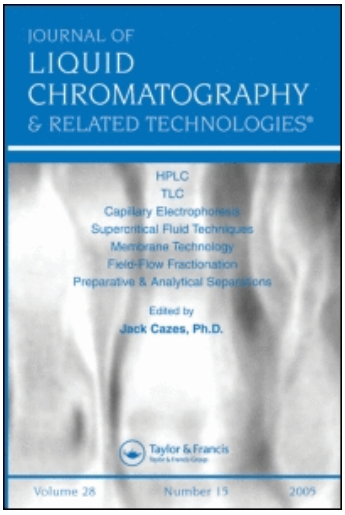

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

OPTIMIZING ENANTIOSEPARATION OF PHENYLTHIOHYDANTOIN AMINO ACIDS WITH POLYMERIZED SODIUM N-UNDECANOYL L-VALINATE IN CHIRAL ELECTROKINETIC CHROMATOGRAPHY

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Online publication date: 18 May 2000

To cite this Article Agnew-Heard, Kimberly A. , Shamsi, Shahab A. and Warner, Isiah M.(2000) 'OPTIMIZING ENANTIOSEPARATION OF PHENYLTHIOHYDANTOIN AMINO ACIDS WITH POLYMERIZED SODIUM N-UNDECANOYL L-VALINATE IN CHIRAL ELECTROKINETIC CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 23: 9, 1301 — 1317
To link to this Article: DOI: 10.1081/JLC-100100415
URL: http://dx.doi.org/10.1081/JLC-100100415

J. LIQ. CHROM. & REL. TECHNOL., 23(9), 1301–1317 (2000)

OPTIMIZING ENANTIOSEPARATION OF PHENYLTHIOHYDANTOIN AMINO ACIDS WITH POLYMERIZED SODIUM N-UNDECANOYL L-VALINATE IN CHIRAL ELECTROKINETIC CHROMATOGRAPHY

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ABSTRACT

Chiral separation of phenylthiohydantoin (PTH)-amino acid derivatives by electrokinetic chromatography (EKC) using poly(sodium N-undecanoyl L-valinate) (poly(L-SUV)) was investigated. Seven PTH-DL-amino acids (alanine, valine, norvaline, norleucine, methionine, serine, and tryptophan) were separated and each enantiomeric pair was resolved. Chiral separation was also achieved for (±)-5-(p-hydroxyphenyl)-5-phenylhydantoin. Sodium dodecyl sulfate was added to the EKC buffer to improve peak shapes and enantioselectivity. Addition of methanol and urea as organic modifiers were also investigated under slightly acidic buffer conditions, but no improvement in chiral selectivity was observed. Comparisons were made between poly(sodium N-undecanoyl L-norvalinate) (poly (L-SUNV)) and poly(L-SUV) to determine how changing the amino acid head group affects analyte-micelle complexes involved in chiral recognition.

INTRODUCTION

In the fields of biology and biochemistry, determining the amino acid sequence of proteins and peptides is very important. The Edman degradation reaction[1] is a fundamental and automated[2] application used to characterize amino acid residues produced from biological and synthetic systems. With the Edman method, truncation occurs at the N-terminal amino residue of the peptide which results in individual phenythiohydantoin (PTH) amino acids. Each antipode of the derivatized amino acid produces a different optical rotation and bioactivity.[3,4] To better understand these differences, separation and identification of each D- and L-amino acid residue produced during Edman degradation is essential.

Individual PTH-DL-amino acids are neutral in charge, similar in structure (e.g., PTH- valine and PTH-norvaline) and have a broad polarity range when grouped in a mixture. The aforementioned characteristics make separation via free zone capillary electrophoresis (CZE) very difficult because analyte elution is based on differences in charge-to-mass ratios. One alternative is electrokinetic chromatography (EKC). This branch of CE was first introduced in 1984 by Terabe et al.[5,6] as a way of separating neutral and charged species simultaneously. These researchers found that by adding a surfactant, sodium dodecyl sulfate (SDS), above its critical micelle concentration (CMC) to the background electrolyte (BGE), a pseudostationary phase would be created in a fused-silica capillary. Therefore, nonionic or electrically neutral analytes can be separated in a single run due to the differential partitioning between the aqueous and micellar phase. Using this method, an achiral mixture of 22 PTH amino acids was separated.[7]

Since all of the PTH-DL-amino acids are chiral, with the exception of glycine, enantioseparation of these neutral molecules with EKC can be accomplished by adding a charged chiral micelle to the BGE. Chiral recognition of PTH-DL-amino acids using micellar EKC (MEKC) has been demonstrated with the addition of sodium *N*-dodecanoyl-L-amino acidates,[8-13] digitonin,[9,11] saponin, and β-escin.[14-16] In all of these studies, the chiral surfactants were used at concentrations above their CMC in the form of unpolymerized micelles. In addition, if higher concentrations of these monomeric surfactants were used, then chiral separation may be compromised due to the dynamic equilibrium between the surfactant monomer and its micelle.[17] The possibility of forming polydispersed micelles was eliminated by polymerizing the surfactant above its CMC. Once polymerized, the "micelle polymer" can be used well below its CMC without disassociating into individual surfactants. Therefore, the dynamic equilibrium between the surfactant and micelle has been eliminated. The polymerized surfactant is very stable, rigid, and can withstand high concentrations of organic solvents.[18,19] One such example of polymerized chiral surfactant is poly(sodium *N*-undecanoyl-L-valinate) [poly(L-SUV)]. By polymerizing

L-SUV above its CMC, the alkene side chains were cleaved and a single bond was formed to covalently link the hydrocarbon tail on the surfactant. This polymerized surfactant was used for chiral separation via EKC and was reported independently by Wang and Warner,[17] as well as Hara and Dobashi.[20] Recently, four papers, one by Dobashi et al.[21] and three by Warner's group[22-24] have made extended use of poly(L-SUV) for chiral separation.

This manuscript reports the use of poly(L-SUV) in the enantioseparation of seven PTH-D,L-amino acids along with (±)-5-(p-hydroxyphenyl)-5-phenylhydantoin. Attempts were made to enhance optical resolution by varying the polymerized surfactant concentration and pH of the BGE. In addition, the role of modifiers (SDS, methanol and urea) on enantioseparation was investigated. All of these conditions were studied to observe their effect on chiral resolution, effective mobility, and chiral selectivity. Also, poly(sodium *N*-undecanoyl-L-norvalinate) [poly (L-SUNV)] was compared to poly(L-SUV) to determine the role of steric hindrance and hydrophobic interaction on chiral recognition.

EXPERIMENTAL

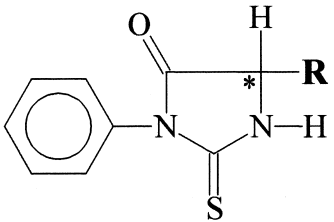
Materials and Reagents

The chiral polymerized surfactant, poly(L-SUV), was synthesized as reported earlier by Wang and Warner.[17] The seven PTH-DL-amino acids, alanine (Ala), methionine (Met), norleucine (Nle), norvaline (Nva), serine (Ser), tryptophan (Trp), and valine (Val), were purchased from Wako Chemical USA (Richmond, VA) with a purity greater than 99%. The hydantoin derivative, (±)-5-(p-hydroxyphenyl)-5-phenylhydantoin (Hyd) (99%) and L-PTH amino acids (Nle, Nva, Trp and Val) were purchased from Sigma Chemical (St. Louis, MO). These L-PTH-amino acids were utilized to determine the migration order of the D,L-enantiomers. The structures of these chiral analytes are provided in Figure 1. Urea (99.9%) and SDS (99%) were obtained from Amresco (Solon, OH). All compounds were used as received.

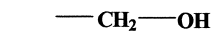
Capillary Electrophoresis

A Biofocus 3000 automated CE system (Bio-Rad Laboratories, Hercules, CA) with a multiwavelength UV absorbance detector was used for the EKC experiments. Separations were performed with an uncoated fused-silica capillary of 50 μm i.d. and a total length of 60 cm (55.5 cm to detector window) purchased from Polymicro Technologies (Phoenix, AZ). The capillary was thermostated at 25°C with an aqueous coolant containing 10-20% (v/v) methanol. Separations were accomplished by applying a constant voltage of +20 kV. An output wavelength of 254 nm was utilized for absorbance detection. For all

PTH-DL-A.A. Derivative Side Chains (R):

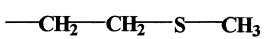


Phenylthiohydantoin-DL-Amino Acid (PTH-DL-a.a.)

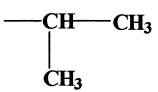


Serine (Ser)

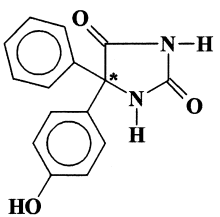
Alanine (Ala)



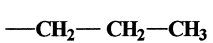
Methionine (Met)



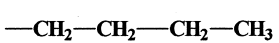
Valine (Val)



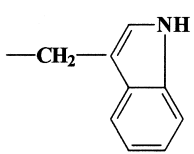
5-(p-Hydroxyphenyl)-5-Phenylhydantoin (Hyd)



Norvaline (Nval)



Norleucine (Nle)



Tryptophan (Trp)

Figure 1. Chemical structures of the phenylthiohydantoin derivatives.

experiments the BGE consisted of 25 mM dibasic sodium phosphate. The pH values of 5.6 and 7 were achieved by adding hydrochloric acid to the BGE, while no acid or base was added to the pH 9 buffer solution. To ensure reproducibility, the capillary was purged with 0.1N sodium hydroxide, followed by water, and then BGE for two minutes before each run. However, purging with sodium hydroxide was not performed at pH values lower than 7.0 to prevent pH hysteresis. Samples were prepared in methanol at concentrations of 0.5 mg/mL and were introduced into the anodic end of the capillary by applying 2 psi·sec pressure injections.

Calculation

The capacity factor (k') and resolution (R_s) were calculated using the following equations[4]:

$$k' = \frac{t_r - t_o}{t_o} \quad (1)$$

$$R_s = \frac{t_{r_2} - t_{r_1}}{w_{1_{1/2}} + w_{2_{1/2}}} \quad (2)$$

where t_0 is the migration time of the unretained species (methanol) which migrates with the electroosmotic flow (EOF) and t_r is the migration time of the enantiomer. The D and L enantiomers are denoted by the subscript "1" and "2", respectively, and $w_{1½}$ and $w_{2½}$ represents the peak widths at half-height.

RESULTS AND DISCUSSION

Effect of Varying Poly(L-SUV) Concentration on Enantiomeric Separation

Since the amount of chiral surfactant is a critical parameter in the optimization of chiral separation, we first studied the influence of poly (L-SUV) concentration on the enantioresolution of all seven PTH-DL-amino acids and Hyd (Figure 2). The concentration of poly(L-SUV) was varied from 0.50% to 1.25% (w/v). As anticipated, the resolution of PTH-DL-amino acids increased as the amount of poly(L-SUV) was increased up to 1.25% (w/v). The relatively more hydrophobic analytes ((±)-Hyd, PTH-DL-Nle and -Trp) provided baseline separation ($R_s > 1.5$) at all of the poly(L-SUV) concentrations. Some degree of enantioseparation was also observed for PTH-DL- Met, -Val, and -Nva using 0.50% (w/v) [or 16 mM equivalent monomer concentration (EMC)] of poly(L-SUV). These results are especially significant since the CMC of L-SUV has been reported to be about 21 mM.[17] It seems that since the surfac-

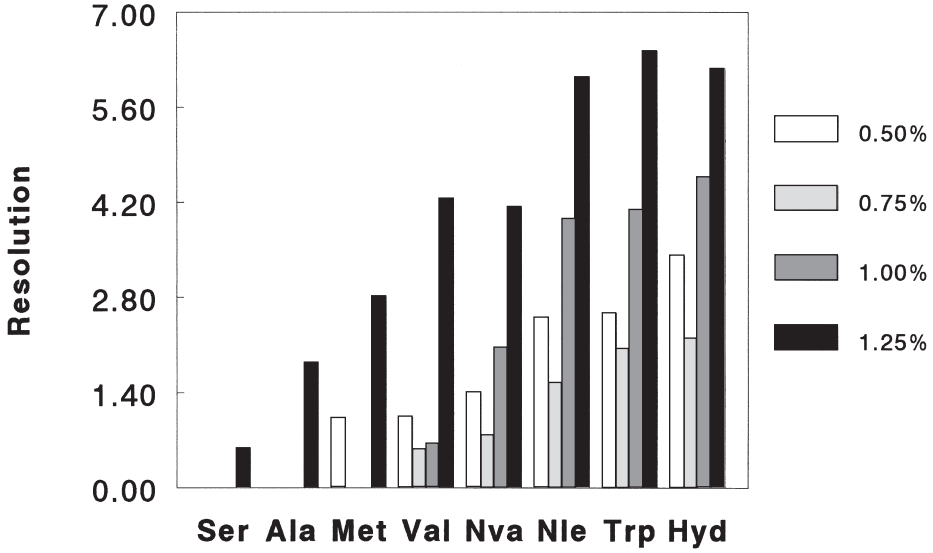


Figure 2. Effect of poly(L-SUV) concentration on chiral resolution of Hyd and PTH-D,L-amino acid derivatives. EKC conditions: 0.50-1.25% (w/v) poly(L-SUV) with 25 mM dibasic phosphate buffered at pH 5.6; capillary, 50 μm x 60 cm (55.5 cm effective length); applied voltage, +20KV; detection, 254 nm.

tants are covalently linked, the analytes are able to interact with the hydrophobic core of micelle polymer to allow chiral recognition at a concentration below its CMC. At 1.25% (w/v) (41 mM EMC) poly(L-SUV), enantioseparation was obtained for all of the racemates. Higher concentration of the polymerized surfactant enables a greater degree of hydrogen bonding and hydrophobic interaction, thereby creating an environment for better chiral recognition.

Separation parameters, such as selectivity (α), resolution (R_s) and effective electrophoretic mobility (μ_e) for (±)-Hyd and each PTH-DL-amino acid are summarized in Table 1. Generally, all of the parameters increased with an increase in analyte hydrophobicity. For example, the α was 1.11 for PTH-DL-Ala versus 1.29 for PTH-DL-Nle with 1.25% (w/v) poly(L-SUV). A larger increase in R_s was noticed as well, i.e. R_s values of 1.84 and 6.43 were obtained in the separation of PTH-DL-Ala and PTH-DL-Nle, respectively. Also, the analytes with the larger negative effective electrophoretic mobilities are more hydrophobic, and entangle to a larger extent with poly(L-SUV). Since all PTH-DL-amino acids are neutral at pH 5.6, chiral recognition is probably due to hydrogen bonding, dipole/dipole and hydrophobic interaction.

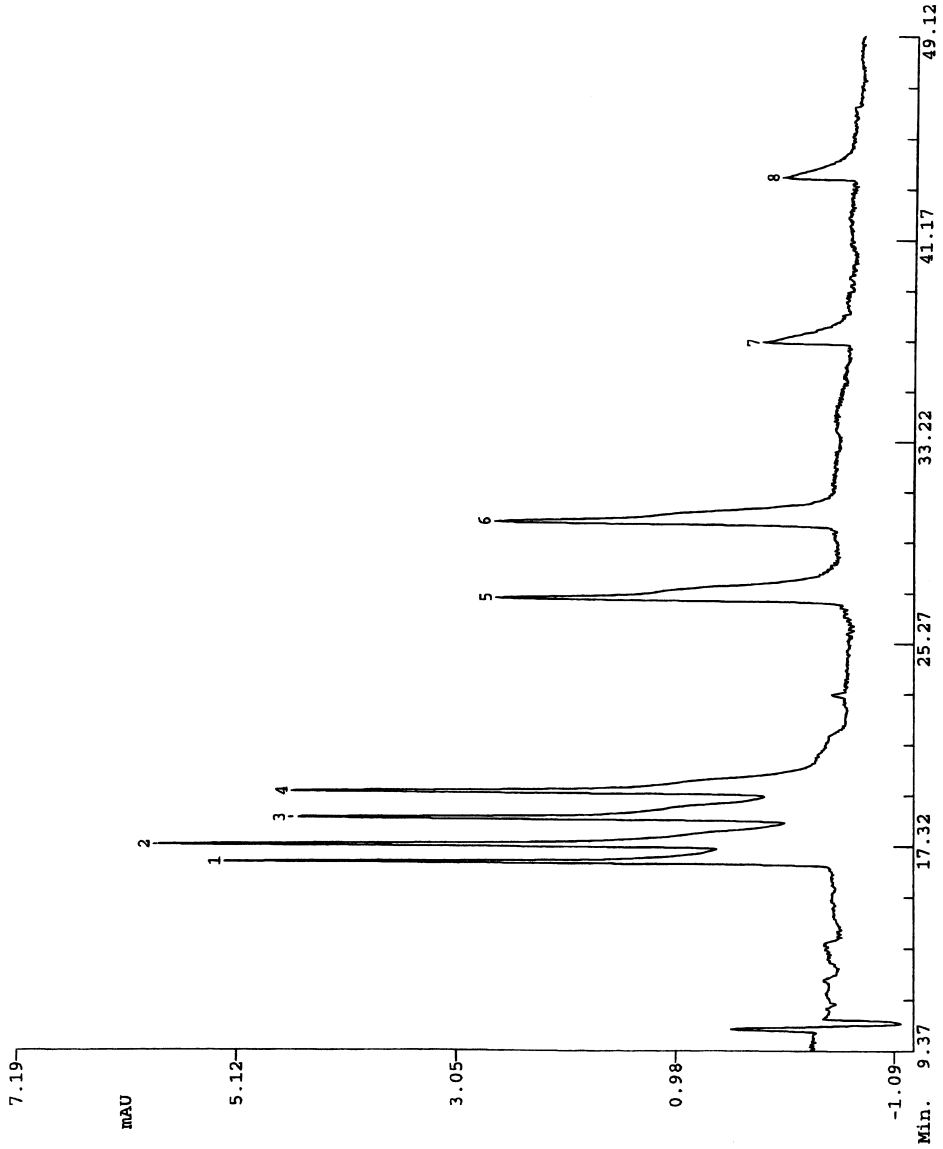
Table 1

Selectivity Factor, Resolution and Effective Mobility of Racemic Mixtures of PTH-DL-Amino Acids and Hyd[a]

	Concentration of Poly(L-SUV)					
	0.50%(w/v)		0.75%(w/v)		1.25%(w/v)	
Analyte	α Rs	μ_{el}[b] μ_{e2}[b]	α Rs	μ_{el}[b] μ_{e2}[b]	α Rs	μ_{el}[b] μ_{e2}[b]
PTH-DL-Ser	-- --	-0.22 --	-- --	-0.31 --	1.06 0.58	-0.84 -0.87
PTH-DL-Ala	--- ---	-0.34 ---	--- ---	-0.44 ---	1.11 1.84	-0.55 -0.59
PTH-DL-Met	1.11 1.39	-0.68 -0.73	--- ---	-0.92 ---	1.15 2.81	-1.14 -1.28
PTH-DL-Val	1.09 1.15	-0.62 -0.66	1.09 0.57	-0.84 -0.90	1.13 4.25	-1.01 -1.08
PTH-DL-Nva	1.10 1.46	-0.74 -0.80	1.10 0.77	-1.00 -1.08	1.16 4.13	-1.16 -1.25
PTH-DL-Nle	1.14 2.60	-1.19 -1.28	1.11 1.54	-1.53 -1.65	1.29 6.04	-1.60 -1.70
PTH-DL-Trp	1.20 2.81	-1.28 -1.41	1.13 2.04	-1.68 -1.81	1.26 6.43	-1.75 -1.89
DL-Hyd	1.18 4.19	-1.40 -1.52	1.12 2.20	-1.80 -1.93	1.29 6.17	-1.95 -2.07

[a] CE conditions: 25 mM dibasic phosphate at pH 5.6; applied voltage, +20 kV; detection, 254 nm. [b] μ_e 10^{-4} cm^2/V-sec.

Figure 3 shows an electropherogram for the simultaneous separation of a racemic mixture containing (±)-Hyd and PTH-DL-Val, -Nva, and -Nle using 1.25% (w/v) poly(L-SUV). Severe peak tailing was observed at this concentration. However, at lower poly(L-SUV) concentrations (0.50% (w/v)) peak tailing was even more severe (data not shown). One possibility for the poor peak shapes could be due to the adsorption of the analytes and/or polymerized surfactants to the capillary wall.[11]



Effect of pH

Poor solubility inhibited the use of poly(L-SUV) at pH values lower than 5.6.[23] There have been reports of PTH-DL-amino acids optically separated in acidic conditions, i.e., pH 3.0.[13-15] These experiments were conducted with mixed micelles containing SDS with digitonin, β-escin, saponin, glycyrrhizin annoniacal hydrate, α-CD, β-CD and TM-β-CD. As reported previously,[19, 23] the BGE pH has a large effect on micellar chiral recognition using polymerized surfactants. Figure 4 demonstrates the influence of pH on the chiral resolution of PTH-DL-amino acids at pH 5.6, 7.0, and 9.0 using 1.25% (w/v) poly(L-SUV). At the lowest buffer pH, all PTH-DL-amino acids, along with (±)-Hyd was baseline resolved. The only exception noted was PTH-DL-Ser.

It seems that increasing the pH to 7.0 improved the chiral resolution further for the more hydrophobic analytes (PTH-DL-Val, -Nval, -Nle, and -Trp, as well as (±)-Hyd). Conversely, the less retained PTH-DL-amino acids (Ala and Met) showed a drop in resolution. Surprisingly, there was also an increase in chiral separation of PTH-DL-Ser. We simultaneously separated a mixture of the more hydrophobic analytes (PTH-DL-Val, -Nleu, -Trp and (±)-Hyd) within 12 minutes at pH 7 using 0.50% (w/v) (16 mM) poly(L-SUV), data not shown. It was only at concentrations above 1.00% (w/v) (33 mM EMC) were the rest of the amino acids, except PTH-DL-Met, resolved.

Under more basic media, pH 9.0, using 1.25% (w/v) the resolution decreased for all the enantiomers. However, the larger polymer concentration enables greater chiral recognition for some PTH-DL-amino acids at higher pH values. For example, PTH-DL-Val and -Trp, in conjunction with (±)-Hyd were baseline resolved. Attempts were made by other researchers to obtain better peak shape by increasing the micelle concentration, using more basic conditions (pH 9 and 11), and changing the micelle head groups from SDVal to SDGlutamate or SDSerine.[10-13] In the pH range of 7-9, the electrophoretic and electroosmotic velocities of the micelle were essentially constant and chiral recognition resulted from electrostatic and hydrophobic interaction.[25,26] It appears that as the pH of the BGE was increased, the negative charge on the carboxyl head group of the poly(L-SUV) increases. This increase in electrostatic repulsion between anionic head groups causes the polymerized surfactant to organize in a looser conformation[27] allowing a greater chance for hydrophobic interaction between the polymerized surfactant and racemates.

Figure 3. Enantioseparation of Hyd and PTH-D,L-amino acid derivative mixture. Corresponding amino acids: 1 = D-Val, 2 = L-Val, 3 = D-Nval, 4 =L-Nval, 5 = D-Nleu, 6 = L-Nleu, 7 = D-Hyd, and 8 = L-Hyd. EKC conditions: 1.25% (w/v) poly(L-SUV) with 25 mM dibasic phosphate buffered at pH 5.6. Other conditions same as Figure 2.

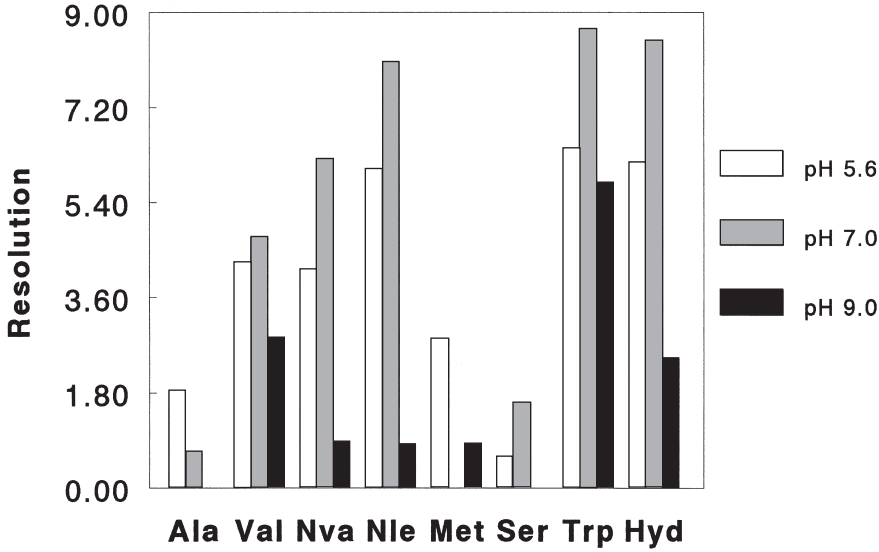


Figure 4. Effect of pH on chiral resolution of Hyd and PTH-D,L-amino acid derivatives with 1.25% (w/v) poly(L-SUV). EKC conditions same as Figure 2, except 25 mM dibasic phosphate was buffered at pH 5.6, 7.0 and 9.0.

Effect of Methanol

In order to improve enantioseparation, an attempt was made to increase the migration time window by adding methanol to the BGE. The use of methanol creates a less polar bulk phase, decreases hydrophobic interactions of the analytes with the pseudostationary, and reduces the electroosmostic velocity.[28] Figure 5 shows the electropherograms of enantiomeric mixture containing PTH-DL-Val, -Nva, and -Nle, along with (±)-Hyd using various concentrations of methanol with 0.50% (w/v) of poly(L-SUV) at pH 5.6. When the amount of methanol was increased from 0% to 20% (v/v), the EOF decreased, while the peak shape and tailing increased, and all the peaks began to co-elute. Although the selectivity factors remained relatively constant, the electrophoretic mobilities decreased with increasing methanol concentrations (data not shown). Similar results were obtained when 25 mM SDVal was used at pH 7 with 10% (v/v) methanol.[8]

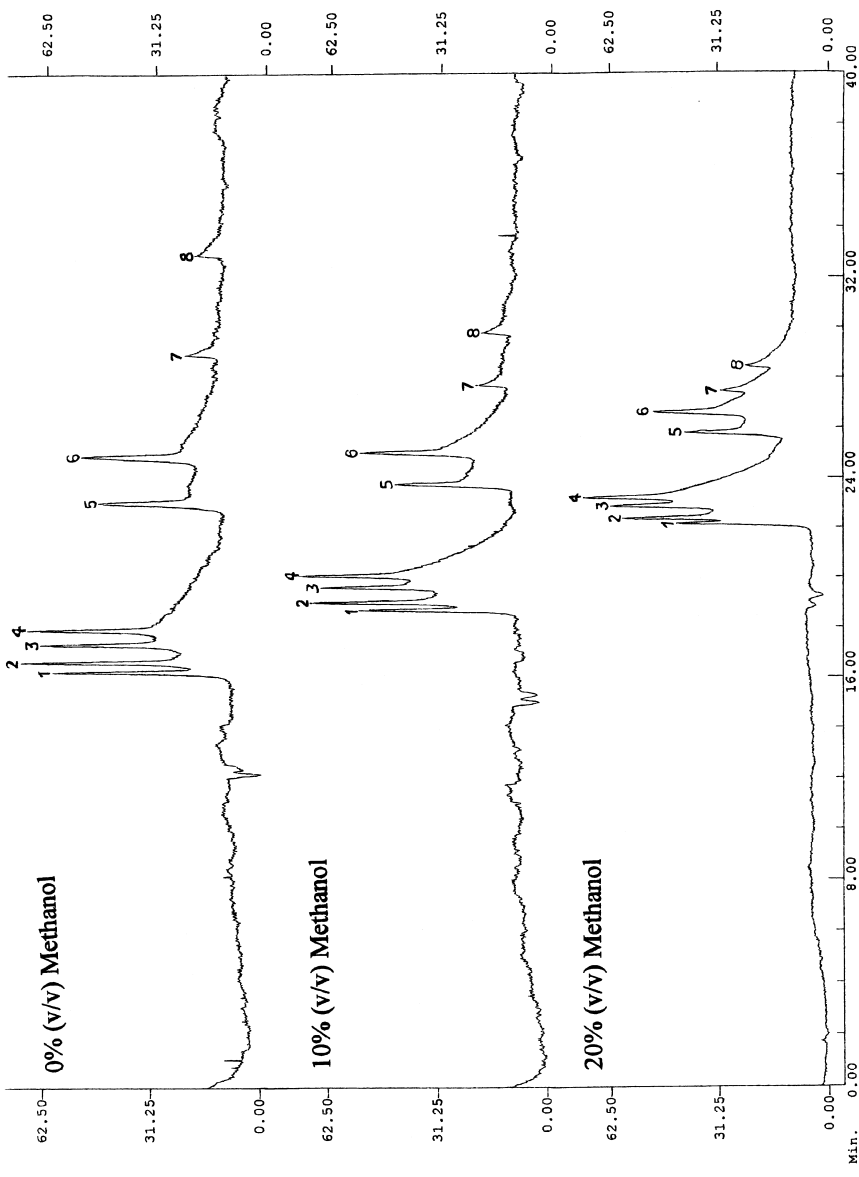


Figure 5. Effect of methanol concentration on chiral resolution, selectivity and analysis time of H yd and PTH-D,L-amino acid derivatives. EKC conditions: 0.50% (w/v) poly(L-SUV) buffered at pH 5.6.

Effect of Achiral Surfactant

Next, we investigated if chiral recognition could be improved using an achiral, anionic surfactant. Therefore, to increase selectivity, SDS was added to the BGE containing 0.50% (w/v) poly(L-SUV) at pH 7.0 (shown in Figure 6). The addition of SDS created a negatively charged mixed micelle media. Previously, it had been reported that the combined use of SDS and unpolymerized micelle reduced the electroosmotic velocity, extended the migration-time window, and thus increased chiral recognition.[8] To determine if the same were true with poly(L-SUV), we added 30 and 50 mM of SDS. With the increased concentration of SDS, all of the enantiomers were retained longer. Also, peak shape and separation deteriorated and (±)-Hyd migrated before PTH-DL-Nle. Overall, enantioseparation deteriorated and the effective mobility increased. Separation with 50 mM SDS was so poor that 1 M urea had to be added to see if the peak shape could be improved. Usually, urea increases the solubility of the micelles and decreases analyte interaction with the capillary wall; hence, interaction of the analytes with the micelles should be increased. In our experiments, the use of urea further decreased the enantioselectivity. Various other combinations of urea and methanol were added to the BGE but peak shape and resolution did not improve.

Applications with Poly (L-SUNV)

Studies were conducted to compare the chiral recognition of poly(L-SUV) with poly (L-SUNV). The two polymerized surfactants differ by the structure of their chiral amino acid head groups. The α-carbon of SUV is attached to an isopropyl group, while that of SUNV is attached to n-propyl group. The chiral resolution of a racemic mixture containing (±)-Hyd and PTH-DL-Val, -Nva, -Nle, and -Trp with 0.5% (w/v) poly(L-SUV) and 0.5% (w/v) poly(L-SUNV) were compared at pH 5.6, 7.0 and 9.0 (see Figures 7a and b). Using the branch-chained polymerized surfactant, poly(L-SUV), all of the five racemates were optically resolved at pH 5.6 and pH 7.0. At pH 5.6, separation with poly(L-SUNV) is slightly worse than that of poly(L-SUV); however, the opposite occurred at pH 9.0. The straight-chained polymerized surfactant was able to separate a mixture containing (±)-Hyd and PTH-DL-Nva, -Nle, and -Trp, something that was not accomplished with the branched-chained polymer under the more basic conditions. At all pH conditions, poly (L-SUNV) allowed shorter migration times. The differences in enantioselectivity, retention and resolution indicates that the structure of the head group can influence the racemates binding to the polymerized surfactants but not necessarily enhance chiral recognition. There are at least two reasons why better chiral separation was achieved with poly (L-SUNV) under neutral and basic conditions: (1) the straight-chained amino acid

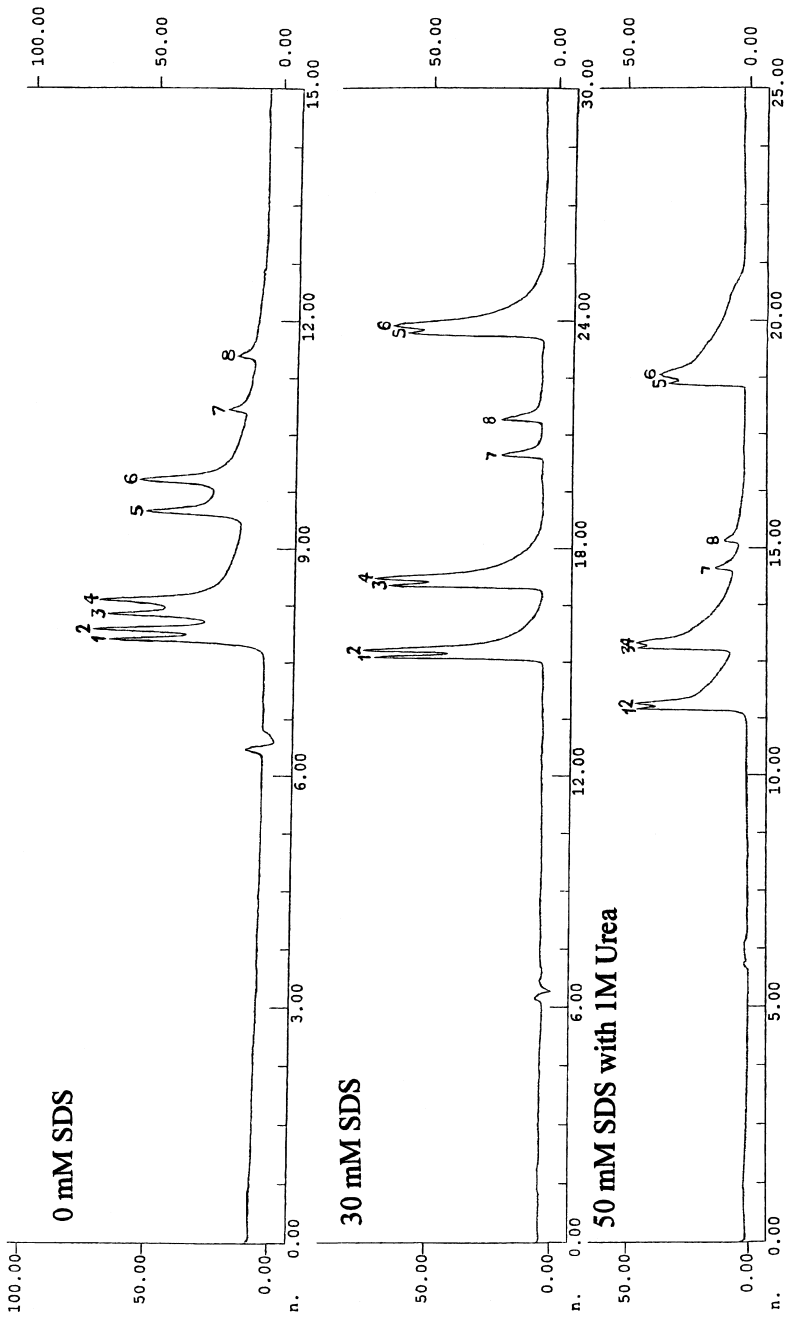


Figure 6. Effect of SDS concentration on chiral resolution, selectivity and analysis time of Hyd and PTH-D,L-amino acid derivatives. EKC conditions: 0.50% (w/v) poly(L-SUV) buffered at pH 7.0.

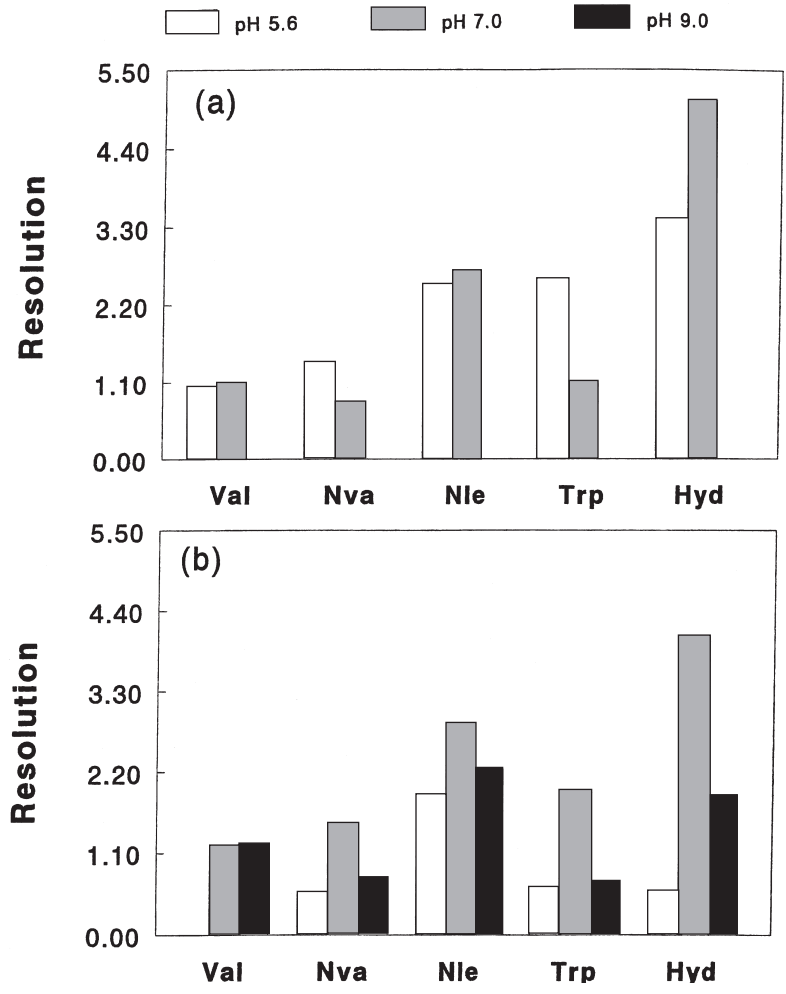


Figure 7. Effect of enantiomeric resolution of Hyd and PTH-D,L-amino acid derivatives with (a) 0.50% (w/v) poly(L-SUV) and (b) 0.50% (w/v) poly(L-SUNV). EKC conditions same as Figure 4.

allowed easier access to the hydrophobic core and the hydrophilic, polar head group of the polymer due to less steric hindrance and (2) poly (L-SUNV) was able to adapt a much looser conformation in a basic media better than poly(L-SUV).

CONCLUSION

The use of polymerized surfactant, poly(L-SUV), in EKC proved to be a versatile micelle. Chiral recognition was achieved for (±)-Hyd and seven PTH-DL-amino acid derivatives. With 1.25% (w/v) polymer under neutral BGE conditions, better chiral separation was accomplished. The addition of modifiers (SDS, urea and methanol) did not significantly improve chiral recognition. When poly (L-SUNV) was compared with poly(L-SUV), better chiral separation and shorter retention times were obtained at pH 9.0, whereas reverse was true at pH 5.6. More studies are underway to improve chiral recognition of PTH-DL-amino acids using various types of polymerized surfactants. Once the best buffer system and polymerized surfactants are identified for optimum enantioseparation, a protein sequencer will be interfaced to CE. This combination will create a powerful tool to identify and to obtain chiral separation of the derivatized amino acids using chiral EKC.

ACKNOWLEDGMENT

The authors would like to thank Montserrat Sánchez Peña for her insightful discussions regarding this work. This work was supported through a grant from the National Institutes of Health (GM39844). I. M. Warner also recognizes the Philip W. West endowment for partial support of this research.

REFERENCES

1. P. Edman, Acta Chem. Scand., **4**, 283-293 (1950).

2. P. Edman, G. Begg, Eur. J. Biochem., **1**, 80-91 (1967).

3. D. H. Coy, A. J. Katin, A. V. Schally, O. Mortin, N. G. Carton, F. Labrie, J. M. Walker, R. Fertel, G. G. Berntson, C. A. Sandman, Biochem. Biophys. Res. Commun., **73**, 632-638 (1976).

4. V. Erspamer, P. Melchiorri, G. F. Erspamer, L. Negri, R. Corsi, C. Severini, D. Barra, M. Simmaco, G. Kreil, Proc. Nat. Acad. Sci. U.S.A., **86**, 5188-5192 (1989).

5. S. Terabe, K. Otsuka, K. Ichikawa, T. Ando, Anal. Chem., **56**, 111-113 (1984).

6. S. Terabe, K. Otsuka, T. Ando, Anal. Chem., **57**, 834-841 (1985).

7. K. Otsuka, S. Terabe, T. Ando, J. Chromatogr. A, **332**, 219-226 (1985).

8. K. Otsuka, S. Terabe, Electrophoresis, **11**, 982-984 (1990).

9. K. Otsuka, S. Terabe, J. Chromatogr. A, **515**, 221-226 (1990)

10. K. Otsuka, J. Kawahara, K. Tatekawa, S. Terabe, J. Chromatogr. A, **559**, 209-214 (1991).

11. K. Otsuka, M. Kashihara, Y. Kawaguchi, R. Koike, T. Hisamitsu, S. Terabe, J. Chromatogr. A, **652**, 253-257 (1993).

12. K. Otsuka, K. Karuhaka, M. Higashimori, S. Terabe, J. Chromatogr. A, 680, 317-320 (1994).

13. K. Otsuka, H. Kawakami, W. Tamaki, S. Terabe, J. Chromatogr. A, **716**, 319-322 (1995).

14. Y. Ishihama, S. Terabe, J. Liq. Chromatogr., **16**, 933-944 (1993).

15. Y. Kurosu, K. Murayama, N. Shindo, Y. Shisa, N. Ishioka, J. Chromatogr. A, **752**, 279-286 (1996).

16. Y. Kurosu, K. Murayama, N. Shindo, Y. Shisa, Y. Satou, N. Ishioka, J. Chromatogr. A, **771**, 311-317 (1997).

17. J. Wang, I. M. Warner, Anal. Chem., **66**, 3773-3776 (1994).

18. C. P. Palmer, S. Terabe, Anal. Chem., **69**, 1852-1857 (1997).

19. S. A. Shamsi, C. Akbay, I. M. Warner, Anal. Chem., **70**, 3078-3083 (1998).

20. S. Hara, A. Dobashi, Jpn Pat # 04149205 Chem. Abstr. (1993) p118, p394052.

21. A. Dobashi, M. Hamada, Y. Dobashi, Anal. Chem., **67**, 3011-3017 (1995).,

22. J. Wang, I. M. Warner, J. Chromatogr. A, **711**, 297-304 (1995).

23. K. A. Agnew-Heard, M. Sánchez Peña, S. A. Shamsi, I. M. Warner, Anal. Chem., **69**, 958-964 (1997).

24. H. Yarabe, S. A. Shamsi, I. M. Warner, Anal. Chem., **71**, 3992-3999 (1999).

25. S. Terabe, H. Utsumi, K. Otsuka, T. Ando, T. Inomato, S. Kuze, Y. Hanaoka, J. High Resolut. Chromatogr., **9**, 666-670 (1986).

26. K. Otsuka, S. Terabe, J. Microcolumn Sep., **1**, 150-154 (1989).

27. D. Y. Chu, T. K. Thomas, Macromolecules, **24**, 2212-2216 (1991).

28. A. Dobashi, T. Ono, S. Hara, J. Yamaguchi, J. Chromatogr. A, **480**, 413-420 (1989).

Received October 30, 1999 Author's Revisions December 24, 1999
Accepted November 29, 1999 Manuscript 5194