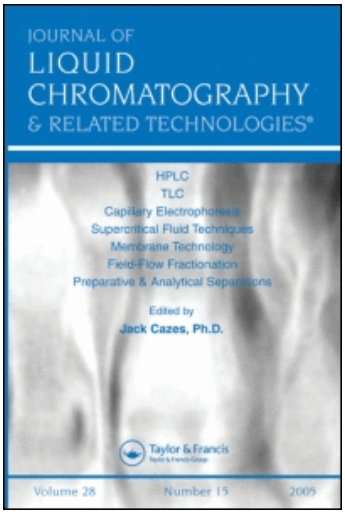

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CHIRAL HPLC SEPARATION OF PROTECTED AMINO ACIDS

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ABSTRACT

This work reports the direct separation of twenty one Fmoc, Boc, Trt, and Pmc, single and double protected amino acids (PAA) using protein-based and macrocyclic antibiotic-based chiral columns. This group of PAAs represents a cross-section of commonly employed reagents in peptide synthesis with diverse structures and protecting groups. The Ultron Ovomucoid (OVM) chiral column was found to be extremely versatile in the resolution of these molecules without the need of any chemical modification. Compounds not separated in the Ovomucoid column, were resolved using Human serum albumin (HSA) and Chirobiotic T (Teicoplanin) columns.

Several interesting structure-related factors were found to affect the enantiomeric separations in the OVM column. The relative position and nature of the protecting groups seems to have great influence on the separations, also it was observed that compounds containing more protecting groups were easier to resolve. In spite of the relatively low efficiency of OVM columns, it was possible to determine amounts as low as 0.15% of the unwanted isomer (usually D isomer) in commercial samples. The separations developed have been applied to the selection of commercial suppliers of protected amino acids (PAA), and to the analysis of research samples obtained from peptide-synthesis reactions. Five PAAs previously reported to require some chemical modification to achieve enantioresolution Fmoc-Lys-(Boc)-OH, Fmoc-Asn-(Trt)-OH, Fmoc-Ser-(tBu), Fmoc-Cys-(tBu)-OH, and Fmoc-Thr-(tBu)-OH), were separated without any derivatization step.

INTRODUCTION

PAAs constitute the building blocks involved in peptide synthesis. Therefore, their chemical and optical purities are important considerations in the screening of suppliers. Peptide synthesis is a multistep process in which each reaction is carried out with one protected amino acid at a time. In this process, it is important to carry out each step to maximum completion, and any loss of yield due to impurities or unwanted isomers will have an impact on the overall purity and recovery of the final product. Chiral HPLC is a valuable technique which can be used to develop separations capable of detecting the unwanted enantiomer at low levels in PAAs.

The two most commonly employed amine protecting groups (N-Protection) are; Fmoc (9- Fluorenylmethyloxycarbonyl) and Boc (t-Butyloxycarbonyl). Groups useful for side chain protection are; Trt (Triphenylmethyl, or Trityl), and Pmc (2,2,5,7,8, Pentamethylchroman-6-sulfonyl). Some protected amino acids contain protecting t-butyl ester groups on the carboxyl terminus (C-protection). The tBu group is also frequently employed to protect the hydroxyl or thiol groups on the side chains of amino acids.

Most of the literature references regarding chiral separations of PAAs involve the use of achiral and chiral derivatization reactions and the separation of the derivatives in chiral or reversed-phase HPLC columns. Miyazawa et al[1] developed the separation for many methyl esters of N-benzyloxycarbonyl

derivatives of non-protein amino acids using a Chiracel OD column. G. Szokan et al[2] employed Marfey's reagent to form stable diastereomers of many amino acids which were separated in a Hypersil ODS reversed phase column. Ch. Spondlin et al[3] reported the chiral separation of seventeen FMOC protected amino acid reagents employed in peptide synthesis, a derivatization (esterification) step was included in the sample preparation and the resulting derivatives were separated in a Chiralcel OD column. The authors studied the separation of isopropyl and methyl ester derivatives but did not mention whether it was possible to obtain enantioresolution without the esterification step.

Few examples of direct enantioresolution (without the formation of derivatives) of protected amino acids can be found in the literature. Zukowski et al[4] has reported the direct separation of Fmoc imino acids on a β acetylated Cyclodextrin column. S. C. Chang et al[5] reported the separation of twenty five Boc-protected amino acid enantiomeric pairs using a (2)-Hydroxypropyl -β Cyclodextrin column. Recently CE was employed by D. Riester et al to separate the enantiomers of Fmoc-Tyr, Trp and Lys using a buffer system containing γ cyclodextrin as a chiral selector.[6] The authors also briefly mentioned that the same selector could separate Fmoc-Tyr-(tBu) and Fmoc-Trp-(Boc), but not Fmoc-Lys-(Boc). This last compound was resolved only when the Boc group was selectively cleaved and a chiral crown ether was added to the buffer system.

The enantioselective properties and the synthesis of OVM columns has been studied in detail. J. Haginaka et al[7,8,9] determined the retention properties of several kinds of ovomucoid-based columns prepared on silica material of different porosities and bonded to their surfaces through different spacer-groups. They also explored the selectivity of OVM columns cross linked with different aldehydes.[10,11] T.C. Pinkerton et al[12] prepared OVM columns using different fractions of turkey ovomucoid protein and tested their selectivities. J. Iredale et al[13] studied the effect of mobile phase pH and of several alcohol organic modifiers on the resolution of acidic, basic and neutral molecules.

EXPERIMENTAL

Materials

Most of the individual D and L PAAs employed were purchased from Bachem Bioscience, King of Prussia Pennsylvania. Fmoc-Thr-OH, Fmoc-D-

Table 1

Compounds Separated and Typical Conditions Employed

Compound	First Isomer To Elute	k'_L	k'_D	α	Mobile Phase** %ACN/Buffer pH
Fmoc-Trp-(Boc)-OH	L	1.90	2.79	1.47	23/6.5
Fmoc-Trp-OH	D	8.58	7.45	1.15	35/3.5
Fmoc-Tyr-(tBu)-OH	L	1.59	3.49	2.2	23/6.5
Fmoc-Tyr-OH	L	2.14	4.33	2.02	23/6.5
Fmoc-Ser-(tBu)-OH*	D	4.07	3.55	1.15	30/5
Fmoc-Glu-(OtBu)	D	11.74	9.74	1.23	15/6.5
Fmoc-Glu-(OtBu)-OH	L	2.35	2.81	1.20	20/65
Fmoc-Met-OH*	D	6.34	5.34	1.19	28/3.5
Fmoc-Lys-(Boc)-OH	L	6.05	7.87	1.30	25/3.5
Fmoc-Lys-OH	L	1.78	2.17	1.22	10/3.5
Fmoc-Orn-(Boc)-OH	L	2.50	3.72	1.49	20/6.5
Boc-Orn-(Fmoc)-OH	D	9.82	7.84	1.25	12/6.5
Fmoc-Gln-(Trt)-OH	D	5.43	3.53	1.54	30/5
Fmoc-Cys-(tBu)-OH	L	6.47	8.60	1.33	28/5
Fmoc-Asp-(OtBu)-OH	D	11.82	9.09	1.30	15/6.5
Fmoc-Asn-(Trt)-OH	D	6.92	2.98	2.32	30/5
Fmoc-Thr-(tBu)-OH	D	3.62	2.33	1.56	30/5

* Elution order reverses at pH 6.5.
**Buffer - 0.02M NaH_2PO_4.

Asn-(Trt)-OH, and Fmoc-Ser-(tBu)-OH, were obtained from Sygena, Cambridge Massachusetts. Fmoc-Trp-(Boc)-OH, and Fmoc-D-(Glu)-(OtBu)-OH were obtained from Advanced Chemtech, Louisville Kentucky. Research samples of Fmoc-Glu, and Fmoc-Asp were from investigations of activation and racemization of protected amino acids to attach the first amino acid to the resin support used in solid phase synthesis. All the protected amino acids were refrigerated at 4°C while not in use.

Mobile phase reagents, NaH_2PO_4, NaOAc, Na_2HPO_4, Acetonitrile (ACN), Methanol (MeOH), n-Propanol, Triethyl amine (TEA), H_3PO_4, and NaOH were all HPLC grade or Analytical Reagent quality obtained from Fisher Scientific, Pittsburgh Pennsylvania. Water of 18 megohm resistivity from a Barnstead purification system was employed. The Ultrom ES-OVM (Ovomucoid) columns (150 x 4.6mm, 5 μm particle size, 120 angstrom porosity) were

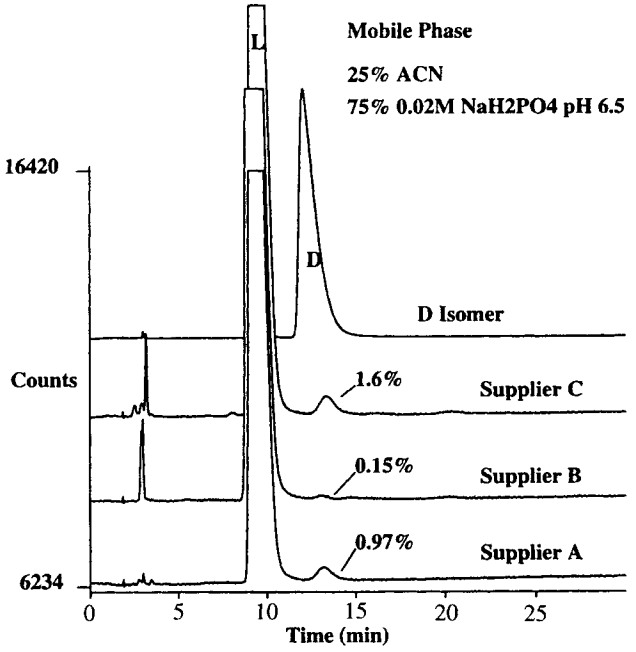

Figure 1. Chiral analysis of Fmoc-Trp-(Boc)-OH from different suppliers in an OVM column.

purchased from Mac Mod Analytical Inc. Chadds Ford Pennsylvania. OVM guard columns (10 x 4.6mm, 5 μm particles) were obtained from the same supplier. The HSA (Human Serum Albumin) (100 x 4 mm 5μm), β CD-RSP (hydroxypropyl ether) (250 x 4.6mm 5μm) and Chirobiotic T (Teicoplanin) (150 x 4.6mm 5 μm) columns were obtained from ASTEC, Whippany, New Jersey.

Mobile phases were generated by pump-mixing water or buffer and organic modifier (ACN, MeOH, or n-propanol) at different proportions. Buffer molarities were expressed as concentrations in the aqueous portion of the mobile phases.

The aqueous NaH_2PO_4, Na_2HPO4 and TEA buffers were pH adjusted with diluted NaOH or H_3PO4. The NaOAc buffer was pH adjusted with diluted acetic acid.

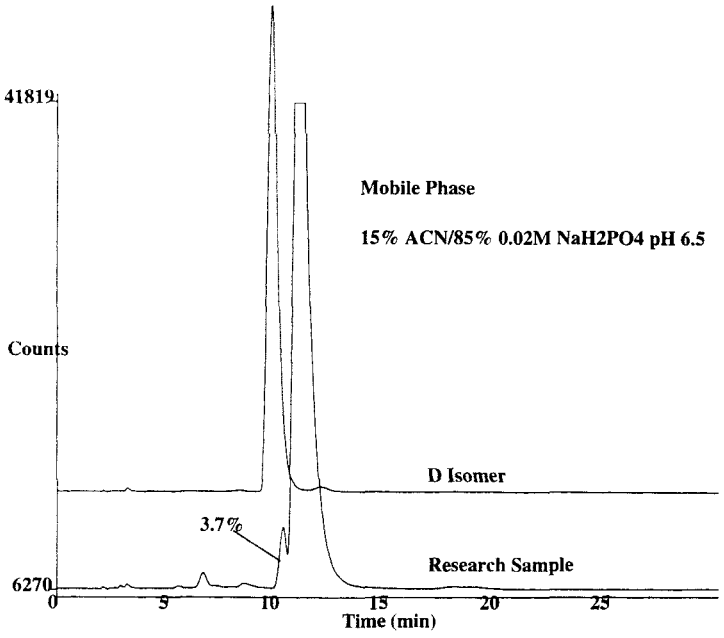

Figure 2. Chiral analysis of Fmoc-Asp-OH research samples in an OVM column.

Liquid Chromatograph and Data System

A Hewlett Packard HP 1090L ternary gradient system liquid chromatograph, equipped with photodiode array detector, and autosampler was employed. The detector's output was set at 265 nm, 4 nm bandwidth (0.002 AU/mv). The signals were processed by a Perkin Elmer Nelson A/D interface and data system using Accesschrom software. Unless otherwise noted the standard operating conditions were; flowrate 1 mL/min, injection 25 μL, run time 30 minutes, and room temperature. Quantitative determinations were expressed as relative Area % values in reference to the total area measured for both isomers.

Sample Preparation

Concentrated stock solutions of each individual PAA enantiomer were prepared in ACN at a concentration of about 1 mg/mL. Working solutions of the PAA enantiomers were prepared by diluting 100 μL of the stocks with 900

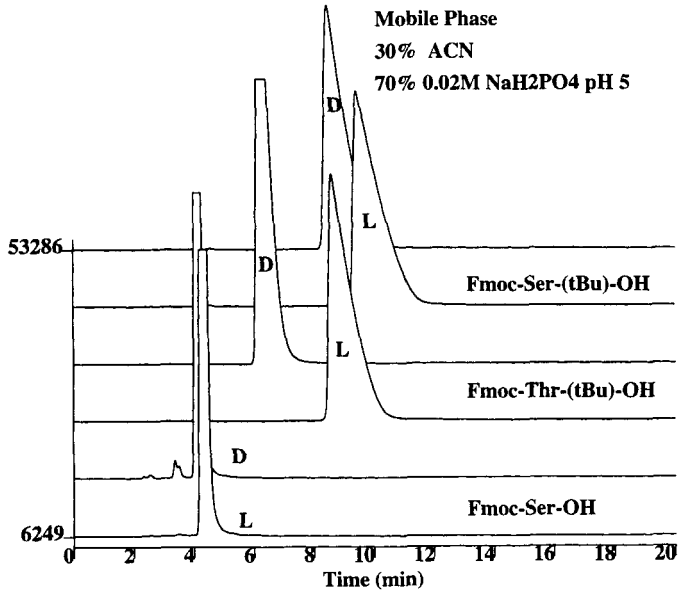

Figure 3. Enantiomeric separation of Fmoc -Ser-(tBu)-OH and Fmoc-Thr-(tBu)-OH in an OVM column.

μL of the mobile phase employed. The retention of PAAs were measured by injection of the individual D and L enantiomers and the retention times were determined by the data system. Each enantiomer solution was injected twice to confirm retention. The research samples studied were prepared in mobile phase solvent at concentrations of about 0.5 mg/mL.

RESULTS AND DISCUSSION

The main objective of this study was to study the performance of several chiral columns in the separation of PAAs and the development of separations which would allow determination of small amounts of the unwanted isomers (usually D isomer) in protected amino acids. In order to do this, the retention and selectivity characteristics of the OVM chiral stationary phase were studied varying the pH and organic content of the mobile phases.

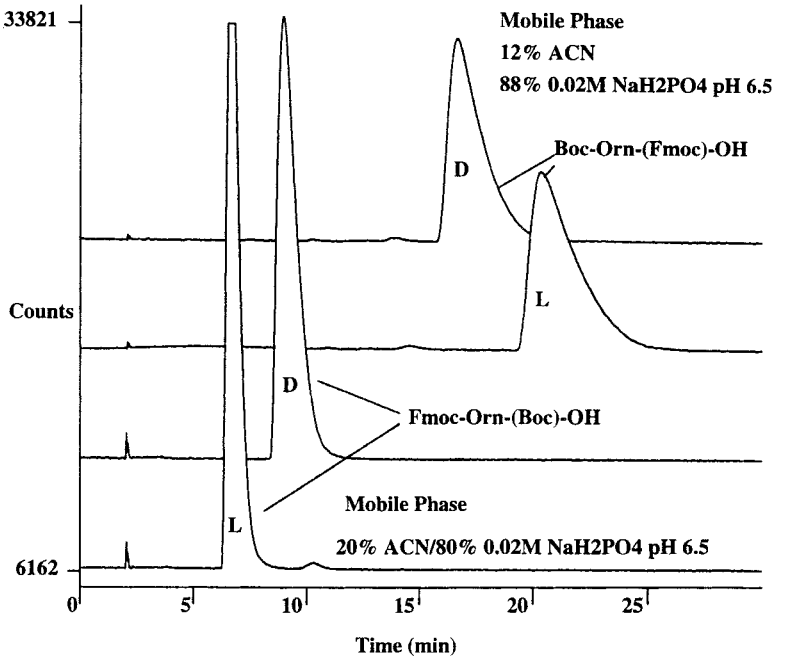

Figure 4. Enantiomeric separation of Boc-Orn-(Fmoc)-OH and Fmoc-Orn-(Boc)-OH in an OVM column.

Three pH values were studied, 6.5, 5, and 3.5. Of these, only 3.5 was below the isoelectric point of the ovomucoid protein (pI 3.9). The ACN contents covered in the study were between 10% and 35% depending on the compounds involved.

The retention behavior of the OVM column was found to be similar to that of standard reversed phase systems. tr (s) increased by decreasing the amount of organic modifier in the mobile phase and vice versa. The retention was found to follow a consistent trend increasing from higher to lower pH, i.e. retention was shorter at pH 6.5 than it was at either pH 5 or 3.5. Similarly, the retention changes between pH 5 and pH 3.5 followed the same trend, except in the cases of Fmoc-Thr-(tBu)-OH, Fmoc-Glu-(OtBu), and Fmoc-Tyr-OH where the retention at pH 3.5 was less than at pH 5. The elution order more frequently observed was the L isomer first.

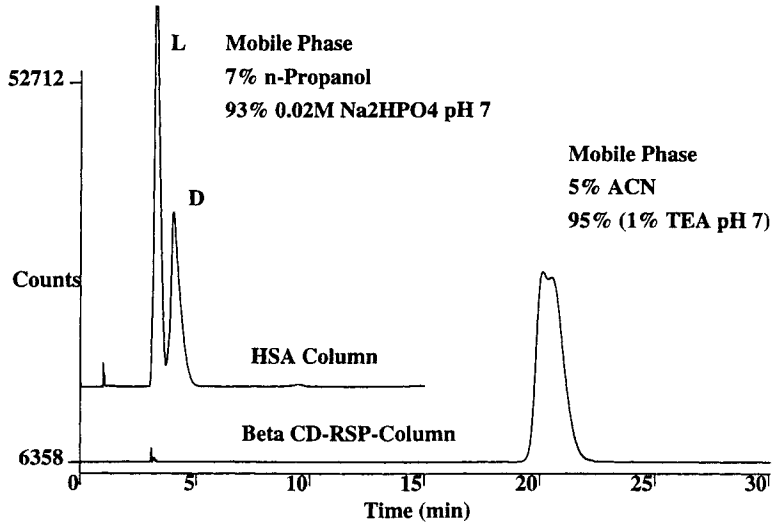

Figure 5. Chiral separation of Fmoc-Glu-OH isomers in HSA and β CD-RSP columns. the separation in the HSA column was conducted at 35°C.

Elution reversal between high pH (6.5) and low pH (5 or 3.5) conditions was observed in the cases of Fmoc-Met-OH and Fmoc-Ser-(tBu)-OH. This probably indicates a change in the OVM protein configuration which affects the chiral recognition mechanism.

Table 1 shows the compounds tested and some typical results obtained. Only conditions which gave α values greater than 1.1 at k' values between 1.5 and 12 are listed. Because of the relatively low efficiency of the OVM column (7000 N/m measured for Fmoc-His at k' 4.4) retentions shorter than k' 1.5 or larger than 12 yield elution signals which were too close to each other or too broad to be of use. Two PAAs tested which could not be separated in the OVM column were; Fmoc-Arg-Pmc-OH and Fmoc-Ser-OH. In these two cases k' values as high as 12 could be obtained but the α values measured indicated no separation. On the other hand; five compounds which have been reported in the literature as possible to separate only after some type of chemical modification; Fmoc-Lys-(Boc)-OH , Fmoc-Asn-(Trt)-OH, Fmoc-Ser-(tBu), Fmoc-Cys-(tBu)-OH, and Fmoc-Thr-(tBu)-OH;[3,14] were all easily resolved in the OVM column without derivatization (See Table 1).

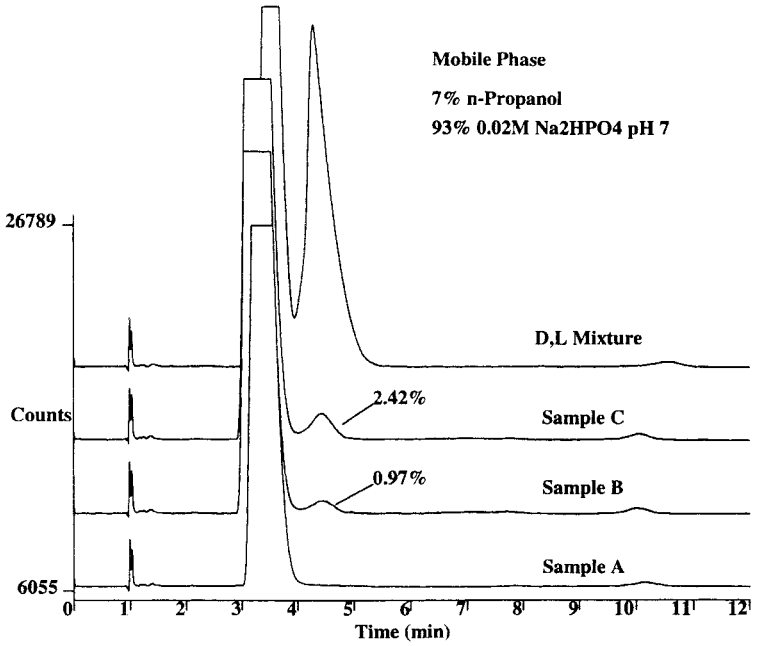

Figure 6. Chiral analysis of Fmoc-Glu-OH research samples in a HSA column, temperature 35°C.

Three additional compounds tested, Fmoc-His-(Boc)-OH, Fmoc-His-Fmoc-OH and Fmoc-D-Gln-OH presented additional problems. The first one showed instability in aqueous solvents generating a signal which matched the retention of Fmoc-His, and the others were not readily soluble in ACN at the desired concentration levels. For these reasons those compounds were not studied any further.

In general terms the OVM column was found to exhibit good selectivities in the separation of PAAs; out of nineteen compounds tested seventeen were separated. In spite of the low column efficiency and the often encountered less than ideal order of elution (L isomer first), the column can be very useful determining small amounts of undesired D isomers.

A good example of this type of application is seen in Figure 1 where three Fmoc-Trp-(Boc)-OH lots from different suppliers are separated. The results indicate that supplier B provides a better enantiomerically-pure material with only 0.15% of the unwanted isomer. Figure 2 shows a second example.

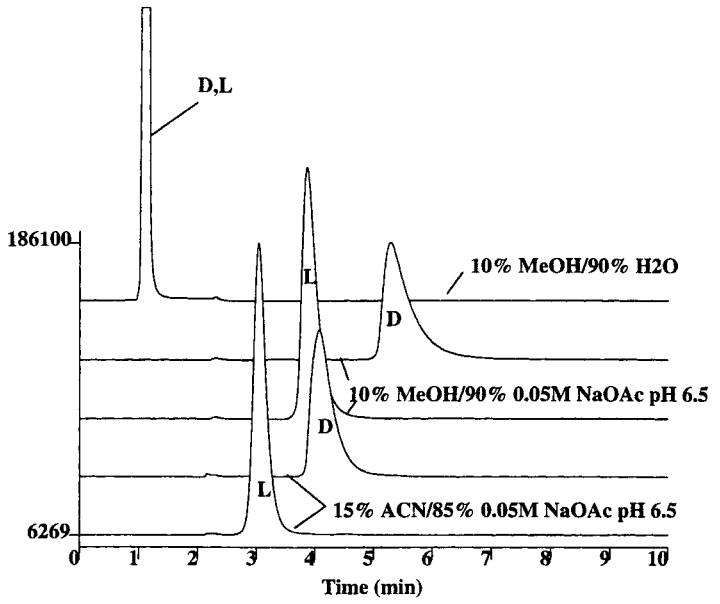

Figure 7. Chiral resolution of Fmoc-Ser-OH in a chirobiotic T column.

In this case the detection of Fmoc-D-Asp-OH acid in research samples of Fmoc-Asp-OH was facilitated by the ideal elution order (D isomer first). Similar molecules such as the side-chain protected Fmoc-Asp-(OtBu)-OH and Fmoc-Glu-(OtBu)-OH acids were also easily separated (See table I). The separations obtained were adequate to the purposes of our studies, and therefore additional operation parameters such as temperature and buffer concentration were not studied. However, it is possible that further optimization of the conditions here reported can yield enhanced separations and better detection limits for the unwanted isomers.

Structure-related factors, such as the number of protecting groups and their relative position, seem to influence the retention and separations obtained in the OVM column. Figure 3 shows the separation of Fmoc-Ser-(tBu)-OH and Fmoc-Thr-(tBu)-OH , these molecules are homologs which differ only in one carbon atom. Surprisingly, the Thr enantiomeric pair, which would be expected to be slightly more hydrophobic, is less retained and better resolved. For comparison, the case of Fmoc-Ser-OH is also shown, these enantiomers could not be separated and illustrates the fact that more protected or substituted molecules may be easier to resolve.

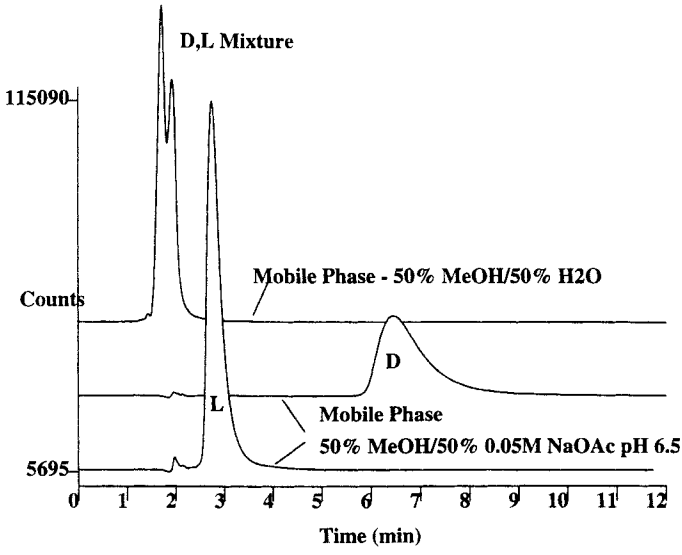

Figure 8. Chiral separation of Fmoc-Arg-(Pmc)-OH in a chirobiotic T column.

Another example of different retention and selectivity shown for seemingly similar molecules is the case of Fmoc-Orn-(Boc)-OH and Boc-Orn-(Fmoc)-OH (Figure 4). Both enantiomeric pairs can be resolved, but with different elution order. Also, Fmoc-Orn-(Boc)-OH is significantly more retained, the k' values shown in Figure 4 for that compound are k'_L 2.5 and k'_D 3.72. Under the same mobile phase conditions the corresponding values for Boc-Orn-(Fmoc)-OH were 1.06 and 0.94.

The enantiomers of Fmoc-Asp-OH, Fmoc-Asp-(OtBu)-OH, Fmoc-Glu-(OtBu)-OH, and Fmoc-Glu-(OtBu), were all separated (see Table I and Figure 2). However, no resolution was obtained for Fmoc-Glu under any of the conditions described in the Experimental section, something that is surprising since these molecules are structurally similar. This lack of success prompted the search for an alternate column.

Figure 5 shows the results obtained with β-CD-RSP and HSA chiral columns. There was only a slight indication of resolution in the cyclodextrin column which could not be improved any further. Attempts to achieve the same separation in a β-CD column were unsuccessful.

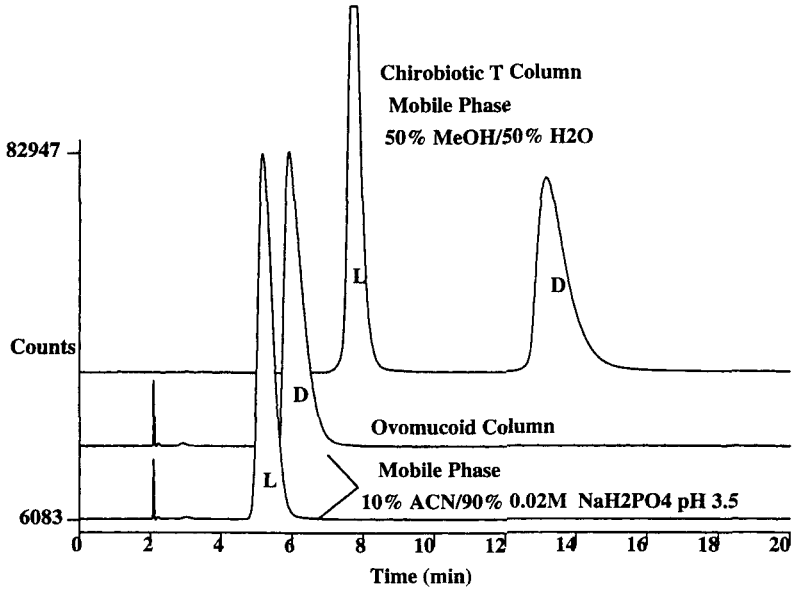

Figure 9. Chiral separation of Fmoc-Lys-OH in OVM and chirobiotic T columns.

The separation achieved in the HSA column was rather surprising since prior to these results it was tried, without success, in the resolution of several Fmoc and Boc protected amino acids. In all instances, poor peak shapes and extremely low column efficiencies were found without any indication of separation. In spite of its limitations the HSA column adequately resolved the Fmoc-Glu enantiomers and was employed in the analysis of research samples as shown in Figure 6. These results confirm the observation that molecules containing more protection or substitution are easier to resolve.

The use of a Chirobiotic T column for the enantiomeric separation of peptides and amino acids has been recently described.[14] One of the advantages mentioned in that publication was that many separations could be achieved without the use of buffers; therefore, sample recovery could be greatly facilitated. The two PAAs which could not be resolved in the OVM column Fmoc-Ser-OH and Fmoc-Arg-(Pmc)-OH, were easily separated in Chirobiotic T column. Figures 7 and 8 show results obtained. In these cases contrary to previously reported results,[14] the presence of a buffer seems to b essential or very important for resolution to be achieved. The same separatior were attempted in a Chirobiotic V (Vancomycin) column without success.

An interesting comparison between the OVM and Chirobiotic T columns is the separation of Fmoc-Lys-OH. This molecule was extremely difficult to retain and resolve in the OVM system requiring the most retentive conditions tried for any PAA tested, 10% ACN and pH 3.5 buffer (See Table I), to show separation. In contrast to this, the Chirobiotic T column was very retentive, requiring as much as 50% MeOH to achieve adequate retention, also, it gave a much better resolution of the enantiomers as can be observed in Figure 9. The good results obtained with the Chirobiotic T column suggest that it deserves further study in this type of application.

The examples and results described, clearly indicate that it is feasible to resolve PAAs without any derivatization step, and determine small amounts of the unwanted isomer in commercial or research samples. This greatly facilitates the study of peptide- synthesis research samples and the selection of PAA suppliers. No single chiral column is likely to resolve all the PAAs commonly employed in peptide synthesis, but the ovomucoid and macrocyclic antibiotic chiral stationary phases are capable of resolving a large number of these molecules.

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