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N-hexadecay-L-proline이 코팅된 역상 원심 액체크로마토그래피에 의한 아미노산 이성질체의 분리

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Centrifugal Liquid Chromatography with Application of the N-hexadecyl-L-proline Coated Reversed Phase for Separation of Amino Acid Enantiomers

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요 약. 간단하고, 경제적이고, 다양한 응용이 가능한 시스템을 이용하여 거울상 이성질체를 분리 및 분취하였다. 소수성 아미노산 이성질체를 N-hexadecyl-L-proline이 코팅된 역상 원심 액체크로마토그래피로써 분리, 분취하였다. 아미노산 이성질체의 용리 및 분리에 영향을 주는 구리(II) 농도, pH, 이동상의 유기용매 종류 및 농도, 원심 액체크로마토그래피의 원형 컬럼의 회전속도 등에 대하여 연구하였다. 수 mg의 소수성 아미노산을 분취하였다. 모든 다른 아미노산을 분리 및 분취하려면, 다양한 코팅 리간드에 대하여 연구하여야 한다.

ABSTRACT. We prepared a simple, economic and versatile preparative system for an enantiomeric separation. Hydrophobic amino acid enantiomers were resolved in the preparative scale by a centrifugal liquid chromatography on the N-hexadecyl-L-proline coated reversed phase. The factors controlling the retention and resolution of racemic amino acids such as the concentration of Cu(II), pH of the eluent, the type and content of organic modifier, and rpm of CLC were examined. Several mg of hydrophobic amino acid enantiomers were separated preparatively. To separate all of different amino acid enantiomers, various coating material will be investigated.

INTRODUCTION

Separation of racemic amino acids by use of liquid chromatography has been of great interest in recent years. The common approach is the ligand exchange chromatography based on the formation of diastereomeric complexes between a metal ion and amino acids. As the analytical resolution of enantiomers becomes more common, demand for an analogous preparative sale separation is growing. Research on a preparative scale chiral separation is currently under way in several laboratories[1~5]. Many preparative separations may use specifically designed CSPs that have large selectivity factors, good reproducibility and good regeneration properties for a given separation. However the preparation of a specifically designed CSPs requires a considerable experience. In this sense, coated chiral stationary phase is an alternative to carry out a preparative scale separation. Chiral modification of commercially available high perfo-

rmance liquid chromatographic columns by adsorption of an appropriate chiral ligand was investigated in several laboratories[6~9]. One of these coated CSPs was prepared by Bernauer *et al.* very early[10,11]. These authors prepared the ion exchange resins saturated with optically active anionic complexes and used them as packings in the chromatography of racemates. Besides practical results, these papers are also of interest in the other respect: it was 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[12~19]. 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 the 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 the reversed phase column in HPLC[7]. 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 chiral agent on the hydrophobic interface layer of the reversed phase packing material. N-alkyl-L-histidine also have been used as a resolving agent[8]. Kimura *et al.*[20] applied this procedure to the preparation of the crown ether coated packings. Even L-phenylalanine was found[21] to adsorb from aqueous solutions on a reversed phase silica gel in quantities sufficient to transform the packing into a chiral ligand exchanger.

In this work, we prepared simple, analytical and preparative coated stationary phase systems that are composed of N-hexadecyl-L-proline coated reverse phase for liquid chromatography and centrifugal liquid chromatography (CLC), respectively. The new preparative scale system for the enantiomeric separation has a number of practical merit. First, CLC is a very convenient system to change packing materials and substitute the fixed chiral resolving ligand. Second, coated CSP remains stable for a period of 4 months by keeping the mobile phase with the organic modifier composition of less than 30%. Third, it is inexpensive to prepare coated CSP compared with purchasing a chiral column. Finally, it shows an excellent resolution for separation of amino acid enantiomers, especially the amino acids with a hydrophobic side chain. A specific objective of this work is the preparative enantiomeric separation of amino acids upto several mg with CLC.

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), methionine (Met) and threonine (Thr) were obtained from Sigma (St. Louis, Mo, USA). Hexadecyl bromide was 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. Octadecyl silica gel (C_{18}, Nucleosil, 10 μm) was obtained from Alltech (Deerfield, IL, USA).

Apparatus. Waters Model 510 pump was used to provide mobile phase flow. Model U6K injector was used. Waters Model 490E UV detector was employed to monitor a column effluent. The chromatograms were recorded with Shimadzu Model C-R4A data station. A centrifugal liquid chromatograph system consisting of Hitachi Model CLC-5 and Model 056 recorder was used to separate the amino acids for a preparative scale. 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) spectrophotomer (Bruker, WP80-SY, Germany) and elemental analyzer (EA, Carlo erba, 1106, Italy) were used to identify the chiral ligand which was synthesized. Free amino acids were detected at 254 nm since the species eluted were mostly in the form Cu(II) complexes having intense absorption bands in the UV and visible regions.

Preparation of chiral ligand. The ligand, N-hexadecyl-L-proline, was synthesized by treating L-proline with hexadecyl bromide under a basic condition. 60 m*l* water containing 5.61 g (0.1 *M*) potassium hydroxide and 11.51 g (0.1 *M*) L-proline, and 40 m*l* ethanol containing 0.12 *M* hexadecyl 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 hexadecyl bromide, the aqueous solution was extracted with ethyl ether. The ethyl ether solution was discarded. After vaporization of the water in the extracted aqueous solution, the residue was dissolved in dichloromethane and then the unreacted proline was precipitated. After 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 was 25%.

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 the organic modifier added.

Coating procedure of coated chiral stationary phase. Coating of the N-hexadecyl-L-proline on the reversed phase support was accomplished by following $10^{-4}M$ copper chelate in methanol through the column for about 1 hour, followed by conditioning with $10^{-4}M$ cupric acetate in MeOH/water (15/85, v/v) for more than 5 hours. To get optimum selectivity and retention time, the extent of coating was controlled by adjusting the time of coating. In general, the higher the concentration of loaded N-hexadecyl-L-proline, the lower the retention times of amino acids. Thus, an appropriate amount of coating was chosen by measuring the retention of an amino acid. After coating the N-hexadecyl-L-proline, 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-hexadecyl-L-proline remains stable for a period of about 4 months by using the mobile phase with the organic modifier composition of less than 30%.

CLC apparatus. In the centrifugal liquid chromatography, a separation disk was used in place of a column. As shown in *Fig.* 1, the separation disk is composed of an uppr disk made of a tempered glass with stainless steel rim, an under disk with a function required to fix the separation disk to the rotary shaft promptly, and a porous spacer with a function required to keep a certain space between uppr and under disk and to hold the packing materials but allow the eluent to flow.

Packing of reverse phase support of CLC. While the separation disk is rotated on the rotary

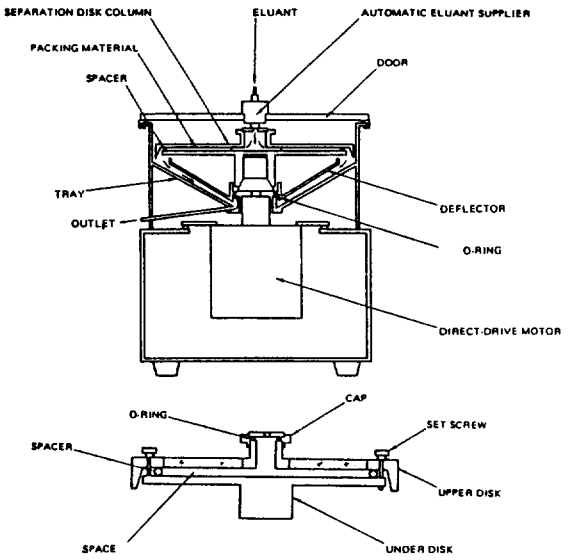


Fig. 1. Structure of CLC-5 and separation disk column.

shaft, the packing material prepared as a slurry of octadecylsilane in MeOH/water (50/50, v/v) is introduced from the central port of disk column. The packing material is separated from the solvent by a centrifugal force and filled into the space between upper and under disk, making a doughnut-like solid layer inward from the outer circumference. Solvent is discharged from the spacer to the outside of the separation disk. The slurry is repeatedly supplied to fill the space formerly occupied by the solvent, then the ideal column free of voids or chennels, which is commonly found in the conventional column chromatography, is formed in the space.

Measurement with CLC. The amino acid sample is introduced into the central port of the disk column through which eluent is continuously introduced with the rotation of the disk column. The sample component is separated into the doughnut-like rings as the solvent moves and is discharged successively from the disk. A chromatogram is obtained by the UV detection at 254 nm and any desired sample is collected by a fraction collector.

RESULTS AND DISCUSSION

On a given N-hexadecyl-L-proline coated reversed phase support, the interactions between the amino acids to be resolvedd and the stationary phase can be understood in terms of combination of complexation and hydrophobic interaction. The complex formation is considered as the dominant factor in the interaction process. 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 hyd-

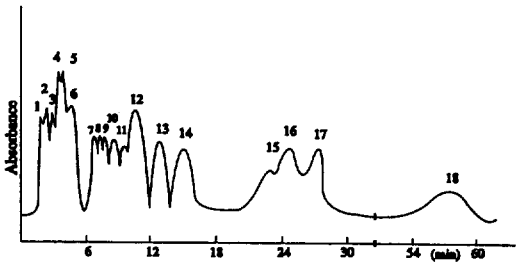
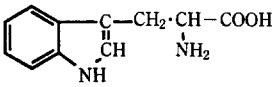
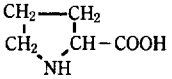


Fig. 2. Separation of 9 racemic amino acids on N-hexadecyl-L-proline coated reversed phase, C_{18} column in the HPLC. Mobile phase: $1.0\times10^{-4}M$ $Cu(ac)_2$ in MeOH/water of 15/85 (v/v) at pH 5.58. Flow rate: 1.0 ml/min. Detection: 254 nm. 10 μl of $1.0\times10^{-3}M$ amino acids was injected. 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.

Table 1. Representative capacity factor (k_L and k_D'), enantioselectivity (α) and resolution (R_S) of free amino acdis by LC

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
Isoleucine	Ile	$CH_3CH_2CH(CH_3)CH(NH_2COOH$	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
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
Methionine	Met	$CH_3S(CH_2)_2CH(NH_2)COOH$	2.61	5.81	2.23	2.83
Proline	Pro		0.69	3.55	5.15	2.86

The experimental conditions are the same as in *Fig.* 2.

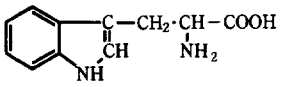
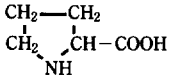
roorganic eluent, and extent of coating.

Liquid chromatograph. As shown in *Fig.* 2, the octadecyl column, coated with N-hexadecyl-L-proline, is effective enough to provide a separation of 9 racemic amino acids. The data in *Table* 1 and *Fig.* 2 indicate that the hydrophobic interations contribute much to the retention of mobile ligands. Tyr, Phe, Leu and Ile appear to be more retained relatively due to their greater hydrophobicity than the others. The elution orders of all D- and L- amino acids except His and Asn are consistent, L form eluting first. The enantioselectivity and resolution for amino acids with hydrophobic side chain are observed to be extremely high, thus permitting a preparative scale resolution. From these results, an application of centrifugal liquid chromatography could be considered for the enantiomeric separation of the amino acids.

Fig. 3. Separation of 5 racemic amino acids on N-hexadecyl-L-proline coated reversed phase, Nucleosil C_{18}, in the CLC. Mobile phase: $7.5\times10^{-4}M$ $Cu(ac)_2$ in MeOH/water of 15/85 (v/v) at pH 5.0. Flow rate: 5.0 ml/min. Detection: 254 nm. 1 ml of $2.0\times10^{-2}M$ amino acids was injected. 80 g of Nucleosil was loaded in the separation disk column using 3 mm spacer. Rotation speed: 1000 rpm. Elution sequence: 1. L-Pro, 2. L-Val, 3. L-Tyr, 4. D-Pro, 5. L-Ile, 6. D-Val, 7. L-Leu, 8. D-Tyr, 9. D-Leu, 10. D-Ile.

Centrifugal liquid chromatography. In view of the results of LC, the hydrophobic amino acid enantiomers can be resolved in the preparative scale by a centrifugal liquid chromatography on the N-hexadecyl-L-proline coated reversed phase. A typical chromatogram of the preparative separation of D- and L- amino acids on the N-hexadecyl-L-proline coated reversed phase in the centrifugal liquid chromatogrphy is shown in *Fig.* 3. The enantioselectivity and resolution of the hyrophobic amino acid enantiomers are sufficiently high, thus permitting prepartive scale resolution as shown in *Table* 2. The retention behaviours are the same

Table 2. Representative retention behaviours of free amino acids by CLC

Amino acid D L	Abbrev.	Structural formula	V_R(ML)	α	R_S
Alanine	Ala	$CH_3CH(NH_2)COOH$	114.0 106.0	1.08	0.14
Valine	Val	$(CH_3)_2CHCH(NH_2)COOH$	296.5 166.5	1.75	1.26
Leucine	Leu	$(CH_3)_2CHCH_2CH(NH_2)COOH$	612.0 300.0	2.04	1.66
Isoleucine	Ile	$CH_3CH_2CH(CH_3)CH(NH_2COOH$	660.0 282.0	2.34	1.99
Tyrosine	Tyr	$HOC_6H_4CH_2CH(NH_2)COOH$	370.0 237.0	1.56	1.02
Histidine	His		115.0	0.96 (1.04)	0.09
Methionine	Met	$CH_3S(CH_2)_2CH(NH_2)COOH$	318.0 224.0	1.42	0.70
Proline	Pro		251.0 133.0	1.89	1.37

The experimental conditions are the same as in *Fig.* 3.

Table 3. Retention values, enantioselectivity (α) and resolution (R_S) as the addition of the organic modifier at pH 5.5

Free-AA		ACN/H_2O : 5/95[a]			ACN/H_2O : 30/70[b]			MeOH/H_2O : 15/85[c]		
		V_R(ML)	α	R_S	V_R(ML)	α	R_S	V_R(ML)	α	R_S
Ala	D	110.4	1.08	0.11	83.2	1.22	0.17	105.5	1.06	0.06
	L	102.0			68.0			99.9		
Val	D	232.8	1.71	0.62	104.0	1.13	0.11	224.8	1.62	0.64
	L	136.2			93.6			100.8		
His	D	106.8	0.83	0.24	86.4	0.96	0.04	100.8	0.96	0.05
	L	128.4	(1.20)		89.6	(1.04)		105.5	(1.05)	
Met	D	252.0	1.39	0.41	102.4	1.07	0.06	247.9	1.41	0.45
	L	181.2			96.0			175.8		
Pro	D	160.8	1.46	0.44	96.4	1.12	0.11	187.8	1.71	0.77
	L	110.4			84.0			110.1		

Concentration of $Cu(ac)_2$: $10^{-3}M$. Flow rata: [a]6 ml/min, [b]3 ml/min, [c]4.6 ml/min. Other experimental conditions are the same as in *Fig.* 3.

Table 4. Retention values, enantioselectivity (α) and resolution (R_S) as a function of pH

Free-AA		4.0			5.0			6.0		
		V_R(ML)	α	R_S	V_R(ML)	α	R_S	V_R(ML)	α	R_S
Ala	D	92.5	1.03	0.05	116.0	1.02	0.02	125.5	1.05	0.06
	L	90.0			114.0			120.0		
Val	D	144.5	1.29	0.54	220.0	1.55	0.64	265/0	1.49	0.56
	L	112.0			142.0			178.0		
His	D	90.0	0.96	0.08	101.0	0.96	0.05	150.0	0.99	0.01
	L	94.0	(1.04)		105.0	(1.04)		151.0	(1.01)	
Met	D	174.0	1.29	0.56	230.0	1.41	0.54	280.0	1.33	0.53
	L	135.0			163.0			210.0		
Pro	D	139.0	1.37	0.61	178.0	1.59	0.67	228.0	1.50	0.59
	L	101.5			112.0			152.0		

Concentration of $Cu(ac)_2$: $10^{-3}M$. Other experimental conditions are the same as in *Fig.* 3.

as those in LC with CCSP. Resolution value of Val, Leu, Ile, Tyr and Pro is high enough for the preparative separation. These compounds are usually retained much longer so the amino acids with a hydrophillic side chain were chosen for the optimization of the preparative separation.

In order to determine the optimum eluent composition, the concentration of methanol and acetonitrile in the eluent was varied. As shown in *Table* 3, 15% methanol gives the better result. By modifing the mobile phase with the aqueous organic solvent, the chromatographic properties are varied. This indicates that the kind and composition of mobile phase are responsible for the retention and enantioselectivity. Thus it is necessary to control the solvent strength in order to obtain the desired retention behavior.

Variation of the capacity factor and the enantio-

Table 5. Retention values, enantioselectivity (α) and resolution (R_S) as a function of $Cu(ac)_2$

Free-AA		$2.0\times10^{-3}M$			$1.0\times10^{-3}M$			$7.5\times10^{-4}M$		
		V_R(ML)	α	R_S	V_R(ML)	α	R_S	V_R(ML)	α	R_S
Ala	D	101.0	1.03	0.06	116.0	1.02	0.02	114.0	1.08	0.14
	L	98.0			114.0			106.0		
Val	D	191.0	1.42	0.58	222.0	1.55	0.64	296.5	1.75	0.26
	L	135.0			142.0			166.5		
His	D	97.0	0.96	0.07	101.0	0.96	0.05	115.0	0.96	0.09
	L	101.0	(1.04)		105.0	(1.04)		120.0	(1.04)	
Met	D	205.0	1.34	0.46	230.0	1.41	0.54	318.0	1.42	0.70
	L	153.0			163.0			224.0		
Pro	D	161.0	1.39	0.53	178.0	1.59	0.67	251.0	1.89	1.37
	L	116.0			112.0			133.0		

pH 5.0. Other experimental conditions are the same as in *Fig.* 3.

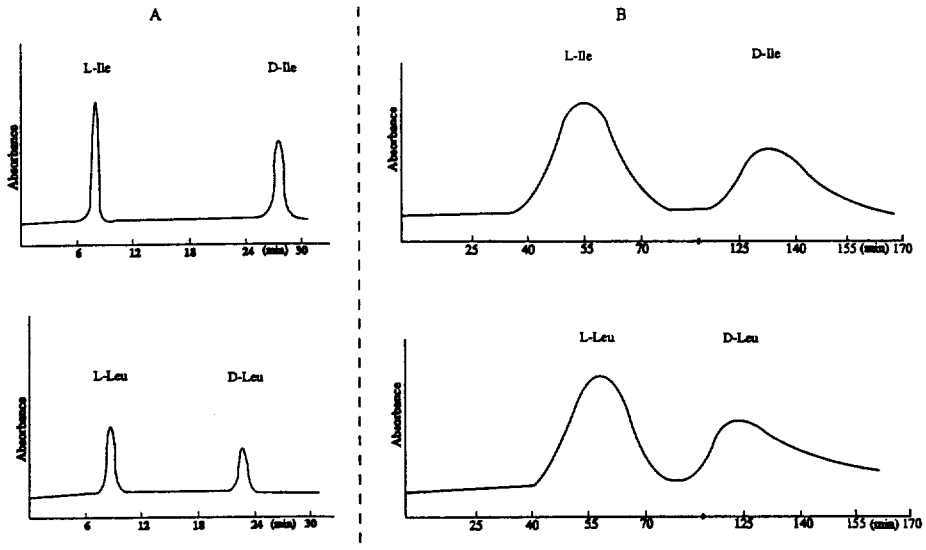


Fig. 4. Chromatograms showing analytical (A) and preparative (B) separation of D,L-Leu and D,L-Ile. A: 1.3 μg was injected. The experimental conditions are the same as in *Fig.* 2. B: 2.6 mg was injected. The experimental conditions are the same as in *Fig.* 3.

selectivity of the free amino acids with changing pH from 4.0 to 6.0 in the 15% methanol mobile phase are shown in *Table* 4. The optimum resolution and enantioselectivity is obtained at pH 5.0. Because the concentration of the ternary complex formation is increased with increase in pH, the retention increases with pH. But in higher pH, the chiral separation becomes worse due to the peak broadening and long retention.

Table 5 shows that the resolution and enantioselectivity also varied with Cu(II) concentration. It can be seen from the retention that the concentration of the ternary complex formation instead of the binary complex formation increases with decreasing Cu(II) concentration. The lower is the Cu(II) concentration, the higher are enantioselec-

tivity and resolution obtained. This also supports the idea that the chiral recognition occurs by the virtue of the ligand exchange reaction[12~19].

For the preparative scale separation, 80 mg of Nucleosil was loaded in the separation disk column using 3 mm spacer and the separation disk is rotated at speed of 1000 rpm. The system described demonstrated an excellent enantioselectivity in the preparative scale separation, as shown in *Table* 2. The potential of the preparative separation is investigated for the separation of the amino acid enantiomers. Separation of D,L-Ile and D,L-Leu is carried out up directly to a 2.6 mg injection through a disk column of CLC, as shown in *Fig.* 4. As expected, the resolution and the column efficiency by the column LC were better than those by the plane chromatography, CLC.

In conclusion, it was found that both ligand exchange reaction and hydrophobic interaction are responsible for the chiral recognition of the enantiomeric separation. CLC data demonstrate that the hydrophobic amino acid enantiomers can be resolved preparatively in CCSP mode by CLC with N-hexadecyl-L-proline, which is a simple, economic and versatile preparative system. With this system, we were able to resolve the hydrophobic amino acid enantiomers upto several milligrams with 3 mm spacer of disk column at rotation speed of 1000 rpm in CLC. To perform a larger scale preparative separation, it is only necessary to increase the thickness of spacer and power of rotatory motor. To separate all of different amino acid enantiomers, a various coating material may be used. For the preparative separation of amino acid enantiomers with a polar side chain, the other chiral stationary phase with a greater enantioselectivity is required. The coated CLC can be a versatile preparative system for the enantiomeric separation of amino acids.

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