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Impact of triethylamine as a mobile phase additive on the resolution of racemic amino acids on an (+)-18-crown-6-tetracarboxylic acid-derived chiral stationary phase

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Abstract

Recently, a new HPLC chiral stationary phase (CSP) prepared by covalently bonding (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid on silica gel was successfully employed in resolving various racemic natural and unnatural amino acids containing a primary amino group. Current work details on-going efforts to improve the effectiveness of this type of material. The analytes used in this study included various substituted phenylalanines, phenylglycine homologues and other primary amino acids. In an attempt to increase enantioselectivity, the effect of methanol and triethylamine modifiers was evaluated in an aqueous mobile phase containing sulfuric acid. In general, retention time increased with increasing methanol and triethylamine concentration. In addition, highest enantioselectivities were obtained with high methanol and high triethylamine; however, these conditions produced excessively long retention. All of the analytes were well resolved on the CSP with a mobile phase of 20% methanol containing 14.3 m*M* triethylamine and 10.0 m*M* sulfuric acid. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Triethylamine; Amino acids; Crown ether

1. Introduction

Enantioseparations using chiral stationary phases (CSPs) in high-performance liquid chromatography (HPLC) are the subject of continuing interest. One example of a chiral ligand used in this field is a chiral crown ether containing a dinaphthyl moiety [1,2]. This type of chiral selector has proven successful for separations of many primary amine compounds. More recently, a CSP based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (1) was reported by Hyun and co-workers [3–6] and Machida et al. [7].

The CSP in Fig. 1 was made and successfully employed for the resolution of investigational quinolone antibacterials [3,6], amines and amino alcohols [4] and various racemic amino acids and their amide or ester derivatives [5]. It should be noted that the derivatives successfully resolved in these studies all contained a free primary amine in close proximity to the stereogenic center.

In the present study, the CSP in Fig. 1 was employed in resolving aromatic halogen substituted phenylalanines, phenylglycine homologues and other

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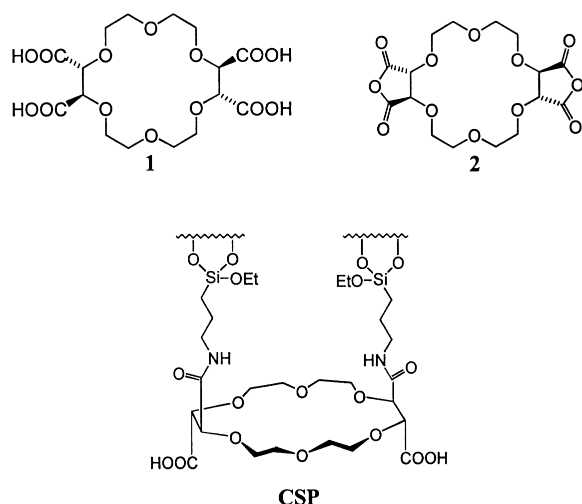

Fig. 1. Structure of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (1) and immobilized chiral selector (CSP).

charged amino acids such as DL-α-amino-2-thiophenacetic acid, *O*-phospho-DL-tyrosine, 4-amino-DL-phenylalanine and DL-homocysteic acid. In addition, the impact of the mobile phase composition (e.g., methanol content, presence of triethylamine) on enantioselectivity and retention was investigated using series of closely related amino acids.

2. Experimental

2.1. Chemicals

The various amino acids were obtained from Aldrich (Milwaukee, WI, USA), Sigma (St. Louis, MO, USA), and Lancaster (Windham, NH, USA). HPLC-grade methanol, water, sulfuric acid, and triethylamine were obtained from Fischer Scientific (St. Louis, MO, USA). (+)-(18-Crown-6)-2,3,11,12-tetracarboxylic acid, acetyl chloride, and other solvents were purchased from Aldrich, and Acros Organics (Fairlawn, NJ, USA). The aminopropyl silica (Kromasil, 5 μm, 100 Å, 312 m^2/g) was obtained from EKA Chemicals (Marietta, GA, USA).

2.2. Preparation of the chiral stationary phase and column packing

The CSP was prepared by bonding (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid, 1, to aminopropyl silica gel via the previously reported method [6] using 2,6-lutidine instead of triethylamine. The CSP was submitted for elemental analysis (Galbraith Labs., Knoxville, TN, USA). The surface concentration of the chiral selector was calculated according to the following equation:

$$\text{Surface concentration } (\mu\text{mol/m}^2) = \frac{\%\text{C} \cdot 10^6}{1200 N_c - \%\text{C}M} \cdot \frac{1}{S}$$

where %C is the percent carbon (w/w) obtained from elemental analysis, N_c is the total number of carbons in the crown ether ligand, S is the surface area of the silica and M is the molecular mass of the bonded ligand [8].

The bonded sorbent was slurry packed into a 150×4.6 mm stainless steel column. The column was evaluated chromatographically and results are presented in the tables.

2.3. Equipment

The HPLC system used for these experiments consisted of a Shimadzu LC-10AT and an SPD-10A UV–Vis detector interfaced to a personal computer data system via an Ampersand ADC instrument. The chromatographic data handling was accomplished using Chrom&Spec software. The flow-rate was typically 0.5 or 1.0 ml/min. Supporting evidence for chiral separation was supplied by repeating the separation at different wavelengths. Chromatographic experiments were conducted at 20°C.

3. Results and discussion

3.1. Bonding results

Using the %C data, the surface concentration of the chiral selector was found to be ~0.8 μmol/m^2. Direct comparison of surface concentration of the chiral ligand with previously synthesized materials was not possible because of insufficient information regarding the physical parameters of the previously employed silica.

3.2. Chromatographic results

Various mobile phase conditions were investigated in the chromatographic evaluation of the CSP. The effect of the concentration of the organic modifier as well as the impact of the presence of triethylamine on the chromatography was explored. The elution orders were determined by injecting pure enantiomer with known configuration. For the analytes for which pure standards were available, the observed elution orders were the same as those previously reported on a similar column (e.g., the D enantiomers eluted second) [5]. The CSP was successfully employed in enantioseparation of various amino acids including some not previously reported as enantioresolved on this type of CSP. The results are summarized in the tables.

The multifunctional character of the CSP confounded selection of an unretained solute. Therefore, the void time (t_0) was calculated using the system peak for each set of mobile phase conditions.

Representative chromatograms for DL-2-fluorophenylanine are shown in Fig. 2. The six chromatograms in Fig. 2 were selected to demonstrate the effect of methanol and triethylamine concentrations on retention and enantioselectivity. These representative chromatograms were obtained using 20% or 80% aqueous methanol with 10 m*M* sulfuric acid mobile phases and triethylamine (1.43 m*M* and 14.3 m*M*) at a flow-rate of 0.5 ml/min.

3.3. Effect of methanol concentration

As can be seen from Table 1, in general, retention times increased for all solutes when the methanol concentration increased from 20% to 80%. In 12 out of 15 cases, better enantioselectivity (α) was observed with lower methanol. However, the other three analytes had better resolution using 80% aqueous methanol with 10 m*M* sulfuric acid mobile phase.

For the substituted phenylalanine compounds, it may be instructive to examine enantioselectivity (α) and retention for the enantiomers with the highest affinity for the stationary phase (k'_2). At low methanol concentrations, retention for both enantiomers correlated with increased size (e.g., $k'_F < k'_{Cl} < k'_{Br}$); however, at the higher concentration of methanol, this trend was less apparent. A fluorine substituent in the ortho position seemed to enhance the interaction between the second eluting enantiomer and the chiral ligand more than a *meta* or *para* substituent. However, as can be seen from Table 1, this increased retention does not result in increased enantioselectivity. This may be the result of additional non-stereospecific binding (e.g., association with residual linker ligands). This implies that chiral selectivity, in this case, may be related more to steric inhibition of binding of the first eluting enantiomer rather than enhanced affinity of the second eluting enantiomer. In 20% aqueous methanol, a fluoro and chloro substituent in the ortho position produces high enantioselectivity but in 80% aqueous methanol with 10 m*M* sulfuric acid mobile phase, confers low enantioselectivity.

3.4. Effect of triethylamine

Certainly, the presence of residual silanols on any type of silica-based stationary phase is known to pose complications for amines [9–11]. However, CSPs, in general, are plagued with poor efficiencies and peak shapes, presumably, in part, because of the presence of various functionalities on these phases. For instance, in the present case, the presence of residual aminopropyl ligands might be expected to degrade peak shape for analytes with carboxylic acid moieties; similarly, the analyte amines may be expected to have fairly strong interactions with the carboxylic acid groups on the chiral ligand. The use of an acidic mobile phase is necessary for protonation of the primary amine facilitating inclusion in the crown ether cavity. Fortuitously, an acidic mobile phase should inhibit ionization of the carboxylic acid groups on both the bonded ligand as well as on the analytes (e.g., the amino acids), thereby minimizing their contribution to poor peak shape.

Amines have been added to various mobile phases to improve peak shape [9–12]. An amine added to the mobile phase may be strongly associated with any deprotonated acid as well as any residual silanols. Thus, triethylamine was investigated as a potential mobile phase additive.

As can be seen from Table 2, at a low concentration (1.43 m*M*) of triethylamine, the pH of the aqueous component of the mobile phase was only

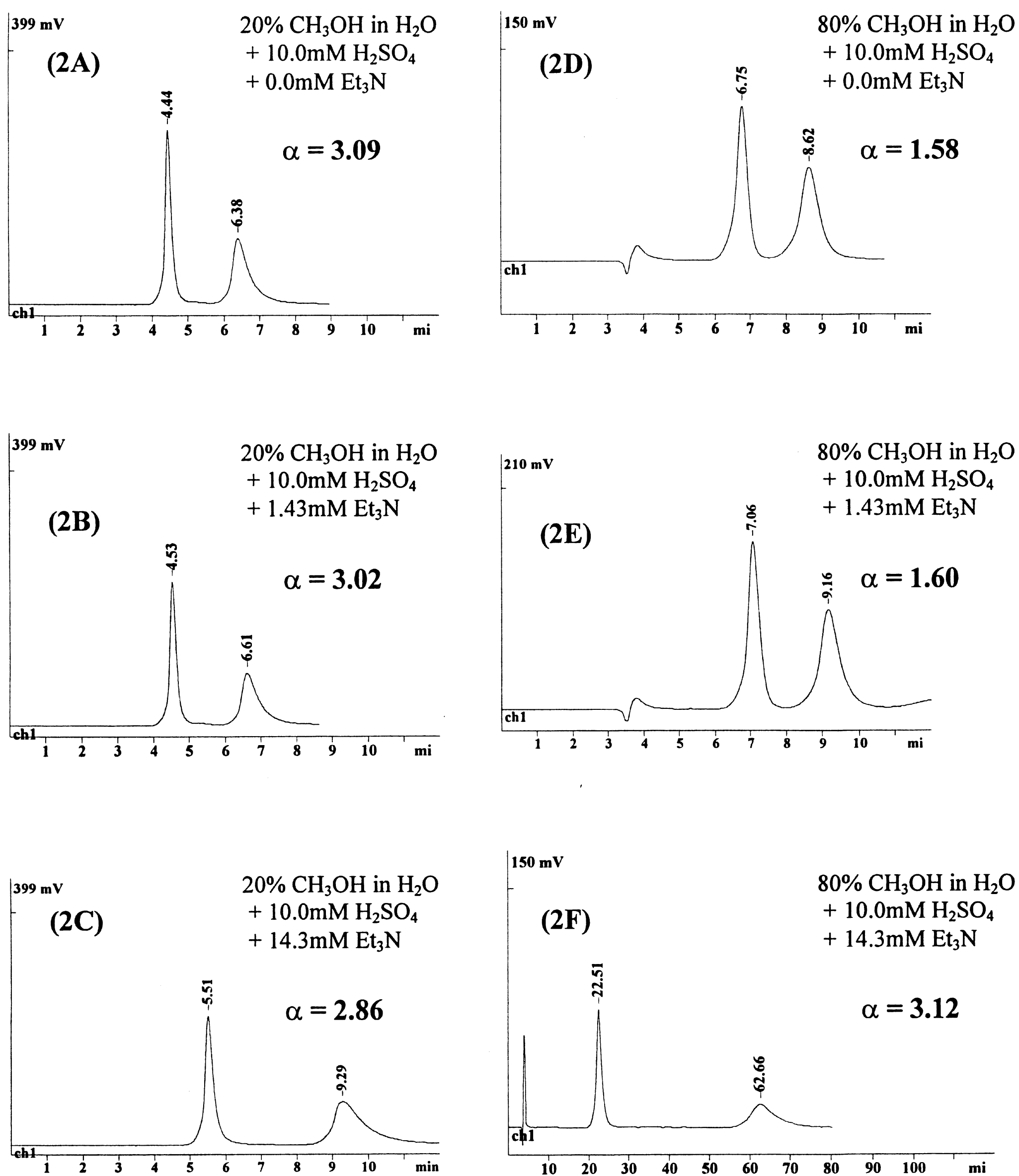

Fig. 2. Representative chromatograms for the resolution of DL-2-fluorophenylalanine on the CSP. (Note: the time axis for 2F is 10× for the other chromatograms).

0.1 pH unit higher than that containing only the sulfuric acid. Not surprisingly, the presence of a low concentration of the base produced only a slight increase in retention. However, at higher concentrations (e.g., 14.3 m*M*), the pH of the aqueous component of the mobile phase increased to pH 2.6. Retention times increased dramatically although this increase in retention was dampened by reduced methanol concentration. The pK_a values of the carboxylic acids on the crown ether have been

Table 1
Effect of methanol on enantioseparation of selected amino acids on the crown ether chiral stationary phase[a]

Analyte	80% CH_3OH in water+10.0 m*M* H_2SO_4[b]				20% CH_3OH in water+10.0 m*M* H_2SO_4[c]			
	k_1'[d]	k_2'[e]	α[f]	R_s[g]	k_1'[d]	k_2'[e]	α[f]	R_s[g]
DL-Phenylglycine	2.04	5.08	2.49	6.42	0.57	1.50	2.63	3.34
DL-Phenylalanine	0.56	0.99	1.76	1.88	0.15	0.41	2.74	1.75
DL-Homophenylalanine	0.99	1.75	1.77	2.56	0.73	1.14	1.56	1.65
DL-3-Amino-3-phenylpropionic acid	4.55	4.55	1.00	0.00	0.75	0.84	1.11	0.44
DL-2-Fluorophenylalanine	0.91	1.44	1.58	1.72	0.26	0.82	3.09	2.24
DL-3-Fluorophenylalanine	0.74	1.29	1.74	2.13	0.26	0.67	2.54	2.17
DL-4-Fluorophenylalanine	0.62	1.05	1.70	1.93	0.23	0.54	2.38	1.82
DL-2-Chlorophenylalanine	0.94	1.44	1.53	1.67	0.45	1.15	2.54	2.43
DL-3-Chlorophenylalanine	0.91	1.57	1.73	2.25	0.59	1.30	2.22	2.36
DL-4-Chlorophenylalanine	0.80	1.33	1.66	2.15	0.56	1.19	2.12	2.29
DL-4-Bromophenylalanine	0.88	1.45	1.65	2.10	0.75	1.59	2.13	2.22
DL-α-Amino-2-thiopheneacetic acid	1.80	5.12	2.85	4.75	0.57	1.55	2.71	3.39
O-Phospho-DL-tyrosine	2.97	4.82	1.62	1.77	0.72	1.17	1.63	1.45
4-Amino-DL-phenylalanine	8.60	12.50	1.45	0.88	0.58	0.88	1.51	0.89
DL-Homocysteic acid	4.21	6.72	1.60	1.04	0.54	0.77	1.42	1.12

[a] Detection: UV at 210 nm. Flow-rate: 0.5 ml/min. Temperature: 20°C.
[b] Void time (t_0)=3.54 min.
[c] Void time (t_0)=3.51 min.
[d] Capacity factor for the first eluted enantiomer.
[e] Capacity factor for the second eluted enantiomer.
[f] Separation factor.
[g] Resolution.

Table 2
Effect of triethylamine on capacity factor (k_1') of selected amino acids on the crown ether chiral stationary phase[a]

Analyte	k_1' using 80% CH_3OH in water+ 10.0 m*M* H_2SO_4+*x* m*M* Et_3N			k_1' using 20% CH_3OH in water+ 10.0 m*M* H_2SO_4+*x* m*M* Et_3N		
	0.00 m*M* (pH 2.0)	1.43 m*M* (pH 2.1)[b]	14.30 m*M* (pH 2.6)[b]	0.00 m*M*	1.43 m*M*	14.30 m*M*
DL-Phenylglycine	2.04	2.18	10.92	0.57	0.60	0.95
DL-Phenylalanine	0.56	0.59	4.15	0.15	0.17	0.43
DL-Homophenylalanine	0.99	0.99	6.90	0.73	0.78	1.35
DL-3-Amino-3-phenylpropionic acid	4.55	5.04	38.73	0.75	0.81	1.64
DL-2-Fluorophenylalanine	0.91	0.98	5.32	0.26	0.29	0.58
DL-3-Fluorophenylalanine	0.74	0.82	4.84	0.26	0.30	0.59
DL-4-Fluorophenylalanine	0.62	0.68	4.21	0.23	0.25	0.53
DL-2-Chlorophenylalanine	0.94	1.05	4.98	0.45	0.49	0.85
DL-3-Chlorophenylalanine	0.91	1.01	5.56	0.59	0.64	1.09
DL-4-Chlorophenylalanine	0.80	0.90	5.05	0.56	0.61	1.05
DL-4-Bromophenylalanine	0.88	0.99	5.44	0.75	0.78	1.33
DL-α-Amino-2-thiopheneacetic acid	1.80	1.98	9.71	0.57	0.60	0.87
O-Phospho-DL-tyrosine	2.97	3.17	7.02	0.72	0.73	0.81
4-Amino-DL-phenylalanine	8.60	10.07	DNE	0.58	0.65	1.60
DL-Homocysteic acid	4.21	4.21	6.44	0.54	0.56	0.58

[a] Detection: UV at 210 nm. Temperature: 20°C; Flow-rate: 1.0 (80% methanol with 14.3 m*M* triethylamine) or 0.5 ml/min.
[b] pH measured before methanol added. DNE: did not elute.

reported to be in the range of 2.1–4.9 [13] while the pK_a values of the carboxylic acids of amino acids are typically in the range of 1.7–2.3 [14]. Thus, the addition of a small amount (1.43 m*M*) of triethylamine does not appreciably change the pH of the mobile phase and hence, the ionization state of the immobilized chiral selector or the analytes. However, at higher (14.3 m*M*) triethylamine concentration, the pH 2.6 is closer to the range in which significant changes in ionization are taking place for both the analyte and the ligand. Indeed, the highest retention was observed when using high (80%) methanol and high (14.3 m*M*) triethylamine mobile phases.

Longer retention was also reported by Machida et al. on a silica-based crown ether CSP using mobile phases with low perchloric acid concentrations [7]. They attributed this longer retention to increased ionization of residual carboxylic acid moieties on the chiral bonded ligand leading to increased electrostatic interactions between the analyte cationic amines and the ligand anionic acids. While deprotonation of the analyte carboxylic acid might also enhance nonstereospecific interactions with residual amines that would be parasitic to the chiral separation, the presence of HSO_4^- ions may help mitigate this effect by masking the primary amines on the surface.

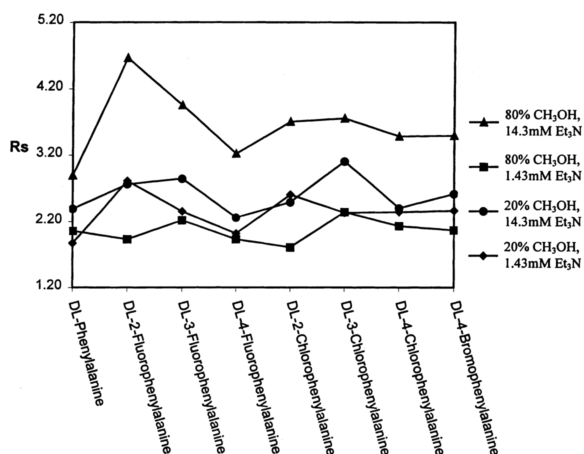

Fig. 3. The effect of triethylamine on resolution for substituted phenylalanines on the CSP.

In general, with respect to enantioselectivity, it can be seen from Table 3 for 80% aqueous methanol, chiral recognition increased with increasing triethylamine; however, this trend only held for about half the analytes in the low concentration methanol mobile phase. Increased deprotonation of the chiral ligand may enhance chiral interactions by promoting the association between the chiral analyte and the chiral ligand. As can be seen Fig. 3, resolution was

Table 3
Effect of triethylamine on enantioselectivity (α) of selected amino acids on the crown ether chiral stationary phase[a]

Analyte	α using 80% CH_3OH in water + 10.0 m*M* H_2SO_4 + *x* m*M* Et_3N			α using 20% CH_3OH in water + 10.0 m*M* H_2SO_4 + *x* m*M* Et_3N		
	0.00 m*M*	1.43 m*M*	14.30 m*M*	0.00 m*M*	1.43 m*M*	14.30 m*M*
DL-Phenylglycine	2.49	2.46	2.72	2.63	2.59	2.52
DL-Phenylalanine	1.76	1.79	2.47	2.74	2.73	2.36
DL-Homophenylalanine	1.77	1.80	2.32	1.56	1.57	1.63
DL-3-Amino-3-phenylpropionic acid	1.00	1.00	1.16	1.11	1.11	1.16
DL-2-Fluorophenylalanine	1.58	1.60	3.12	3.09	3.02	2.86
DL-3-Fluorophenylalanine	1.74	1.74	2.49	2.54	2.50	2.34
DL-4-Fluorophenylalanine	1.70	1.71	2.33	2.38	2.30	2.13
DL-2-Chlorophenylalanine	1.53	1.54	2.81	2.54	2.51	2.53
DL-3-Chlorophenylalanine	1.73	1.71	2.52	2.22	2.27	2.34
DL-4-Chlorophenylalanine	1.66	1.67	2.33	2.12	2.13	2.15
DL-4-Bromophenylalanine	1.65	1.64	2.33	2.13	2.12	2.23
DL-α-Amino-2-thiopheneacetic acid	2.85	2.81	2.67	2.71	2.69	2.61
O-Phospho-DL-tyrosine	1.62	1.64	2.58	1.63	1.63	1.79
4-Amino-DL-phenylalanine	1.45	1.48	DNE[b]	1.51	1.51	1.48
DL-Homocysteic acid	1.60	1.60	1.66	1.42	1.41	1.45

[a] Detection: UV at 210 nm. Temperature: 20°C; Flow-rate: 1.0 (80% methanol with 14.3 m*M* triethylamine) or 0.5 ml/min.
[b] Second enantiomer did not elute (t_R >300 min at 1.0 ml/min).

also generally highest using 14.3 m*M* triethylamine in 80% aqueous methanol with 10 m*M* sulfuric acid mobile phase. However, it should be noted that this increase in resolution came with the cost of greatly increased analysis time.

As in the case of methanol content, it may be instructive to consider for the substituted phenylalanine compounds the influence of the position of the aromatic substituent on the separation factor (α). As can be seen from Table 3, the extent of the influence of the site of aromatic substitution on the observed enantioselectivities was highly dependent upon mobile phase conditions. Interestingly, the impact of triethylamine concentration was most dramatic for the 80% aqueous methanol with 10 m*M* sulfuric acid mobile phase case. Not only was improved enantioselectivity observed for most analytes with the addition of triethylamine (14.3 m*M*) in 80% aqueous methanol with 10 m*M* sulfuric acid mobile phase case but the differences in selectivity resulting from substituent position were also amplified in this mobile phase relative to the addition of triethylamine (1.43 m*M*) in 80% aqueous methanol with 10 m*M* sulfuric acid mobile phase. In general, the highest enantioselectivity was observed for the ortho substituted analyte and the lowest selectivity was observed for the *para* substituted analyte, for both the fluoro and chloro substituted phenylalanines.

Resolution for the various phenylalanine analytes was plotted in Fig. 3. Again, resolution, R_s, was highest in the highest organic and triethylamine mobile phase.

The effect of proximity of the chiral center to the aromatic ring may be gleaned from examining the enantioselectivities for phenylglycine homologues (e.g., phenylglycine, phenylalanine and homophenylalanine). As shown in Table 2, the capacity factors for the first eluted enantiomer did not correlate with distance between the aromatic ring and the chiral center. However, as can be seen from Table 3, with the exception of no and low triethylamine, the enantioselectivity did decrease with increasing methylenes between the chiral center and the aromatic ring on the analyte. Interestingly, as can be seen from the comparison between the separation of DL-3-amino-3-phenylpropionic acid and DL-phenylalanine in Table 3, the crown ether chiral selector seemed to be more enantioselective for α-amino acids than β-amino acids suggesting that proximity to the carboxylic acid moiety may be more important than proximity to an aromatic group.

Finally, as can be seen from *O*-phospho-DL-tyrosine, 4-amino-DL-phenylalanine, and DL-homocysteic acid in Tables 2 and 3, amino acids with additional ionic groups were also resolved on the CSP. For instance, DL-homocysteic acid was the only analyte resolved in this study, which did not have an aromatic group. Indeed, one advantage of the chiral crown ether column is its ability to resolve non-aromatic chiral primary amines [4,5]. These types of analytes present challenges for many of the currently available CSP. As can be seen from Table 2, 4-amino-DL-phenylalanine, which contains an additional cationic amino group, was the most retained on the column when using the highest concentration of triethylamine. It is likely that the presence of this additional cationic group on the analyte may help promote association with the crown ether but, as the aromatic amine is positioned at the opposite end of the molecule, its presence does little for enantioselectivity.

4. Conclusions

The covalent immobilization of a chiral crown ether ligand on a silica substrate allows the use of methanol-rich mobile phases. Retention generally increases with increasing methanol. The addition of triethylamine also generally increases retention. Highest enantioselectivities were observed in the presence of high methanol, high triethylamine concentrations. However, the enhanced selectivity came at the expense of greatly increased retention times.

Acknowledgements

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