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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PEPTIDE AND AMINO ACID STEREOISOMERS

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SUMMARY

Several high-performance liquid chromatographic (HPLC) methods are described for separation of peptide stereoisomers which are not well resolved by traditional reversed-phase chromatography. These chiral HPLC methods include investigations with a β-cyclodextrin column, a Pirkle D-Phenyl Glycine column and a Chiral-Pak WH column. A method based on derivatization of dipeptides with a chiral reagent, N-acetyl-L-cysteine and *o*-phthalaldehyde, is also discussed. A series of linear and cyclic dipeptides and modified amino acids were chromatographed on the four systems. Resolution varied for the four different systems depending on the types of compounds that were chromatographed.

INTRODUCTION

Separation of chiral peptides and enantiomers of modified amino acids has gained importance recently due to regulatory requirements and the bioactivity profiles of the resolved isomers [1]. The pharmaceutical industry increasingly needs methods of analysis and separation for these enantiomeric compounds. Separation of peptide and amino acid stereoisomers has been performed chromatographically by several different methods including chiral stationary phases, derivatization with chiral reagents, and chiral eluents [2–4].

Gradient reversed-phase high-performance liquid chromatography (HPLC) has traditionally been used to resolve chiral peptides containing several residues. However, modified amino acids and short peptides have often required derivatization to diastereomers for resolution. A chromatographic separation that did not require derivatization would have advantages in preparative work.

A chiral HPLC method discussed by Armstrong and DeMond [5, 6] is based

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on a cyclodextrin cyclic oligomer covalently linked to a silica support. Cyclodextrins containing six to twelve glucose units connected in (α-1, 4) linkages have previously been used in solution as well as immobilized on a variety of supports [7, 8] to form inclusion complexes with enantiomers. The stereospecific separation phenomena require molecules of the appropriate size to form an inclusion complex with the toroidal shaped pocket of the cyclodextrin. A wide variety of compounds including barbiturates, dansyl amino acids, and prostaglandins have been resolved into their respective enantiomers using cyclodextrin; one commercial manufacturer of these columns is ASTEC [9]. Dipeptides have been separated by Isaaq [10].

Derivatization of amino acids and peptides with *o*-phthalaldehyde (OPA) and a thiol reagent such as mercaptoethanol or mercaptopropionic acid is a well established analytical technique [11–15]. HPLC systems with automated derivatization are commercially available. Resolution of amino acid enantiomers by substitution of mercaptoethanol with a chiral mercaptan such as N-acetyl-L-cysteine (NAC) has been studied [16]. The present study investigates the chiral derivatization of modified amino acids and small peptides.

Ligand exchange chromatography has been used extensively for separation of unmodified amino acids [17] with methods that generally use bound amino acids and a mobile phase containing a metal ion such as copper. One such column evaluated in this study is manufactured by Daicel [18].

Pirkle HPLC columns of various chemistries have been utilized for resolving many classes of organic molecules. Recent papers describe resolution of chiral amino acids as their N-3,5-dinitrobenzoyl (DNB) derivatives [19, 20]. D-Phenyl Glycine columns are commercially available and investigations were made on separation of enantiomers of modified amino acids and small peptides.

The present investigation discusses methods for analytical and preparative resolution of diastereomers of small peptides and enantiomers of modified amino acids. The four methods described above were evaluated to determine which techniques are useful for a particular class of compounds.

EXPERIMENTAL

Instrumental

A Waters Assoc. Trimodule HPLC system (Milford, MA, U.S.A.) with a WISP autosampler and a Waters 481 UV detector were used for chromatography. Fluorescent measurements were made with a Perkin-Elmer Model 241 fluorimeter (Norwalk, CT, U.S.A.).

Reagents

All cyclic dipeptides were prepared from the corresponding linear dipeptides by cyclodehydration using phenol under non-racemizing conditions. This was a modification of the procedure as originally described by Kopple and Ghazarian [21]. Stereoisomer pairs of c(Trp-Tyr) and c(Leu-Pro) were obtained from Bachem (Dubendorf, Switzerland). Most linear dipeptides and substituted amino acid enantiomers were obtained from Sigma (St. Louis, MO, U.S.A.), with the

exception of Leu-Phe, Ala-Phe and Gly-Trp which were obtained from Bachem. α-Phenyl-α-ethylglycine was obtained from Chemical Dynamics (South Plainfield, NJ, U.S.A.). Solvents were all J.T. Baker (Phillipsburg, NJ, U.S.A.) HPLC grade. Water were obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.). OPA and OPA reagent diluent were obtained from Pierce (Rockford, IL, U.S.A.) and NAC from Fluka (Hauppauge, NY, U.S.A.). All chemicals were reagent grade.

Chromatographic conditions

A cyclodextrin-based column called Cyclobond I (25 cm×4.6 mm) (ASTEC, Whippany, NJ, U.S.A.) was used with an isocratic mobile phase of methanol and water. Flow-rates were normally 1 ml/min. The percentage of organic modifier is included under Results. A buffer of 0.05 *M* triethylamine acetate pH 4.0 was also used in experiments to increase retention and resolution. Shallow gradient conditions with acetonitrile were also investigated.

The automated chiral derivatization of substituted amino acids and dipeptides was performed with a Vydac C_{18} column (Separations Group, Hesperia, CA, U.S.A.) (15 cm×4.6 mm, 5 μm) with a 5-cm precolumn filled with small glass beads. The column temperature was 35°C. Buffer A was 0.05 *M* sodium phosphate–sodium acetate pH 7.0 with 2% tetrahydrofuran and 2% methanol added. Buffer B was methanol–water (65:35). The gradient consisted of zero flow in the beginning, then 0.1 ml/min for 1 min followed by a 30-min linear gradient of 0% B to 100% B at 1 ml/min. The derivatizing solution was prepared with 10 mg of OPA in 0.3 ml ethanol, which was diluted with 22 ml of 0.4 *M* borate buffer pH 10. NAC (30 mg) was then added along with a drop of Brij-35 to improve mixing during derivatization. The NAC solution was prepared fresh daily. The automated derivatization was performed with the autosampler first sampling the reagent vial with no flow. Secondly, the peptide or amino acid vial was sampled and a flow-rate of 0.1 ml/min was begun. The OPA reaction was very fast such that with proper mixing the derivatization took place on-line. The normal gradient then began and the fluorescent adducts formed as described were monitored at 338 nm excitation and 425 nm emission. The products were also monitored at 340 nm with the UV detector.

The Pirkle covalent D-Phenyl Glycine column (25 cm×4.6 mm) was obtained from Regis Chemical (Morton Grove, IL, U.S.A.). For normal-phase conditions isocratic mixtures of hexane and isopropanol were used at 1 ml/min. Reversed-phase conditions consisted of isocratic mixtures of methanol and 0.2% sodium carbonate pH 7.0.

The Chiral-Pak WH column (25 cm×4.6 mm) (Daicel, Los Angeles, CA, U.S.A.) was used with an aqueous mobile phase containing 0.25 *M* copper sulfate. The column temperature was 50°C. Flow-rates were normally 2 ml/min, but were studied at 1–3 ml/min.

RESULTS AND DISCUSSION

Diastereomers of linear dipeptides and cyclic dipeptides were investigated with the cyclodextrin (CD) column. Cyclic dipeptides apparently form an inclusion

TABLE I

α VALUES FOR SOME DIPEPTIDES ON CYCLODEXTRIN COLUMN CYCLOBOND I

Peptide	Retention time (min)	Solvent A/solvent B ratio	α
Cyclic dipeptides			
c(L-Leu-Gly)	4.0	90:10	1.0
c(D-Leu-Gly)	4.0		
c(L-Leu-L-Tyr)	9.8	90:10	3.12
c(D-Leu-L-Tyr)	4.3		
c(L-Phe-Gly)	12.2	90:10	1.60
c(D-Phe-Gly)	8.4		
c(L-His-L-Ala)	3.0	90:10	1.00
c(D-His-L-Ala)	3.0		
c(L-His-L-Tyr)	9.3	80:20	2.19
c(D-His-L-Tyr)	5.4		
c(L-His-L-Leu)	4.4	90:10	1.26
c(D-His-L-Leu)	3.5		
c(L-His-L-Phe)	7.3	90:10	1.43
c(D-His-L-Phe)	5.7		
c(L-His-L-Trp)	4.7	90:10	1.58
c(D-His-L-Trp)	3.7		
c(L-α-Phenyl-α-ethyl-Gly-Gly)	14.8	90:10	1.13
c(D-α-Phenyl-α-ethyl-Gly-Gly)	13.3		
c(L-Trp-L-Tyr)	7.7	60:40	2.11
c(D-Trp-L-Tyr)	4.7		
c(L-Leu-L-Pro)	4.0	90:10	1.00
c(D-Leu-L-Pro)	4.0		
Linear peptides			
Gly-L-Phe	8.2	90:10	1.39
Gly-D-Phe	7.8		
L-Leu-L-Tyr	10.5	90:10	2.12
D-Leu-L-Tyr	6.0		
L-Kynurenine	3.9	90:10	1.35
D-Kynurenine	3.4		
L-Leu-L-Phe	18.5	50:50	1.44
D-Leu-L-Phe	13.4		
L-Leu-L-Leu	12.4	90:10	2.00
D-Leu-L-Leu	7.2		

complex with the β-cyclodextrin bound to silica and provide good resolution of the cyclic dipeptide diastereomers. Table I lists α values as well as solvent conditions for these compounds. The inclusion pocket of the β-cyclodextrin seems to have the appropriate dimensions for discrimination between cyclic dipeptide stereoisomers. The side-chains attached to the α-carbon of the constituent amino acids tend to increase the resolution of the isomers depending on size. Cyclic dipeptides containing α-disubstituted amino acids or aromatic amino acids have the largest α values. Fig. 1 shows the resolution of c(His-Tyr) with a large α of 2.19. The stereoisomers of linear dipeptides and modified amino acids were typically not resolved on the CD column, except for several leucine-containing dipep-

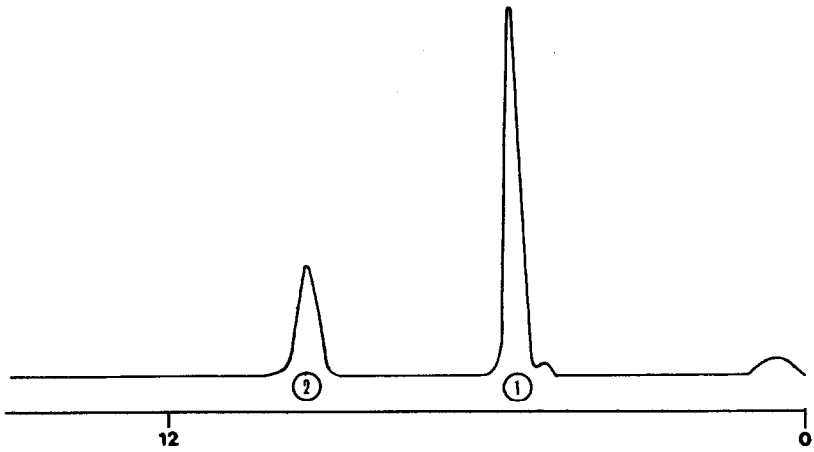

Fig. 1. Resolution of a dipeptide His-Tyr on Cyclobond I. Chromatographic conditions: mobile phase, water–methanol (80:20); flow-rate, 1 ml/min. Peaks: 1 = c(D-His-L-Tyr); 2 = c(L-His-L-Tyr).

tides, i.e. Leu-Tyr with an α of 3.12. Leucine peptides are often resolved on C_{18} columns, and the CD column might be expected to resolve those as well. Cyclic dipeptides with smaller side-chains might be resolved better on the α-CD column while larger side-chains might prefer the γ-CD column, both of which are commercially available.

Experiments to improve resolution with combinations of methanol–water were performed with solvent ratios ranging from 10:90 to 50:50. As in reversed phase HPLC, the higher the percentage of organic modifier, the faster the elution. With the β-CD column ethanol and acetonitrile will generally elute compounds faster than methanol. Ethanol can also be used for regeneration of the CD columns. Experiments using an ion pair such as triethylammonium acetate (10 m*M*, pH 4.00) in the aqueous portion of the elution solvent did not improve resolution, although retention times were shortened. Methanol gradients from 5 to 25% were also investigated, but did not improve resolution of the stereoisomers tested. The retention order of the cyclic dipeptides is D, then L; thus a compound with two asymmetric centers would elute D,D; D,L; L,D; L,L. Armstrong [5] has observed that enantiomers with a chiral center at the second carbon attached to a ring such as the barbiturate butabarbital would not be well resolved on the CD columns, while compounds with the chiral center in the barbiturate ring such as mephobarbital were resolved. The peptides that were available for this investigation all contained a chiral center that was attached directly to the diketopiperazine ring so this observation could not be tested.

Semi-preparative separations of several hundred milligrams of the cyclic analogue of α-phenyl-α-ethyl-Gly-Gly were attempted on the 1-in. CD column, but resolution of the isomers as checked by an analytical CD column was poor. Higher cyclodextrin loadings with increased column efficiencies have now been achieved according to ASTEC. This should improve both analytical and preparative separations.

Stereoisomers of His-containing cyclic dipeptides were readily separated on

the cyclodextrin column. With a pK_a for His of 6.0 the ion-pair reagents or pH adjustments might improve resolution, but these experiments were not done. This method could be useful as an analytical technique for monitoring histidine racemization during synthesis of small peptides.

The automated technique of chiral derivatization using NAC–OPA was investigated with a series of modified amino acids and dipeptides. These compounds formed fluorescent adducts readily and could be resolved into their respective stereoisomers. Table II contains the resolved compounds with their α and resolution (R_s) values. The α values are based on capacity factors (k') and the resolution is calculated using peak widths (pw) with the formula:

$$R_s = (t_1 + t_2)/0.5(pw_1 + pw_2)$$

Halogenated amino acids, α-substituted amino acids and dipeptides were all resolved with about the same α. Trp-containing compounds had slightly better resolution than the others. The α values are small because of the large k' values, although the resolution is sufficient for detecting minor enantiomer impurities.

This method allows for resolution of all four stereoisomers of dipeptides such as Leu-Leu and Ala-Phe (see Fig. 2), as well as enantiomers of racemic amino acids. The order of elution of the derivatized compounds is: L,L; L,D; D,L; D,D. This order of elution was followed by all compounds tested with this method. The elution order of positional isomers was determined by the amino terminus component, the L eluting before D.

Certain derivatized amino acids, especially Lys or Gly, have been reported to suffer from stability problems when derivatization is performed manually with the OPA–mercaptoethanol system. Using a longer-chain thiol seems to enhance the stability of these derivatives, e.g. mercaptopropionic acid–OPA derivatives are quite stable. Preliminary experiments with Gly-L-Trp and Gly-D-Trp and Standard H (amino acid standard from Pierce, data not shown) indicate that OPA–NAC derivatives are also very stable. For a reliable analytical technique the OPA–NAC reagent should not react differentially with either isomer of an enantiomeric pair. The derivatization appears to be complete in that quantitative conversion takes place. Investigations with Gly-L-Trp and Gly-D-Trp indicate that these enantiomers react at equal rates.

One advantage of this derivatization technique is the ease of detection of small amounts of an enantiomer contaminant due to the sensitivity of the method. The detection limit for the OPA method is less than 1 pmol for amino acids. The ease and relative speed of this automated method allows its routine use as an analytical technique.

The investigations with the D-Phenyl Glycine Pirkle column were largely unsuccessful. The one exception was the resolution of c(L-Trp-L-Tyr) and c(D-Trp-L-Tyr) with an α of 1.1 as seen in Fig. 3. The elution order was L, then D. Both normal-phase and reversed phase conditions were attempted, although no experiments were performed with derivatization of a primary amine to the DNB derivative. One possible explanation for the lack of resolution with this Pirkle column is that small peptides and underivatized amino acids are often charged and may not contain the necessary H-bonding sites or participate in the appro-

TABLE II

AUTOMATED CHIRAL DERIVATIZATION WITH OPA AND NAC

For HPLC conditions see Experimental.

	Retention time (min)	α	Resolution
Dipeptides			
Gly-L-Ser	3.70	1.13	0.40
Gly-D-Ser	3.85		
L-Ala-Gly	16.54	1.04	1.47
D-Ala-Gly	17.13		
L-Leu-L-Leu	28.11	1.01	1.40
L-Leu-D-Leu	28.53		
D-Leu-L-Leu	29.05	1.01	1.10
D-Leu-D-Leu	29.38		
L-Leu-L-Phe	31.63	1.01	1.23
L-Leu-D-Phe	32.00		
D-Leu-L-Phe	32.50	1.005	1.00
D-Leu-D-Phe	32.70		
L-Ala-L-Phe	23.75	1.02	1.30
L-Ala-D-Phe	24.27		
D-Ala-D-Phe	24.36	1.004	
Gly-L-Trp	20.74	1.03	1.40
Gly-D-Trp	21.30		
(+)-α-Phenyl-α-ethyl-Gly-Gly	28.45	1.02	1.28
(−)-α-Phenyl-α-ethyl-Gly-Gly	28.90		
Modified amino acids			
α-Methyl-L-Glu	3.18	1.38	0.70
α-Methyl-D-Glu	3.40		
α-Methyl-L-Trp	22.7	1.03	1.50
α-Methyl-D-Trp	23.3		
α-Methyl-L-Leu	26.0	1.01	0.5
α-Methyl-D-Leu	26.2		
α-Methyl-L-Phe	26.0	1.01	0.75
α-Methyl-D-Phe	26.3		
α-Methyl-L-Met	21.6	1.01	0.75
α-Methyl-D-Met	21.9		
6-Fluoro-L-Trp	24.49	1.04	2.11
6-Fluoro-D-Trp	25.39		
Chloro-L-Ala	29.09	1.02	1.27
Chloro-D-Ala	29.60		
L-Trp-ethyl ester	22.24	1.05	5.00
D-Trp-ethyl ester	23.25		
p-Nitro-L-Phe	25.0	1.02	1.25
p-Nitro-D-Phe	25.5		
L-Met-ethyl ester	20.53	1.02	0.85
D-Met-ethyl ester	20.87		
5-Fluoro-L-Trp	23.84	1.04	1.65
5-Fluoro-D-Trp	24.75		
Methoxy-L-Trp	21.36	1.05	1.98
Methoxy-D-Trp	22.35		
5-Bromo-L-Trp	27.94	1.03	1.60
5-Bromo-D-Trp	28.66		
Allyl-L-Gly	17.98	1.02	0.58
Allyl-D-Gly	18.27		

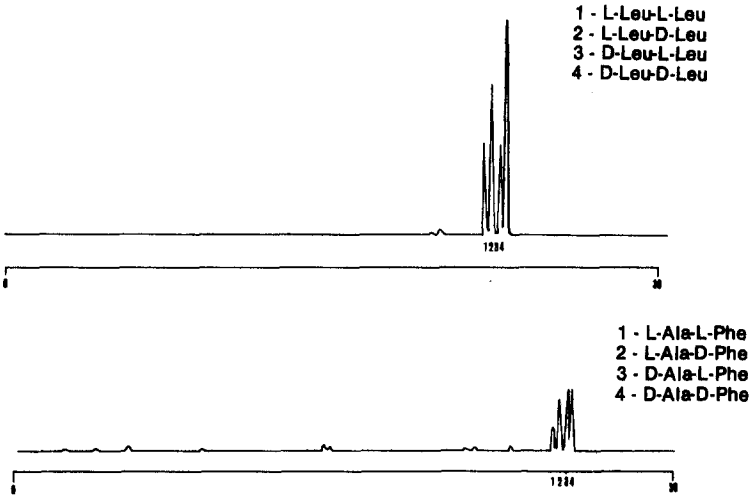

Fig. 2. Automated chiral derivatization and resolution of Leu-Leu and Ala-Phe on Vydac C_{18}. Solvent A: sodium acetate–sodium phosphate pH 7.0 containing 2% tetrahydrofuran and 2% methanol; solvent B: methanol–water (65:35). Gradient: zero flow in the beginning, then 0.1 ml/min for 1 min followed by a 30-min linear gradient of 0% solvent B to 100% solvent B at 1.0 ml/min.

priate π–π interactions. A proposed model for chiral separation includes π–π interactions between aromatic rings of the stereoisomer and DNB-phenylglycine attached to the support. Hydrogen bonding and dipole–dipole interactions are also important in this model for chiral recognition of enantiomers.

A ligand exchange column made by Daicel resolved enantiomers of "normal" amino acids but did not resolve enantiomers of modified amino acids or linear and cyclic dipeptide stereoisomers. Table III shows the results of the experiments. Raising the column temperature above the 50 °C did not reduce the retention time significantly or improve resolution. Higher flow-rates up to 3 ml/min did decrease retention times, but these were still about 40 min per run. The pressure limit of the column was reported to be 46 bar, so higher flow-rates were not attempted. Experiments with increasing or decreasing the concentration of copper sulfate did not affect retention or resolution of the modified amino acids. One amino acid metabolite of tryptophan that was well resolved into D- and L-enantiomers was

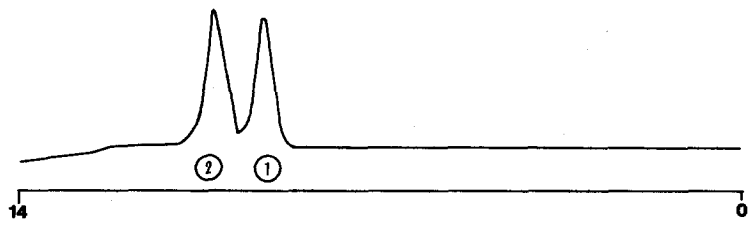

Fig. 3. Resolution of Trp-Trp on Pirkle D-Phenyl Glycine. Chromatographic conditions: mobile phase, sodium carbonate–methanol (50:50); flow-rate, 1 ml/min. Peaks: 1 = c(L-Trp-L-Tyr); 2 = c(D-Trp-L-Tyr).

TABLE III

RESOLUTION ON LIGAND EXCHANGE COLUMN CHIRAL-PAK WH

	Retention time (min)	α
Modified amino acid		
L-Kynurenine	49.0	1.44
D-Kynurenine	35.0	
α-Methyl-L-Leu	57.0	1.52
α-Methyl-D-Leu	36.0	
α-Methyl-L-Met	58.0	1.27
α-Methyl-D-Met	46.0	
L-Allyl-Gly	15.6	1.36
D-Allyl-Gly	12.0	
5-Fluoro-L-Trp	35.2	1.00
5-Fluoro-D-Trp		
Chloro-L-Ala	26.0	1.00
Chloro-D-Ala		
Dipeptides		
L-Leu-L-Leu	15.5	1.68
D-Leu-L-Leu	10.0	
L-Leu-L-Tyr	12.0	1.66
D-Leu-L-Try	8.0	

kynurenine. The D-enantiomer eluted before the L-enantiomer with an α of 1.44, as shown in Fig. 4.

In summary, these conditions indicate that no one chiral separation technique can be used for all diastereomer and enantiomer separations. The cyclodextrin method worked well with the cyclic dipeptides. This method has significantly shorter run times than the other methods and does not require derivatization.

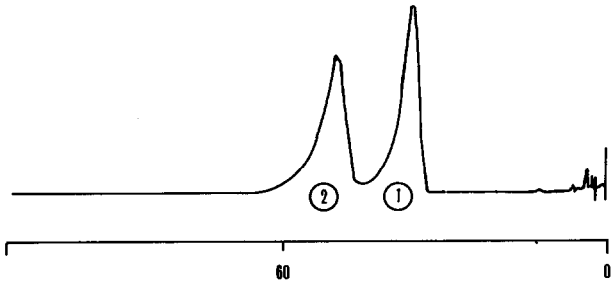

Fig. 4. Resolution of kynurenine on Chiral-Pak WH. Chromatographic conditions: mobile phase, 0.25 *M* copper sulfate; flow-rate, 2 ml/min. Peaks: 1 = D-kynurenine; 2 = L-kynurenine.

Preparative separations are possible since larger columns are available. This cyclodextrin method might be considered superior if resolution is obtained.

The OPA–NAC derivatization technique worked very well with linear dipeptides and modified amino acids. The run times were intermediate, but derivatization probably restricts this interesting technique to an automated analytical procedure. Since the derivatives are fluorescent, other UV impurities will not interfere with this analysis.

The Pirkle column has been used with a number of organic compounds, although it did not work well with peptides or underivatized amino acids in our investigation. The run times are intermediate, but apparently derivatization is required to achieve separation of stereoisomers. Preparative Pirkle columns are available.

The ligand exchange method was very successful for normal amino acids, but not peptides. The run times were quite long, and a small amount of copper sulfate needs to be present in the mobile phase. Several modified amino acids were also resolved into their stereoisomers, although most of the ones investigated were not resolved.

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