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Enantiomeric Separation of Amino Acids Using N-alkyl-L-proline Coated Stationary Phase

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Enantiomeric separation of underivatized amino acids using N-alkyl-L-proline (octyl, dodecyl or hexadecyl) coated HPLC has been accomplished. The anchoring N-alkyl groups of L-proline provides a permanent adsorption of the resolving chiral agent on the hydrophobic interface layer of a reversed phase. The factors controlling retention and enantioselectivity such as the Cu(II) concentration, pH of the eluent, the type and concentration of organic modifier in the hydroorganic eluent, and extent of coating were examined. The elution orders between D and L- amino acids were consistent, L-forms eluting first, except histidine and asparagine. The extremely high enantioselectivity (α up to 13 for proline) is observed. The retention mechanism for the chiral separation can be illustrated by a complexation and hydrophobic interaction.

Introduction

The separation of racemic amino acids by use of a liquid chromatography has been of great interest in recent years. The prevailing approach is the ligand exchange chromatography based on the formation of diastereomeric complexes between a metal ion and amino acids. Using either the chiral bonded phase or the chiral additive, a high chiral recognition is achieved for a number of D,L-amino acids (free or derivatized) with optically active metal chelate system. Chiral modification of commercially available high performance liquid chromatographic columns by adsorption of an appropriate chiral ligand was investigated in several laboratories.[1-4] One of these coated CSPs was prepared by Bernauer *et. al.* very early. The authors mentioned above prepared ion exchange resins, Dowex 1×2 saturated with optically active anionic complexes and used them as packings in chromatography of racemates.[5,6] Besides the practical results, these papers are also of interest in the other respect: it has been demonstrated that an adsorptional modification of a nonchiral sorbent with a chiral ligand or complex is a promising way for obtaining chiral packings.

Chiral modification of commercially available high performance liquid chromatographic columns by adsorption of appropriate chiral ligands combines important advantages of the two approachs (CMPA and CSP) in resolving racemates by means of a ligand exchange chromatography.[7-14] These advantages are the possibility of using available chromatographic sorbents and applying desired chiral coating agents; the high selectivity of chiral phase system; the unique possibility of eluting the modifier, thus regenerating both the column and the resolving agent; and the possibility of preparative resolutions because of the absence of disturbing organic contaminants in the eluted fractions.

N-Alkyl derivatives of L-hydroxyproline have been used to modify reversed phase column, Lichrosorb RP-18.[2] The anchoring N-alkyl groups (n-C_7H_{15}-, n-$C_{10}H_{21}$- or n-$C_{16}H_{33}$-) of L-hydroxyproline provided a permanent adsorption of the resolving agent on the hydrophobic interface layer of the reversed phase packing material. N-Alkyl-L-histiidine also has been used as a resolving agent.[3] Kimura *et al.*[15] applied this procedure to the preparation of crown ether coated packings. The very simple technique of modifying column packings *via* adsorption of chiral ligands can be easily extended to the various new systems. Even L-phenylalanine was found [16] to adsorb from aqueous solutions onto a reversed phase silica gel in the quantities sufficient to transform the packing into a chiral ligand exchanger. The favored application area of chemically and adsorptionally modified silica packings is the enantiomeric analysis of complex mixtures where column efficiency is of utmost importance.

In this work, we prepared an easy, economic and versatile coated chiral stationary phase system that is composed of N-alkyl-L-proline coated reversed phase to LC. The objective of this work is to investigate the enantiomeric separation and its mechanism on the basis of ligand exchange reaction and hydrophobic interaction.

Experimental

Materials. Free amino acids such as alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr), proline (Pro), asparagine (Asn), histidine (His), glutamic acid (Glu), methionine (Met) and threonine (Thr) were obtained from Sigma (St. Louis, MO, USA). Octyl bromide, dodecyl bromide and hexadecyl bromide were obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile, dichloromethane, chloroform, methyl alcohol and ethyl alcohol were purchased from Merck (Darmstadt, F.R. Germany). Solvents for the mobile phase were HPLC garde. Triply distilled water was used for the preparation of mobile phase.

Instrument. Waters Associates Model 510 pump was used to provide the mobile phase. Model U6K injector was used. Waters Associates Model 490E UV detector was employed to monitor the column effluent. The chromatograms were recorded with Shimadzu Model C-R4A data station. The analytical column used was Spherisorb ODS-2 (Alltech, 150 mm×4.6 mm, 3 μm). Fourier transform-infrared (FT-IR) spectrophotometer (Digilab, FTS-20/80, USA), Fourier transform-nuclear magnetic resonance (FT-NMR) spectrophotometer (Bruker, WP80-SY, Germany) and elemental analyzer (EA, Carlo erba, 1106, Italy) were used to identify N-alkyl-L-proline prepared. The free amino acids eluted were detected at 254 nm since the species eluted were mostly in the form of Cu(II) complexes having intense absorption bands in the UV and visible regions.

Preparation of Chiral Ligands. The ligand, N-alkyl-L-proline, was prepared by treating L-proline with the alkyl bromide under a basic condition. 60 m*l* water containing 5.61 g (0.1 mole) potassium hydroxide and 11.51 g (0.1 mole) L-proline, and 40 m*l* ethanol containing 0.12 M alkyl bromide were mixed and stirred for 5 hours with refluxing. After completion of reaction, water and ethanol were evaporated by a rotary evaporator. The residue was dissolved in water. To remove the unreacted alkyl bromide, the aqueous solution was extracted with ethyl ether and the ethyl ether solution was discarded. After removal of water in the extracted aqueous solution, the residue was dissolved in dichloromethane and then the unreacted proline was precipitated. After the filtration of proline, the product which was dissolved in dichloromethane was recrystallized by adding chloroform. The product was identified with FT-IR, FT-NMR and EA. The yield of N-octyl-L-proline, N-dodecyl-L-proline and N-hexadecyl-L-proline was 40%, 37% and 25%, respectively.

Mobile Phase Preparation. The mobile phase was prepared by dissolving cupric acetate and buffer reagent in HPLC grade water and by adjusting the pH to the desired value with acetic acid or KOH and the desired amount of organic modifier added.

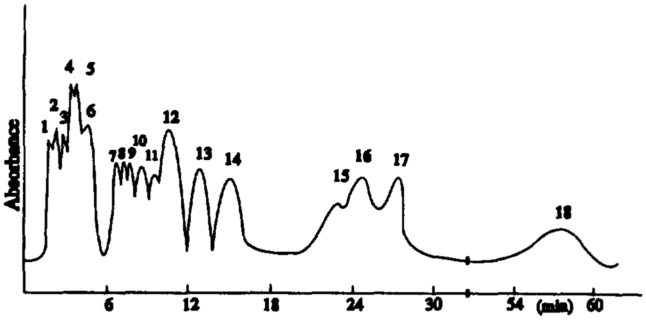


Figure 1. Separation of 9 racemic amino acids on N-hexadecyl-L-proline coated reversed phase, C_{18}, column in the HPLC. Column: Spherisorb ODS-2 (Alltech, 150×4.6 mm, 3 μm). Mobile phase: 1.0×10^{-4} M $Cu(ac)_2$ in MeOH/water of 15/85 (v/v) at pH 5.58. Flow rate: 1.0 m*l*/min. Detection: 254 nm. Elution sequence: 1. D-His, 2. L-His ,3. L-Pro, 4. L-Ala, 5. D-Ala, 6. L-Val, 7. L-Met, 8. L-Tyr, 9. L-Ile, 10. L-Leu, 11. D-Pro, 12. D-Val, 13. D-Met, 14. D-Tyr, 15. D-Leu, 16. L-Phe, 17. D-Ile, 18. D-Phe.

Coating Procedure of Coated Chiral Stationary Phase. Coating of N-alkyl-L-proline on a C_{18} reversed phase was accomplished by flowing 10^{-4} M solution of copper chelate in methanol or MeOH/water through the column with 1.0 m*l*/min for about 1 hour, followed by conditioning with 10^{-4}M solution of cupric acetate in MeOH/water (15/85, v/v) for more than 5 hours. To achieve an effective coating, copper chelate was dissolved in the following solution: $Cu(N\text{-octyl-L-proline})_2$ in MeOH/water (50/50, v/v), $Cu(N\text{-dodecyl-L-proline})_2$ in MeOH/water (50/50, v/v) and $Cu(N\text{-hexadecyl-L-proline})_2$ in pure methanol were used.

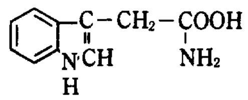
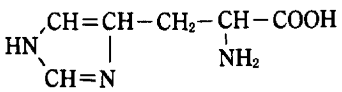
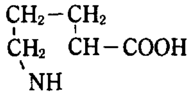
To get optimum selectivity and retention time, the extent of coating was controlled by adjusting the time of the coating. In general, the higher the concentration of the loaded N-alkyl-L-proline, the lower the retention time of amino acids. Thus, the appropriate coating amount would be chosen by measuring the retention of amino acids. After coating, the column was conditioned by flowing at least 100 column volumes of the mobile phase. During the conditioning, the unloaded chelate was removed. The immobilized N-alkyl-L-proline remains stable for a period of about 4 months by using the mobile phase with the organic modifier content below 30%.

Results and Discussion.

As coating a given N-alkyl-L-proline on a reversed phase, the interaction between the amino acids to be resolved and the stationary phase can be understood in terms of superposition of a complexation and hydrophobic interaction process. The complex formation is considered to be the dominant factor in the interaction. The factors controlling the retention and enantioselectivity are the Cu(II) concentration, pH of the eluent, the type and concentration of organic modifier in the hydroorganic eluent, and extent of coating. In this paper, the exact amount of the adsorbed chiral reagent has not been determined.

As shown in Figure 1, the octadecyl column coated with

Table 1. Representative Capacity Factor (k_L' and k_D'), Enantioselectivity (α) and Resolution (R_S) for Free Amino Acids

Amino acid	Abbrev.	Structural formula	k_L'	k_D'	α	R_S
Alanine	Ala	$CH_3CH(NH_2)COOH$	0.60	0.74	1.23	0.16
Valine	Val	$(CH_3)_2CHCH(NH_2)COOH$	1.51	4.86	3.22	2.97
Leucine	Leu	$(CH_3)_2CHCH_2CH(NH_2)COOH$	3.67	11.80	3.22	4.65
Isoluecine	Ile	$CH_3CH_2CH(CH_3)CH(NH_2)COOH$	2.80	13.99	5.00	17.22
Phenylalanine	Phe	$C_6H_5CH_2CH(NH_2)COOH$	12.38	31.12	2.51	3.41
Tryptophan	Trp		39.54	59.82	1.51	2.00
Tyrosine	Tyr	$HOC_6H_4CH_2CH(NH_2)COOH$	3.04	7.17	2.36	3.30
Histidine	His		0.67	0.52	0.78 (1.29)	0.20
Asparagine	Asn	$H_2NCOCH_2CH(NH_2)COOH$	0.51	0.48	0.94	0.04
Methionine	Met	$CH_3S(CH_2)_2CH(NH_2)COOH$	2.61	5.81	2.23	2.83
Threonine	Thr	$CH_3CH(OH)CH(NH_2)COOH$	0.63	0.69	1.10	0.08
Proline	Pro		0.69	2.55	5.15	2.86

N-hexadecyl-L-proline coated RP-18 column was used. Mobile phase: 1.0×10^{-4} M $Cu(ac)_2$ in MeOH/water of 15/85 (v/v) at pH 5.58. Flow rate: 1.0 ml/min. Detection: 254 nm. $\alpha=k_D'/k_L'$

N-hexadecyl-L-proline is effective enough to provide a separation of 9 racemic amino acids. The data in Table 1 show that a noticeable increase in retention of the amino acid enantiomers takes place when the side chain of the mobile ligand changes from Ala to Trp. It indicates that hydrophobic interactions contribute much to the retention of the amino acids. The enantioselectivity and resolution for hydrophobic amino acids capable of having the distinct hydrophobic interactions with the reversed phase are observed to be extremely high. The elution orders between D- and L- amino acids except His and Asn are consistent, L forms eluting first. Especially, this system is better for the enanntiomeric separation of proline than the CMPA method (α=1.00-1.68).[1,7,8] These results are also better than those (Max. α: Val; 1.70) of commercial CSP column (Alltech, Nucleosil Chiral-1, L-hydroxyproline modified silica packing) that are studied in our group.

Enantiomeric separation of amino acids is dependent on the type of ligand as shown in Table 2. But, lacking determination of the exact amount of adsorbed chiral reagent, it is not possible to discuss differences in the chromatographic results obtained on the three columns which could be referred to the N-alkyl- chain length of the chiral ligand. Three coated columns behave similiarly in their retention behaviour of amino acid enantiomers. In this work, N-octyl-L-proline coated column shows the best result among them for the enantiomeric separation.

Separation of the optical isomers is dependent on the Cu (II) concentration in the mobile phase as shown in Table 3 for two N-alkyl-L-proline coated columns. With the decrease of $Cu(ac)_2$ concentration, the amino acid enantiomers are retained longer and the enantioselectivity of amino acids is improved. Polar amino acids such as Asn, Glu and Thr also shows a good separation in contrast with that[2] of N-alkyl-L-hydroxyproline which is hardly resolved. It is believed that the equillibrium between two mono(amino acidato)copper complex and the mixed ligand bis(amino acidato) copper complex is shifted to the left as shown in Eq. (1) and favors the formation of a mixed ligand complex with

Table 2. Capacity Factor (k') and Enantioselectivity (α) on the Type of Ligand at pH 7.0

Free-AA		Type of ligand C_{16}		C_{12}		C_8	
		k'	α	k'	α	k'	α
Ala	D	2.09	1.39	0.48	1.71	1.52	1.81
	L	1.50		0.28		0.84	
Val	D	7.97	2.44	4.66	5.07	31.82	4.94
	L	3.27		0.92		6.44	
Leu	D	18.23	2.54	9.09	1.96	74.59	4.44
	L	7.17		4.64		16.82	
Ile	D	21.70	3.47	12.81	3.29	53.62	2.84
	L	6.25		3.90		18.88	
His	D	4.06	0.92	0.38	0.43	1.10	0.59
	L	4.43		0.88		1.87	
Glu	D	4.34	1.03	0.92	1.24	2.74	1.60
	L	4.22		0.74		1.71	
Met	D	9.27	1.79	6.55	3.03	35.80	2.08
	L	5.17		2.17		17.21	
Thr	D	1.83	1.23	0.41	1.58	1.80	1.02
	L	1.49		0.26		1.02	
Pro	D	16.59	3.00	2.72	3.53	27.37	10.65
	L	2.20		0.77		2.57	

The experimental conditions are the same as in Table 1.

Table 3. Capacity Factor (k') and Enantioselectivity (α) on Concentration of $Cu(ac)_2$

Free-AA		1×10^{-3}		1×10^{-4}		5×10^{-5}	
		k'	α	k'	α	k'	α
Ala	D	0.14	1.56	0.37	1.85	0.50	1.92
	L	0.09		0.20		0.26	
Val	D	2.90	1.67	6.31	1.97	8.83	2.00
	L	1.74		3.20		4.41	
Leu	D	8.41	1.62	16.95	1.88	23.62	1.92
	L	5.18		9.01		12.10	
Ile	D	9.83	2.08	19.96	2.41	27.52	2.35
	L	4.72		8.23		11.72	
Tyr	D	9.23	1.64	17.13	1.95	23.57	1.72
	L	5.64		8.78		13.73	
His	D	0.14	0.56	0.30	0.59	0.53	0.64
	L	0.25		0.51		0.83	
Asn	D	0.02	0.67	0.15	0.79	0.25	0.19
	L	0.03		0.19		1.35	
Glu	D	0.94	1.15	2.79	1.08	3.13	1.14
	L	0.82		2.58		2.75	
Met	D	4.08	1.30	8.46	1.37	12.09	1.36
	L	3.15		6.18		8.92	
Thr	D	0.21	2.10	0.30	1.30	0.44	1.42
	L	0.10		0.23		0.31	
Pro	D	2.55	3.59	5.53	4.35	8.18	5.24
	L	0.71		1.27		1.56	

N-dodecyl-L-proline coated RP-18 column was used. Mobile phase: aqueous solution. pH 7.0. The other conditions are the same as in Table 1.

Table 4. Capacity Factor (k') and Enantioselectivity (α) as a Function of pH

Free-AA		pH			
		5.5		7.0	
		k'	α	k'	α
Ala	D	0.66	1.08	0.94	1.36
	L	0.61		0.69	
Val	D	2.22	2.02	3.97	2.94
	L	1.10		1.35	
Leu	D	4.56	1.98	9.50	2.36
	L	2.30		4.03	
Ile	D	5.25	3.20	11.14	3.35
	L	1.64		3.33	
Phe	D	10.00	2.05	20.59	2.01
	L	4.88		10.26	
Tyr	D	2.43	1.44	4.89	1.46
	L	1.69		3.35	
His	D	0.65	0.69	2.63	0.85
	L	0.94		3.08	
Asn	D	0.53	0.93	0.98	0.93
	L	0.57		1.06	
Glu	D	0.26	1.13	2.46	0.96
	L	0.23		2.56	
Met	D		2.59	1.58	4.99
	L	1.64		2.87	
Pro	D	1.48	2.24	2.77	2.33
	L	0.63		1.19	

The experimental conditions are the same as in Table 1.

Table 5. Capacity Factor (k') and Enantioselectivity (α) on the Addition of the Organic Modifier at pH 7.0

Free-AA		100% H_2O		15% MeOH		15% ACN	
		k'	α	k'	α	k'	α
Ala	D	1.63	1.61	2.09	1.39	0.94	1.36
	L	1.01		1.50		0.69	
Val	D	17.44	3.43	7.97	2.44	3.97	2.94
	L	5.08		3.27		1.35	
Leu	D	54.91	3.37	18.23	2.54	9.50	2.36
	L	16.29		7.17		4.03	
Ile	D	64.65	4.02	21.70	3.47	11.14	3.35
	L	16.10		6.25		3.33	
His	D	1.44	0.74	4.06	0.92	2.63	0.85
	L	1.94		4.43		3.08	
Glu	D	1.57	1.99	4.34	1.03	2.46	0.96
	L	0.81		4.22		2.56	
Met	D	27.92	2.28	9.27	1.79	4.99	1.74
	L	12.26		5.17		2.87	
Thr	D	1.54	1.20	1.83	1.23	0.87	0.92
	L	1.28		1.49		0.95	
Pro	D	17.73	6.07	6.59	3.00	2.77	2.33
	L	2.92		2.20		1.19	

The experimental conditions are the same as in Table 1.

the decrease of $Cu(ac)_2$ concentration.

$$[Cu(AA)(ac)_2] + [Cu(N\text{-alkyl-L-proline})(ac)_2]_s \rightleftarrows [Cu(AA)(N\text{-alkyl-L-proline})]_s + Cu(ac)_4 \qquad (1)$$

Where AA and ac mean amino and acetate ion, respectively and the subscript, s means attached to the stationary phase.

Table 4 shows the influence of pH on the retention behaviours. The capacity factor increase with increasing pH of the eluent. This indicates that with increase in pH, the formation of the copper complexes is favoured. Enantioselectivity also increases with increasing pH of the eluent.

Retention and enantioselectivity of the enantiomers unambiguously depend on the concentration of the organic modifier in the eluent as shown in Table 5. With the increase of the solvent strength, the retention of amino acid enantiomers decreases. As the addition of the organic modifier in the eluent, these solvents make the stationary phase less hydrophobic *via* adsorption on the stationary phase. Thus, the hydrophobic interaction between the amino acid enantiomers and reversed phase is reduced as the addition of the organic modifier in the eluent. The hydrophobic interaction appears to be important enough to cause a noticeable change of enantioselectivity with adding the organic modifier in the eluent. This behaviours demonstrate that the hydrophobic

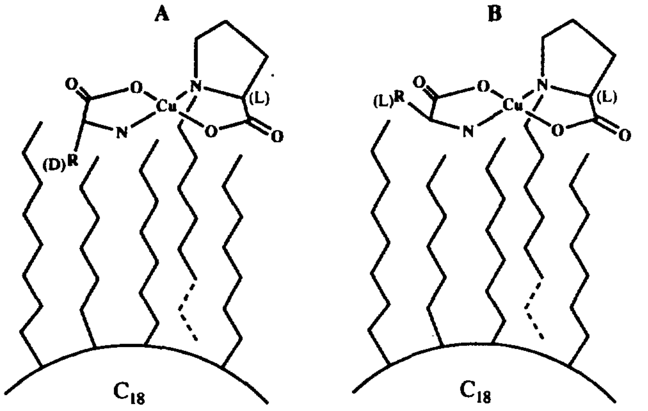


Figure 2. Structure of coated ternary complex containing N-alkyl-L-proline and D-amino acid with planar, *trans* chelate ring (A) L-amino acid with planar, *trans* chelate ring (B).

interactions between the side chain of amino acids and reversed phase column are critical in the retention and chiral recognition of enantiomers.

In conclusion, general retention behaviour using N-alkyl-L-proline is similar to that of N-alkyl-L-hydroxyproline. But the enantiomeric separation of the amino acids with a polar side chain by this system is better than that by the N-alkyl-L-Hydroxyproline coated system (α: Asn; 1.00, Thr; 1.02).[2] A separation mechanism capable of illustrating all the retention behaviours can be explained as follows:

First, the complexation process as shown in Eq. (1) is responsible for chiral recognition. The ternary complex plays a role in chiral recognition. The extent of the ternary complex formation affects the retention of the amino acid enantiomers. Second, It is believed that the mixed ligand complex is square planar[17,18] and has *trans* configuration[17,18] as shown in Figure 2. It can explain the retention behaviour that D forms are retained longer than L forms except His and Asn. Third, the retention is influenced by the hydrophobic interaction between the side chain of amino acids and the reversed phase column. To separate all of the different amino acid enantiomers, a various coating material may be used. For the separation of amino acid enantiomers with a polar side chain, the other chiral stationary phase with a greater enantioselectivity is required.

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