, H. Yuki, S. Murata, R. Noyori, and H. Takaya, *J. Am. Chem. Soc.*, 103 (1981) 6971.
47. G. D. Y. Sogah and D. J. Cram, *J. Am. Chem. Soc.*, 98 (1976) 3038.
48. S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.*, 269 (1983) 63.
49. J. Hermansson, *J. Chromatogr.*, 269 (1983) 71.

50. W. H. Pirkle, D. W. House and J. M. Fin, *J. Chromatogr.*, 103 (1980) 143.
51. I. W. Wainer, *A Practical Guide to the Selection and Use of HPLC Chiral Stationary Phases*, J. T. Baker, 1988.
52. D. W. Armstrong, *J. Liq. Chromatogr.*, 7 (1984) 353.
53. R. Dappen, H. Arm and V. Meyer, *J. Chromatogr.*, 373 (1986) 1.
54. I. W. Wainer, *Bioactive Analytes, Including CNS Drugs, Peptides and Enantiomers* (E. Reid, B. Scales and I. Wilson, Eds.), Plenum Publishing Corp., New York (1986).
55. I. W. Wainer, *Tr. Anal. Chem.*, 6 (1987) 125.
56. D. W. Armstrong, *Anal. Chem.*, 59 (1987) 84.
57. S. Yuasa, A. Shimado, K. Kameyama, M. Yasui and K.Adzuma, *J. Chromatogr. Sci.*, 18 (1980) 311.
58. I. W. Wainer, C. Brunner and T. Doyle, *J. Chromatogr.*, 264 (1983) 154.
59. C. Brunner and I. W. Wainer, *J. Chromatogr.*, 472 (1989) 277.
60. A. Alak and D. W. Armstrong, *Anal. Chem.*, 58 (1986) 582.
61. I. D. Wilson in *Bioactive Analytes, Including CNS Drugs, Peptides, and Enantiomers* (E. Reid, B. Scales and I. Wilson, Eds.), Plenum Publishing Corp., New York (1986).
62. S. Weinstein, *Tetrahedron Lett.*, 25 (1984) 985.
63. N. Grinberg and S. Weinstein, *J. Chromatogr.*, 303 (1984) 251.
64. K. Gunther, J. Martens and M. Schickedanz, *Angew. Chem.* (Int. Ed., Engl.), 23 (1984) 506.
65. E. Stahl, *A Quarter of A Century of Thin-Layer Chromatography. An Interim Report, In Chromatography, the State of the Art*; H. Kalasz and L. Ettre, Eds., Akademiai Kiado, Budapest (1985).

66. R. Kaliszan, *Quantitative Structure-Chromatographic Retention Relationships*; J. Wineforder and I. Kolthoff, Eds., John Wiley and Sons (1987).
67. K. J. O'Loane, *Chem. Rev.*, 80 (1980) 41.
68. L. Pasteur, *Ann. Chim. Phys.*, 24 (1848) 442.
69. J. H. Van't Hoff, *Arch. Neerl. Sci. Exactes Natur.*, 9 (1874) 445.
70. J. A. Le Bel, *Bull. Soc. Chim. Paris*, 22 (1874) 337.
71. R. T. Morrison and R. N. Boyd, Organic Chemistry, 4th Ed., Allyn and Bacon, Inc., Massachusetts, 1983.
72. J. M. Bijvoet, *Computing Methods and the Phase Problem in X-Ray Cryst. Analy, X-Ray Cryst. Anal. Lab.*, Dept. Phys., Penna. State Coll., (1952) 75.
73. M. Tswett, *Trav. Soc. Naturalistes Varsovie*, (1903) 14.
74. H. H. Strain, *Chromatographic Adsorption Analysis*, Wiley Interscience, New York, 1942.
75. L. Zechmeister, L. von Cholnoky and E. Ujhelyi, *Bull. Soc. Chim. Biol.*, 18 (1936) 1885.
76. R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, 38 (1944) 224.
77. A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1951) 679.
78. E. Stahl, *Pharmazie*, 11 (1956) 633.
79. J. F. K. Huber and Y. A. R. J. Hulsman, *Anal. Chim. Acta*, 38 (1967) 405.
80. I. Halasz and I. Sebastian, *Angew. Chemie*, 81 (1969) 464.
81. R. Willstatter, *Ber. Dtsch. Chem. Ges.*, 37 (1904) 3758.
82. G. M. Henderson and H. G. Rule, *J. Chem. Soc.*, (1939) 1568.

83. M. Kotake, T. Sakan, N. Nakamura and S. Senon, *J. Am. Chem. Soc.*, 73 (1951) 2973.
84. C. E. Dalgliesh, *J. Chem. Soc.*, (1952) 3940.
85. V. Prelog and P. Weiland, *Helv. Chim. Acta*, 27 (1944) 1127.
86. H. Krebs, J. A. Wagner and J. Diewald, *Chem. Ber.*, 89 (1956) 1875.
87. L. H. Klemn and D. Reed, *J. Chromatogr.*, 3 (1960) 364.
88. E. Gil-Av, B. Feibush and R. Charles-Singer, *Tetrahedron Lett.*, 1009 (1966).
89. S. V. Rogozhin and V. A. Davankov, *Usp. Khim.*, 37 (1968) 1327.
90. E. P. Kyba, K. Koga, L. R. Sousa, M. G. Seigel and D. J. Cram, *J. Am. Chem. Soc.*, 95 (1973) 2692.
91. R. C. Hegelson, J. M. Timko, P. Morean, S. C. Peacock, J. M. Mayer and D. J. Cram, *J. Am. Chem. Soc.*, 96 (1974) 6762.
92. N. Oi, M. Nagase, and T. Doi, *J. Chromatogr.*, 257 (1983) 111.
93. D. W. Armstrong, *J. Liq. Chromatogr.*, 7 (S-2) (1984) 353.
94. K. R. Lindner and A. Mannschreck, *J. Chromatogr.*, 193 (1980) 308.
95. D. W. Armstrong and W. DeMond, *J. Chromatogr. Sci.*, 22 (1984) 411.
96. D. W. Armstrong, *U. S. Patent*, 1985, 4539.399.
97. G. Berendsen, K. A. Pikaart and L. de Galan, *J. Liq. Chromatogr.*, 3 (1978) 561.
98. W. Jost and E. Hauck, *Chromatographia GIT Supplement*, 70 (March 1988).
99. C. Pettersson and G. Schill, *J. Liq. Chromatogr.*, 8 (1985) 269.
100. D. W. Armstrong, Y. I. Han and S. M. Han, *Anal. Chim. Acta*, 208 (1988) 275.

101. S. M. Han, Y. I. Han and D. W. Armstrong, *J. Chromatogr.*, 441 (1988) 376.

102. T. J. Ward and D. W. Armstrong in *Chromatographic Chiral Separations* (M. Zief and L. J. Crane, Eds.), Chromatographic Science Series, Dekker, New York, 20 (1988) 131.

103. D. W. Armstrong, W. Y. Li and J. Pitha, *Anal. Chem.*, 62 (1990) 214.

104. I. W. Wainer in *Chiral Separations in HPLC* (A. M. Krustolvic, Ed.), John Wiley and Sons, New York, (1989) 194.

105. W. H. Pirkle and T. C. Pochapsky, *J. Am. Chem. Soc.*, 108 (1986) 5627.

106. D. W. Armstrong, T. J. Ward, A. Czech, B. P. Czech and R. A. Bartsch, *J. Org. Chem.*, 50 (1985) 5556.

107. T. Shibata, K. Mori and Y. Okamota in *Chiral Separations by HPLC* (A. M. Krustolvic, Ed.), John Wiley and Sons, New York, (1989) 336.

108. W. H. Pirkle and T. C. Pochapsky, *J. Am. Chem. Soc.*, 109 (1986) 352.

109. J. L. Bada in *Chemistry and Biochemistry of Amino Acids* (G. C. Barrett, Ed.), Chapman and Hall, (1985) 399.

110. M. L. Hilton and D. W. Armstrong, *J. Liq. Chromatogr.*, 14 (1991) 9.

111. P. Soupart in *Amino Acid Pools* (J. T. Holden, Ed.), Elsevier, Amsterdam, (1962) 220.

VITA

Jo Dee Duncan was born on November 29, 1960 in Orange, Texas. After graduation from Palestine High School in 1979, she studied at Southwest Texas State University in San Marcos, Texas. In 1981, she began teaching analytical chemistry labs under Dr. William Cude. She was in a near fatal car accident in March 1984 but with the help of her mentor, Dr. Cude, she was able to continue her studies during her hospital stay. She received a Bachelor of Science degree from SWTSU in General Chemistry in August 1984.

In 1986, after successfully completing two years of physical therapy, she was able to continue her education. She began graduate studies at Texas Tech University in Lubbock, Texas. In 1987, she continued her studies at University of Missouri-Rolla in Rolla, Missouri. She graduated from UMR with a Ph.D. in Analytical Chemistry in 1991.