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Chiral separation of 9-fluorenylmethyl chloroformate- and dansyl chloride-derivatized D,L-serine by γ-cyclodextrin-bonded high-performance liquid chromatography

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Abstract

When acetate buffer was used in chiral separation of D,L-serine derivatives using a γ-cyclodextrin (CD) column, both retention factor and resolution were high below the pK_a of acetic acid and decreased sharply as the pH approached the pK_a. A similar result was obtained by increasing the buffer concentration at a fixed pH. These observations suggest that hydrogen bonding interaction between the carboxylate group of the amino acid and the secondary hydroxyl groups at the CD rim plays an important role in chiral separation and is disrupted by the buffer anion. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Serine; Amino acids; Fluorenylmethyl chloroformate; Dansyl chloride

1. Introduction

A recent report [1] that D-serine is a neurotransmitter in mammalian brain prompted us to look into the chiral separation of D,L-serine. In 1996 Imai et al. reviewed the analysis of D-amino acids in biological material and discussed the distribution of D-amino acids in the body, their origin, metabolism and possible roles in human diseases [2].

In 1987 Einarsson et al. reported a reversed-phase liquid chromatographic separation of amino acid enantiomers, including D,L-serine, following derivatization with a chiral agent, (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) [3]. In 1995 Chan et al. reported separation of diastereomeric FLEC-D,L-serine by micellar electrokinetic chromatography (MEKC) using a buffer containing sodium dodecylsulfate (SDS) [4]. In the same paper they showed that D,L-serine derivatized with 9-fluorenylmethyl chloroformate (FMOC) are not separated by MEKC with γ-cyclodextrin (CD) added to the buffer, whereas FMOC-derivatized enantiomers of valine, methionine, leucine, phenylalanine, and tryptophan are. In 1998 Tsai et al. attempted sepaaration of D,L-serine derivatized with 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl chloride) by β-CD modified MEKC; however, the resolution (Rs) was poor [5].

Since results by Chan et al. and Tsai et al. showed that amino acids derivatized with FMOC or by dansylation interact to some extent with CDs, we undertook a systematic investigation of the interac-

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tions between derivatized serine enantiomers and the CDs using CD-bonded high-performance liquid chromatography (HPLC) columns. We used HPLC instead of capillary electrophoresis (CE) because the mode of separation is more straightforward in HPLC. In CE the migration time and the resolution of the enantiomers depend in a complex way on the pH (which affects electroosmotic flow as well as electrophoretic mobility of the analyte), the direction of the electric field, and the concentration of the chiral selectors. On the other hand, in HPLC a fixed amount of the chiral selector is covalently attached to the stationary phase material and the mobility of the analyte in the bulk liquid is not influenced by the pH of the eluent as in CE.

Separation of FMOC-derivatized D,L-serine using a γ-CD-bonded HPLC column was reported in 1993; however, the mechanism of chiral separation was not discussed [6]. Subsequently several reports discussed enantiomeric resolution of derivatized D,L-amino acids using a CD-bonded stationary phase [7,8]. We carried out a systematic investigation of the chiral separation of D,L-serine derivatized with FMOC, dansyl chloride, and dimethylaminoazobenzenesulfonyl chloride (dabsyl chloride) under different pH, buffer and organic modifier conditions. We were particularly interested in the effect of pH on chiral discrimination. In the past the effect of pH has been considered primarily with respect to the change in the charge state of the analyte [9]. In this paper, we demonstrate that the anion concentration of the buffer affects the hydrogen bonding interaction between the analyte and the hydroxyl group of the cyclodextrin and plays a key role, in addition to the hydrophobic interaction responsible for formation of the inclusion complex, in the chiral separation of derivatized D,L-serine.

2. Experimental

2.1. Chemicals

D,L-Serine, L-serine, FMOC, dansyl chloride, and dabsyl chloride were purchased from Sigma (St. Louis, MO, USA). Acetonitrile was HPLC grade. Distilled water was prepared using NANOpure II system (Barnstead, Dubuque, IA, USA).

2.2. Derivatization procedure

FMOC derivatization was carried out as described elsewhere [4]. A 400-μl volume of 1 m*M* aqueous serine solution was mixed with 400 μl of 200 m*M* sodium borate buffer (pH 8.0) and 400 μl of 5 m*M* FMOC solution. The reaction mixture was left for 1 min at room temperature and diluted as necessary with deionized water before injection. Dansylation was carried out as described elsewhere [10]. A 300-μl volume of 500 m*M* sodium hydrogencarbonate in deionized water and 300 μl of 20 m*M* dansyl chloride in acetone were added to 300 μl of 1 m*M* aqueous serine solution. The reaction proceeded in the dark for 40 min at 65°C. Dabsylation was carried out as described elsewhere [11]. A 400-μl volume of a 15 m*M* solution of dabsyl chloride in acetone was added to 400 μl of 100 m*M* serine solution in 0.15 *M* sodium hydrogencarbonate (pH 9.0). The mixture was incubated at 70°C for 15 min. The volume was brought up to 2 ml with ethanol–40 m*M* sodium phosphate buffer (pH 6.5) (1:1, v/v). Subsequently, the mixture was centrifuged at 5000 *g* for 5 min and the supernatant was used in HPLC. To identify the L-serine peak, L-serine was derivatized separately and the derivative was added to the derivatized racemic mixture.

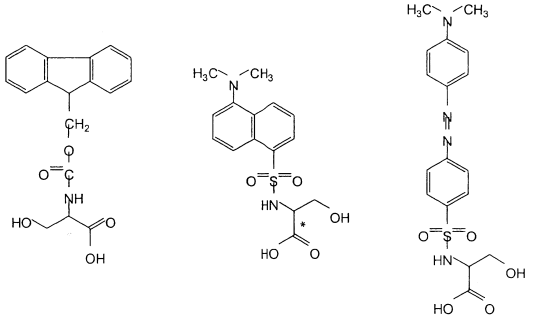
2.3. HPLC

A Hewlett-Packard 1100 Series HPLC system consisting of a quaternary pump, an on-line vacuum degasser, a Rheodyne 7725i injector with a 20 μl loop, and a diode array detector was used. A LichroCART ChiralDex column and a LichroCART ChiralDex GAMMA column (250×4 mm; β- and γ-CD columns, respectively) were purchased from Merck (Darmstadt, Germany). The mobile phase (acetate/phosphate buffer and acetonitrile in different proportions) was filtered through a 0.45-μm membrane filter. Flow-rate was 1.0 ml/min and HPLC was performed at room temperature. FMOC-, dansyl and dabsyl serine were detected at 266, 254, and 436 nm, respectively.

0.10 *M* acetate buffers in the pH 3.6–6.4 range were made by mixing 0.10 *M* acetic acid and 0.10 *M* sodium acetate solution in different proportions to give the desired pH.

3. Results and discussion

As shown below, FMOC- and dansyl serine are characterized by three and two fused rings, respectively. In dabsyl serine two benzene rings are not fused but connected by a diazo linkage. The hydrophobic moieties of the three compounds are distinctively different and the hydrophobic interaction with the CD cavity is expected to be different.



FMOC-serine Dansyl serine Dabsyl serine

In CD-based chiral HPLC, a portion of the solute molecule forms an inclusion complex with the relatively hydrophobic cavity of the CD, and the inclusion complex could be further stabilized by hydrogen bonds and other interactions [12–14]. In the present case the nonpolar moiety of the derivatizing agent is believed to interact with the hydrophobic cavity. The amino acid portion of the derivatized serine, particularly the carboxylate anion, is expected to interact through hydrogen bonding interaction with the hydroxyl groups on the hydrophilic rim of the CD stabilizing the inclusion complex and leading to chiral recognition and separation.

3.1. Order of elution

The order of elution was determined by spiking the mixture of derivatized D,L-serine with derivatized L-serine. The peak eluting later was enhanced by the spiking suggesting that derivatized L-serine interacts more strongly with CD than derivatized D-serine (Fig. 1). Fujimura et al. reported that dansyl L-amino acids are eluted before the D-forms from both β-CD and γ-CD columns [15]. Tsai et al. [5] showed that

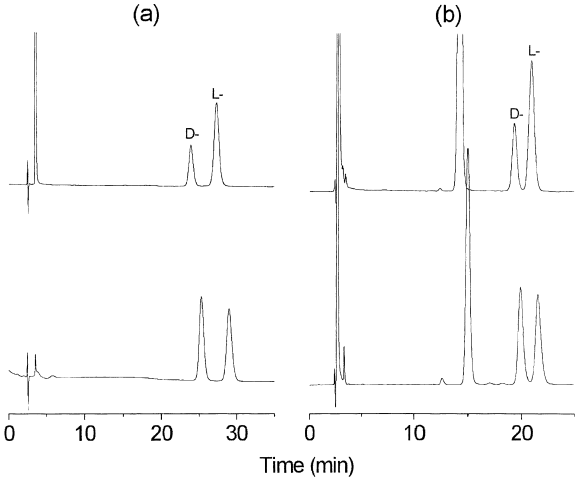


Fig. 1. Identification of (a) FMOC-L-serine peak and (b) dansyl L-serine peak by spiking a racemic mixture with L-serine after derivatization using a γ-CD column. Top, spiked; bottom, not spiked; mobile phase, 0.10 *M* acetate buffer (pH 4.4)–acetonitrile (70:30).

dansyl D-serine migrates faster than the L-serine derivative in β-CD modified MEKC, which means that dansyl D-serine interacts more strongly with β-CD than dansyl L-serine. Contrary to these earlier results, the D-enantiomer was always eluted first in our experiments whether it was FMOC-, dansyl or dabsyl serine. The reason for the discrepancy is unclear. Anyhow, the elution order is not critical in interpreting main results of this paper.

3.2. β-CD vs. γ-CD column

To form an inclusion complex the size of the nonpolar moiety and the diameter of the CD should be compatible. β-CD and γ-CD have different cavity diameters (β-CD: ca. 8.0 Å, γ-CD: ca. 10 Å) and, therefore, the choice of CD will influence retention time and resolution. Generally, β-CD is suitable for separation of compounds having a two-ring moiety such as naphthyl and biphenyl groups. FMOC-serine was eluted much faster than dansyl serine using the β-CD column probably because the FMOC group could not make a snug inclusion complex with the β-CD cavity. As a result, the FMOC-derivatized D,L-serine were not separated using the β-CD column

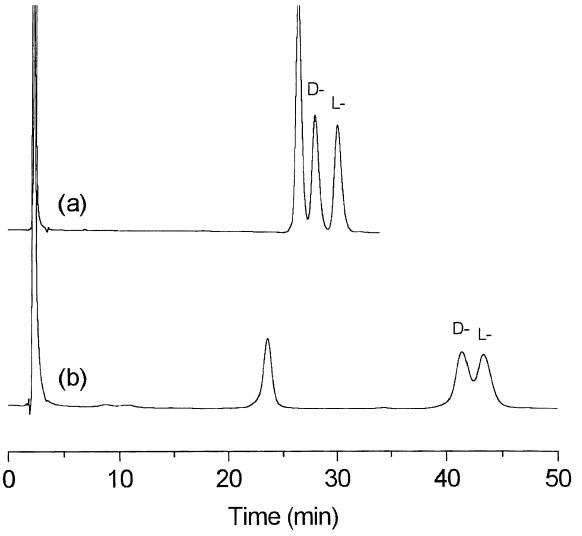


Fig. 2. Chromatogram showing chiral separation of dansyl D,L-serine using (a) γ-CD and (b) β-CD columns; mobile phase, 0.10 *M* acetate buffer (pH 3.6)–acetonitrile (50:50).

under the condition used for separation of dansyl D,L-serine.

Fig. 2 shows separation of dansyl D,L-serine using a β-CD and a γ-CD column. In both cases, the D-enantiomer was eluted before the L-enantiomer. Fig. 2 also shows that the β-CD column yields a higher retention factor (*k*). Fujimura et al. also reported higher retention factor for dansyl serine on the β-CD column than on the γ-CD column [15]. This observation suggests that dansyl serine interacts more strongly with the β-CD column than with the γ-CD column. Longer retention is expected to lead to higher resolution, because more time is available for enantiomeric recognition to take place. However, in this case baseline separation was achieved with the γ-CD column, but not with the β-CD column. A possible explanation is that the inclusion complex with the β-CD cavity is too tight to allow enough mobility of the analyte and to facilitate chiral recognition. The results in Fig. 2 do not represent optimized separation; however, separation of derivatized D,L-serine seemed more promising with the γ-CD column, which yielded a higher resolution at a shorter retention time for dansyl D,L-serine. Dabsyl D,L-serine showed similar results (data not shown). Therefore, further work was carried out with γ-CD column only. Separation could be affected by the spacer and the immobilization ratio. According to the manufacturer of the columns, there was no difference in the spacer and the immobilization ratio between the β-CD and the γ-CD columns.

3.3. Selection of derivatizing agent

Derivatization of serine introduces a chromophore for UV detection. More importantly, it is crucial for formation of an inclusion complex between the analyte and the CD. The size and the hydrophobicity of the derivatizing agent are expected to influence the overall chiral separation. Fig. 3 shows separation of D,L-serine derivatized with FMOC, dansyl chloride, and dabsyl chloride under a typical separation condition [γ-CD column; 0.10 *M* acetate buffer (pH 4.4)–acetonitrile, 70:30]. FMOC-D,L-serine and dansyl D,L-serine show good resolution with similar retention time; however, dabsyl D,L-serine show low resolution with retention time twice as long. This could be explained as following. The elongated shape of dabsyl D,L-serine facilitates formation of an inclusion complex and retention in the CD cavity. However, because the CD cavity has a limited depth, the same elongated shape could lead to reduced interaction between the charged groups around the

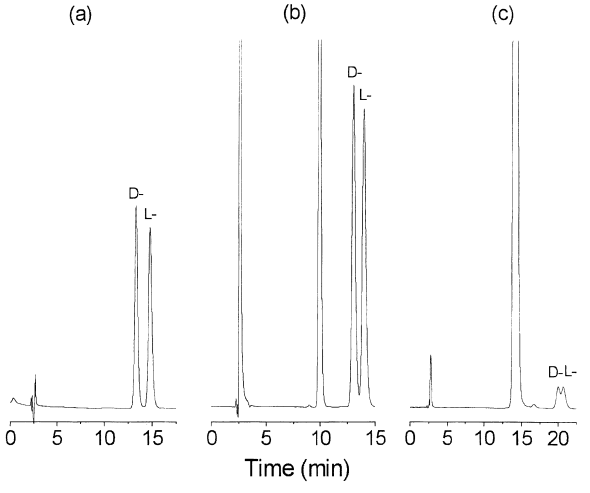


Fig. 3. Chiral separation of (a) FMOC-D,L-serine, (b) dansyl D,L-serine, and (c) dabsyl D,L-serine using a γ-CD column; mobile phase, 0.10 *M* acetate buffer (pH 4.4)–acetonitrile (60:40).

chiral center of dabsyl D,L-serine and the CD rim which is necessary for enantiomeric discrimination. Derivatization with FMOC or dansyl chloride is more straightforward than with dabsyl chloride. Therefore, further experiments were carried out with FMOC- and dansyl D,L-serine only.

3.4. Effect of organic modifier

When the concentration of the organic modifier is increased, the hydrophobic interaction between the analyte and the CD will be affected and the formation of the inclusion complex will be diminished. The situation is analogous to partitioning of an analyte between the hydrophobic surface and the organic modifier in reversed-phase HPLC. Fujimura et al. noted that the elution order of dansyl amino acids from γ-CD column resembles the order from a reversed-phase column [15].

When the acetonitrile content of the eluent (0.10 *M* acetate buffer, pH 4.4) was increased, the retention factor for both FMOC- and dansyl serine decreased as expected (Table 1). Araki et al. also noted that the retention factor for dansyl serine on γ-CD column decreased from 2.23 to 1.18 as the methanol content increased from 20 to 30% [16]. The *k* values for FMOC-serine were more strongly influenced by acetonitrile than those for dansyl serine. FMOC-serine has a larger hydrophobic moiety (three rings fused) than dansyl serine (two rings fused). Therefore, at low acetonitrile concentrations FMOC-serine shows higher *k* values than dansyl serine due to stronger hydrophobic interactions with the cavity. As the acetonitrile content was increased above 60%, the *k* values for FMOC-serine decreased below those for dansyl serine due to a strong hydrophobic interaction between FMOC-serine and acetonitrile. The increased acetonitrile similarly affected resolution. The analytes will spend less time in the cavity as the acetonitrile concentration is increased, which means less time for enantiomeric discrimination to take place. The more dramatic decrease in resolution for FMOC-serine is consistent with greater decrease in the retention factor. These results clearly demonstrate that hydrophobic interaction plays an important role in chiral separation of derivatized serine using a CD column. The retention factor decreased slightly as the acetonitrile concentration was increased (Table 1).

3.5. Effect of buffer anion concentration

Once the role of hydrophobic interaction in formation of the inclusion complex was demonstrated, it was of interest to investigate how the buffer in the eluent affects the separation. The effects of the mobile phase pH on *k* and R_s values of FMOC- and dansyl D,L-serine are summarized in Table 2. When the pH of the 0.10 *M* acetate buffer was increased from 3.6 to 4.4, a significant decrease in the *k* and R_s values was observed for both FMOC- and dansyl D,L-serine. Further increase in pH decreased retention time and resolution further; however, the decrease was not as dramatic. Araki et al. also observed a decrease in retention factor for dansyl serine on γ-CD column (the D,L-forms were not resolved) as the pH of 1.0% triethylammonium acetate buffer (30% methanol) was increased from 5 to 7 [16].

The pK_a value of the carboxyl group of serine is 2.19. In the pH range investigated (3.6–6.4) the carboxyl group would be almost fully ionized to COO^-. Therefore, the strength of the hydrogen bonding interaction between the carboxylate group and the hydroxyl group on γ-CD should not be

Table 1
Effect of acetonitrile on retention factor (*k*), separation factor (α), and peak resolution (R_s) of derivatized D,L-serine using a γ-CD column and 0.10 *M* acetate buffer (pH 4.4)

Eluent (0.10 *M* acetate buffer–acetonitrile)	FMOC-serine				Dansyl serine			
	k (D)	*k* (L)	α	R_s	*k* (D)	*k* (L)	α	R_s
70:30	9.61	10.86	1.13	2.22	7.18	7.86	1.09	1.70
60:40	5.01	5.50	1.10	1.62	4.78	5.19	1.09	1.44
50:50	3.45	3.73	1.08	1.26	2.78	3.01	1.08	1.25
40:60	2.61	2.79	1.07	1.00	2.58	2.79	1.08	1.22
30:70	2.29	2.41	1.05	0.71	2.50	2.68	1.07	1.14

Table 2
Effect of pH on retention factor (k), separation factor (α), and peak resolution (R_s) of derivatized D,L-serine using a γ-CD column and 0.10 *M* acetate buffer–acetonitrile (70:30)

Eluent pH	FMOC-serine				Dansyl serine			
	k (D)	k (L)	α	R_s	k (D)	k (L)	α	R_s
3.6	25.46	28.87	1.13	2.77	18.96	20.72	1.09	1.97
4.4	9.61	10.86	1.13	2.22	7.18	7.86	1.09	1.44
5.5	3.29	3.62	1.10	1.42	2.31	2.55	1.10	1.25
6.4	1.60	1.68	1.05	0.42	0.97	1.06	1.09	0.88

affected by pH above 3.6. Thus, the effect of the pH needs to be explained otherwise. The pK_a of acetic acid is 4.8. Therefore, about a third of the acetate is in the ionized form at pH 4.4, whereas only 6% are ionized at pH 3.6. The acetate anion in the eluent could disrupt the hydrogen bonding interaction and decrease the retention factor in a manner similar to ion exchange. Decreased retention factor would lead to a concomitant decrease in resolution, because less time is available for enantiomeric discrimination. Above pH 5.5 this effect becomes less significant.

To test this hypothesis, the experiment was repeated in the same pH range using phosphate buffer. The pK_a values of phosphoric acid for the first and second ionization are 2.1 and 7.2, respectively. So, in the entire pH range studied (3.6–6.4) most phosphate would exist as monovalent anion at a similar concentration and the effect of pH on retention would be entirely different from that of the acetate buffer if the concentration of the buffer anion affects retention as in anion-exchange chromatography. On the other hand, if pH influences retention and chiral separation only by determining the extent of acid dissociation of the analyte, it would not make any difference whether acetate or phosphate is used. The retention factor for both FMOC- and dansyl D,L-serine changed little when pH was varied from 3.6 to 6.4 using phosphate buffer in sharp contrast with when the acetate buffer was used (Fig. 4). It should be noted that about 14% of the phosphate exists as divalent anion at pH 6.4, which might explain the slightly lower retention factor observed using phosphate buffer. Clearly the observed difference is due to the buffer anion not the hydronium ion or the sodium ion. The results also suggest that the role of hydrophobic interaction is minimal even at 30% acetonitrile. Fujimura et al. investigated the effect of pH on enantioselectivity of dansyl amino acids including dansyl D,L-serine on γ-CD bonded stationary phase [15]. They showed that the k and R_s values of dansyl D,L-serine tend to decrease with decreasing pH of the mobile phase, which is contrary to our observation. They used only two pH values (5.0 and 6.5) and used different column and mobile phase [phosphate buffer ($\mu = 0.2$)–methanol, 80:20]. The reason for the discrepancy is not clear. It would be interesting to investigate the separation below pK_{a1} and above pK_{a2} of phosphoric acid; however, they were outside the operating pH range of the column.

If the major effect of increased pH on retention is

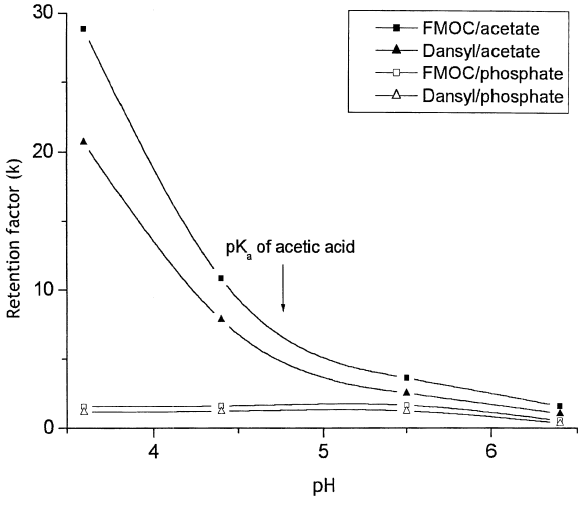


Fig. 4. pH dependence of retention factor for FMOC- and dansyl L-serine on a γ-CD column using sodium acetate and phosphate buffer; mobile phase, 0.10 *M* acetate/phosphate buffer–acetonitrile (70:30).

Table 3
Effect of buffer concentration on retention factor (k), separation factor (α), and peak resolution (R_s) of derivatized D,L-serine using a γ-CD column and acetate buffer (pH 4.4)–acetonitrile (70:30)

Eluent concentration (m*M*)	FMOC-serine				Dansyl serine			
	k (D)	k (L)	α	R_s	k (D)	k (L)	α	R_s
20.0	29.35	32.32	1.10	2.51	21.08	22.89	1.09	2.08
50.0	13.96	15.58	1.12	2.38	9.76	10.62	1.09	1.85
100	9.61	10.86	1.13	2.22	7.18	7.86	1.09	1.70
200	3.33	3.80	1.14	2.05	2.31	2.53	1.10	1.35

through disruption of the hydrogen bonding interaction by the buffer anion, increased buffer concentration at the same pH should have the same effect. Lowering the buffer concentration would have the opposite effect. The effects of buffer concentration on k and R_s are summarized in Table 3. Decreasing the buffer concentration fivefold from 100 m*M* to 20 m*M*, keeping the pH 4.4, increased k approximately threefold for both FMOC- and dansyl D,L-serine. For example, k for FMOC-D-serine increased from 9.61 to 29.35. When the pH of the 100 m*M* acetate buffer is decreased from 4.4 to 3.6, the acetate concentration is decreased about sixfold. In this case, k for the FMOC-D-serine was increased from 9.61 to 25.46 (Table 2). The agreement seems quite good considering the complex nature of the various interactions involved. Overall the situation is analogous with the pH gradient or the concentration gradient in anion-exchange chromatography, where anions are eluted from an anion-exchange column as the anion concentration is increased either by an increase in pH or by an increase in the buffer concentration at the same pH. Thus we conclude that the effect of pH is not the result of affecting the dissociation state of the amino acid but the result of disrupting the hydrogen bonding interaction by the buffer anion.

4. Conclusion

These results clearly indicate that the hydrogen bonding interaction plays a key role in the retention and the resulting chiral separation of the derivatized D,L-serine using a γ-CD column. Disruption of that interaction by the buffer anion decreases the retention factor and the resolution. So, we have an interesting example where hydrophobic interaction anchors the analyte via formation of an inclusion complex. Increased amount of organic modifier decreases formation of an inclusion complex and thus enantiomeric separation. Concurrent hydrogen bonding interaction around the chiral center facilitates separation of the derivatized D,L-serine. The separation is believed to be due to the difference in the free energy between two diastereomeric complexes between the analyte and the enantioselective environment of the CD rim. The overall separation is based on the dual (hydrophobic and hydrogen bonding) interaction. Disruption of any one interaction decreases both the retention factor and the resolution. We are currently extending this work to other FMOC- and dansyl D,L-amino acids. Comparison of the observed retention behavior and enantiomeric resolution with prediction based on computer modeling of the dual interaction would be interesting.

Acknowledgements

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