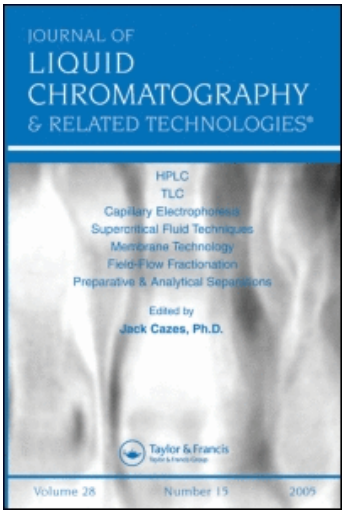

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UREA-FORMALDEHYDE RESIN MICROSPHERES AS A NEW PACKING MATERIAL FOR LIQUID CHROMATOGRAPHY: LIGAND EXCHANGE

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UREA-FORMALDEHYDE RESIN MICROSPHERES AS A NEW PACKING MATERIAL FOR LIQUID CHROMATOGRAPHY: LIGAND EXCHANGE

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ABSTRACT

A new ligand exchange chromatography (LEC) packing material consisting of L-proline modified urea-formaldehyde resin microspheres was prepared and tested. The experiments show that the material has a good enantioselectivity for the chiral separation of amino acid racemates. Solvent acetonitrile and high column temperature are favorable for enhancing the selectivity. There is an optimal ammonia concentration in the eluent for the LEC separation. Among the 17 DL-amino acids separated, 16 have the D-eluted ahead of the L-enantiomers, except for proline.

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INTRODUCTION

Packing materials play a key role in liquid chromatography. Many packing materials are proposed for modern liquid chromatography, in which silica, glass, dextran, agarose, polyacrylamide, polyhydroxyalkyl methacrylate, hydroxylated polyethers, and polystyrene are the most important. Almost all the commercial products are made from these materials. (1) In order to analyze more different substances, much faster, with higher efficiency and lower cost, new packing materials are always needed. Today, one more requirement that the material must be "green" is put forward. This means that the material does not contaminate the environment in any case, from preparing, putting into application, to being discarded as the solid waste. This new requirement is particularly important to large-scale chromatographic separation.

Urea-formaldehyde resin (UF) microspheres were first reported 12 y ago, and weak anion exchange packings were prepared to separate nucleotides and proteins. (2) In preparation, ammonium bifluoride was used to dissolve silica from the UF/silica composite microspheres. In our lab, the preparation was modified. The UF/ferric hydroxide composite microspheres were prepared and inorganic acids were used to wash out ferric hydroxide. The new method makes the preparation much easier and more "green."

Also, UF microspheres were found to have other advantages. Because of high crosslinking density, the microspheres kept constant volume in solvents. The microspheres can stand 1 MPa pressure per cm column length when packed into a column. Many derivatives can be obtained from reactions with the imino group of UF, which include almost all the modes of liquid chromatography.

Among the separation modes of liquid chromatography, ligand exchange chromatography (LEC) is an important technique, which mainly separates the racemic amino acids into the constituent enantiomers on both analytical and preparative scales. In this method, complexes of transition metal ions and enantiomeric molecules are formed. Chiral separation is a result of differences between the free energies of the intermediate diastereomeric complexes formed. Polystyrene, polyacrylamide, polymethacrylate, and silica gel modified with the chiral selector L-proline or L-hydroxyproline, are the main packing materials. (3) The L-amino acid modified silica gel offers high column efficiency and mechanical strength for fast analysis, but the material has low exchange capacity and lower chemical stability. The L-proline, or L-hydroxyproline modified low crosslinked "macronet isoporous" polystyrene, is a good packing material for LEC, which separates most of the racemic amino acids, but the hydrophobic backbone of the material decreases exchange rate and the low crosslinking density prohibits fast analysis. It is believed that the homogeneous size and hydrophilic polymer microspheres are a better choice for LEC.

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In this contribution, we introduce the L-proline via epichlorohydrin modified UF microspheres as a packing material for LEC. The UF and derivative microspheres were prepared. The new support was characterized. Separation conditions of the racemic amino acids are studied.

EXPERIMENTAL

Materials

All the chemicals were of analytical pure grade. Polystyrene standard samples for steric exclusion chromatography (SEC) were prepared at Institute of Chemistry, Academia Sinica. (4) In this article 24 DL-, 22 L-, and 8 D-amino acids were used for LEC. Most of the amino acids were made in China, a few (9 out of 54) were obtained from abroad. The manufacturers and addresses are listed in Table 1. All the amino acids were of biochemical standards grade.

Preparation of UF Microspheres

In 4000 mL of boiling water, a solution of 300 mL water and 80 g ferric chloride was added; a dark red colloid solution of ferric hydroxide was formed immediately. During cooling down of the colloid solution, (60 rpm) 40 g urea was added, with slow stirring, at 50°C. The acidity of the solution was adjusted to pH 2 with 2 N HCl; finally 80 mL formaldehyde (37%) was added at 30°C. The solution turned to turbid in 10 minutes. After being set for 5 hours, the composite microspheres of UF/ferric hydroxide were filtered out. The composite microspheres were washed with 4 N HCl at 80°C till the color of the microspheres changed from red to white; and the white microspheres were washed with water till the filtrate was neutral.

Elemental analysis (gas chromatography of oxidation or reduction products) gave: C 31.4%, H 5.7%, N 35.8%, and O 22.9%. SEM was performed for the microspheres (Figure 1). The average diameter, d, of the microspheres (10.2 μ) and the dispersion coefficient, ε, (0.207) were measured from the SEM micrograph. ε was defined as σ/d, where σ was the standard deviation of the diameter. The specific surface area was determined as 214 m^2/g by gas adsorption. The pore volume was obtained as 0.71 mL/g by water titration.

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Table 1. Sources of Amino Acids

Amino Acid	DL-form	L-form	D-form
1. asparagic acid (Asp)	KD*	KD	
2. glutamic acid (Glu)	KD	KD	Serva
3. serine (Ser)	BT	KD	Sigma
4. threonine (Thr)	BT	KD	IBC
5. tyrosine (Tyr)	IBC	KD	IBC
6. methionine (Met)	BT	KD	IBC
7. ethionine (Eth)	Fluka		
8. cystine (Cys-Cys)	IBC	KD	
9. alanine (Ala)	KD	KD	
10. valine (Val)	IBC	KD	IBC
11. norleucine (Nle)	Sigma	Sigma	IBC
12. isoleucine (Ile)	IBC	KD	
13. leucine (Leu)	KD	KD	IBC
14. phenylalanine (Phe)	Sigma	KD	
15. β-phenylalanine (β-Phe)	IBC		
16. asparagine (Asp)	KD	Aldrich	
17. glutamine (Glu)	IBC	KD	
18. tryptophan (Try)	BT	KD	
19. proline (Pro)	Aldrich	KD	
20. ornithine (Orn)	IBC	KD	
21. lysine (Lys)	IBC	KD	
22. citrulline (Cit)	KD	Sigma	
23. histidine (His)	IBC	KD	
24. arginine (Arg)	BT	KD	

*Aldrich = Aldrich, Milwaukee, WI, USA. BT = Baitai Co., Beijing, China. IBC = Institute of Biochemistry, Academia Sinica, Shanghai, China. KD = Kangda Co., Shanghai, China. Serva = Serva, Heidelberg, Germany. Sigma = Sigma, St. Louis, MO, USA.

Preparation of L-Proline Modified UF Microspheres

One mL concentrated sulfuric acid was added to a mixture of 10 g UF microspheres, 50 mL dioxane, and 30 mL epichlorohydrin. After the reaction proceeded at 95°C for 24 hours with stirring, the microspheres were filtered out and washed 3 times with dioxane. The elemental analysis (Hg(II) titration) gave Cl 0.97%, i.e., 0.27 mmol/g.

The obtained microspheres were again added into a mixture of 40 mL dioxane, 40 mL water, 3 g sodium hydroxide, and 3 g L-proline. After the reaction

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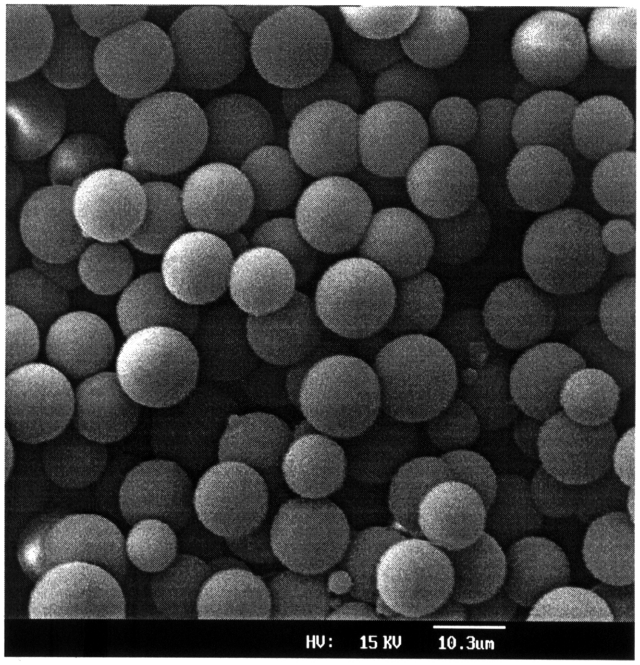

Figure 1. SEM micrograph of UF microspheres.

proceeded with stirring, at 55°C for 24 hours, the final microspheres were filtered out and washed with water till the filtrate was neutral. The proline content was measured as follows. The clean L-proline modified UF microspheres were placed in a 2% copper acetate solution. After 1 day, the blue microspheres were filtered out, washed with water till the filtrate was colorless, and dried in a vacuum oven at 50°C. 200 mg dry blue microspheres were placed in 10 mL 4 N HCl and dissolved into a blue solution at the boiling temperature. Cu(II), i.e., the proline content was determined by using a UV photometer (Model 752, Shanghai Precise Scientific Instrument Co. Ltd., Shanghai, China) at 620 nm as 0.28 mmol/g.

Liquid Chromatography Experiments

The UF microspheres were packed into a column of 10 mm i.d. × 300 mm at 30 MPa pressure with tetrahydrofuran (THF) as the eluent. A Shimadzu 3 A LC apparatus with a refractive index (RI) detector was used for SEC measurement.

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The SEC calibration curve was obtained with polystyrene standard samples at 1 mL/min and room temperature, with the column back pressure 1 MPa.

The L-proline modified microspheres were placed in 100 mL of 2% copper acetate aqueous solution for 1 day in order to form the bonded L-proline-Cu(II) complex. On the second day, the microspheres were packed into a column of 4 mm i.d. × 250 mm at 25 MPa with water as the eluent. A Shimadzu 3 A LC apparatus with a UV254 detector was used for LEC measurement. Samples (20 μL of 0.1% solution) were injected onto the column using a Rheodyne 7725 injector. For the LEC experiments, the flow rate was 0.2 mL/min and the column back pressure was 8 MPa (30°C) and 5 MPa (60°C). DL-amino acids were used to examine the separation capacity of the new packing material. L- and D-amino acids were used to determine the elution order of the two separated enantiomers. The eluents were 0, 0.1, and 0.3 M ammonium hydroxide (NH_4OH) solutions with 0.1, 0.033, and 0.1 mM copper acetate ($Cu(OAc)_2$), respectively. Some experiments were run at two temperatures (30° and 60°C), or with 30 V% acetonitrile (MeCN), or without $Cu(OAc)_2$ in the eluent.

RESULTS AND DISCUSSION

Preparation of Microspheres

UF Microspheres

The preparation of UF/ferric hydroxide composite microspheres is greatly influenced by temperature and acidity of the reaction mixture. In this work, the reaction condition is chosen as 30°C and pH 2. Higher temperature and lower pH value will result in a fast reaction. Often, small and size-inhomogeneous microspheres are obtained in a slow reaction. With the cylinder model of pores, the mean pore size diameter (D) is calculated from the specific surface area (S) and pore volume (V_p) as: $D = 4V_p/S = 131$ angstroms. This is roughly in agreement with the SEC result. The SEC calibration curve (Fig. 2) shows that the microspheres can separate molecules of molecular weight (MW) $100–1 \times 10^5$, which indicates a proper pore size as a packing material.

In the reaction, 1 mole of urea reacts with 1.5 moles of formaldehyde; therefore, almost 1 repeat unit of UF main chain has 1 $-CH_2$-crosslinking point. There should be 1 spare imino group left for further modification. The calculated composition is: C 38.5, H 5.1, N 35.9, and O 20.5 in weight percentage. Compared with the composition determined, we have fewer C (31.4) and more O (22.9). This is because the practical reaction is complicated; there are still $-NH-CH_2-O-CH_2-NH-$ in the UF main chain and $-CH_2OH$ as the end group. (5)

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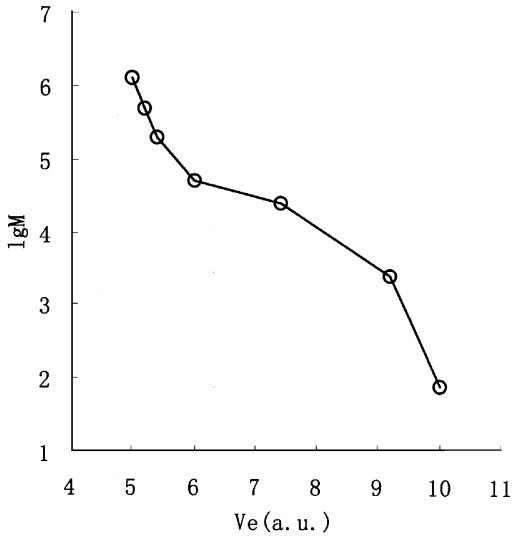
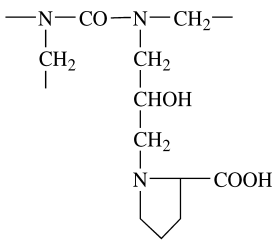


Figure 2. Steric exclusion chromatography calibration curve of UF microspheres column.

L-Proline Modified Microspheres

The preparation follows a literature paper, (6) in which L-proline via epichlorohydrin modified poly(N-2-hydroxylethyl acrylamide) was prepared. With concentrated sulfuric acid as the catalyst, the epoxy group of epichlorohydrin reacts with the imino group of UF resin. In the second reaction, chlorine of epichlorohydrin reacts with the imino group of proline in the basic environment. In the two reactions, excess epichlorohydrin and proline are used. The second reaction is controlled at 55°C, because the higher reaction temperature will induce side reactions.

The final structure is:



If 1 proline molecule per repeat unit of UF is obtained, the proline content should be 4.0 mmol/g. In practice, the obtained proline content is 0.28 mmol/g.

This is because the microspheres have macropores, which are formed from agglomeration of the small UF particles. The high crosslinking density prohibits the imino groups inside the small particles from reacting with epichlorohydrin. So, only 0.27 mmol/g Cl has been found. The reaction of chlorine with proline is complete, which results in the proline content 0.28 mmol/g. It was reported that L-proline content was 0.3 mmol/g for a modified polymethacrylate sorbent (Separon H1000, 10–14 μm). (7)

Ligand Exchange Chromatography

LEC experiments are run at 5 different elution conditions: (1) eluent 0.1 M $NH_4OH + 0.033$ mM $Cu(OAc)_2$ with 30 V% MeCN at 30°C; (2) eluent 1 without MeCN at 30°C; (3) eluent 2 at 60°C; (4) eluent 0.3 M $NH_4OH + 0.1$ mM $Cu(OAc)_2$ with 30 V% MeCN at 30°C; and (5) eluent 4 without MeCN at 30°C. The elution order of the separated enantiomers is determined by comparing the peaks of L-, D-, or DL-mixed with L-amino acids. Table 2 lists the chromatographic results. The enantioselectivity, α is defined as $\alpha = (V_2 - V_0)/(V_1 - V_0)$, where V_1 and V_2 are the retention volume of the two resolved enantiomers, $V_2 > V_1$. V_0 is the retention volume of acetone on the column.

We also tried eluents 0.1 mM $Cu(OAc)_2$/MeCN and H_2O/MeCN (both 70/30, V/V) at 30°C. In the first case, among the 24 DL-amino acids, only a few (Phe, β-Phe and His) were separated with long and changeable retention volumes, others were either not separated (Eth, Nle, Orn, Lys, and Arg) or adsorbed on the column. In the second case, all the DL-amino acids (Asp, Glu, Thr, Nle, Lys, Cyt, and His) injected were adsorbed on the column; no peaks were observed.

Solvent

In this work, the solvent MeCN effect on chiral separation is observed. The eluent 0.1 M $NH_4OH + 0.033$ mM $Cu(OAc)_2$ with 30 V% MeCN resolves 18 DL-amino acids at 30°C, only 2 acidic (Asp and Glu), 1 sulfur containing neutral (Cys-Cys), and 3 other neutral (Ala, Leu, and Gln) are not resolved. The same eluent without MeCN only resolves 8 racemic amino acids. The same trend can be seen with the eluent 0.3 M $NH_4OH + 0.1$ mM $Cu(OAc)_2$, with and without MeCN. Figure 3 is the chromatogram of the DL-amino acids that have baseline resolution on the column.

In general, larger enantioselectivity, α and retention volume are obtained with MeCN in the eluent. This indicates that the solvent stabilizes the coordinate complex of Cu(II) with the selector and analyte amino acids, and the initially stable diastereomeric complex (retention volume) gains more with this

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Table 2. Chiral Separations of Amino Acids

	0.1 M NH_4OH+0.033 mM $Cu(OAc)_2$									0.3 M NH_4OH+0.1 mM $Cu(OAc)_2$					
	+30v%MeCN, 30°C			30°C			60°C			+30v%MeCN, 30°C			30°C		
Amino Acid	V_1	V_2	α	V_1	V_2	α	V_1	V_2	α	V_1	V_2	α	V_1	V_2	α
1. OH-containing															
Ser	18.6	23	1.28				7	8	1.24						
Thr	16.4	19	1.19				6.4	8.4	1.22						
Tyr	22.2	34	1.60	6.2	7.8	1.47	12.4	18.8	1.67	7	9.4	1.55	4.4	5.2	1.50
2. S-containing															
Met	14.8	16.6	1.15												
Eth	16.2	18.2	1.15												
3. Neutral															
Val	20.4	26.6	1.35	12.2	13.2	1.11	7.4	8.2	1.17						
Nle	23.8	27.8	1.19							11.2	12	1.10			
Ile	23.2	29.2	1.29	9.6	10.6	1.15				11	12.2	1.14	5.4	6	1.23
Phe	23	36.6	1.67	22.2	27.6	1.28	16.4	22.2	1.43	10.8	14	1.39			
β-Phe	14	20.4	1.56	22	28	1.31	18.4	22.8	1.28	10.6	13.6	1.38			
Asn	16.6	19.4	1.20				6.4	7.4	1.28						
Trp	28	58.4	2.20							20.4	32	1.65	20.4	24.2	1.22
Pro	29.4	38.4	1.34	15.8	21.4	1.43	15.2	20	1.39	16.2	21	1.35	7.4	8.4	1.22
4. Basic															
Orn	14	15.2	1.11												
Lys	25	29	1.18				9.6	10.4	1.12						
Cyt	15.6	17.8	1.17				8.2	9	1.15						
His	27.4	54.6	2.10	20.2	39.4	2.10	16.2	28	1.77	23.4	34.6	1.54	13.2	19.2	1.58
Arg	21.2	24.4	1.17	13.4	14.2	1.08	12.4	13.6	1.13						
Acetone (V_0)		2.6			2.8			2.8			2.6			2.8	

Note: Retention volume V_1, V_2 and V_0: mL.

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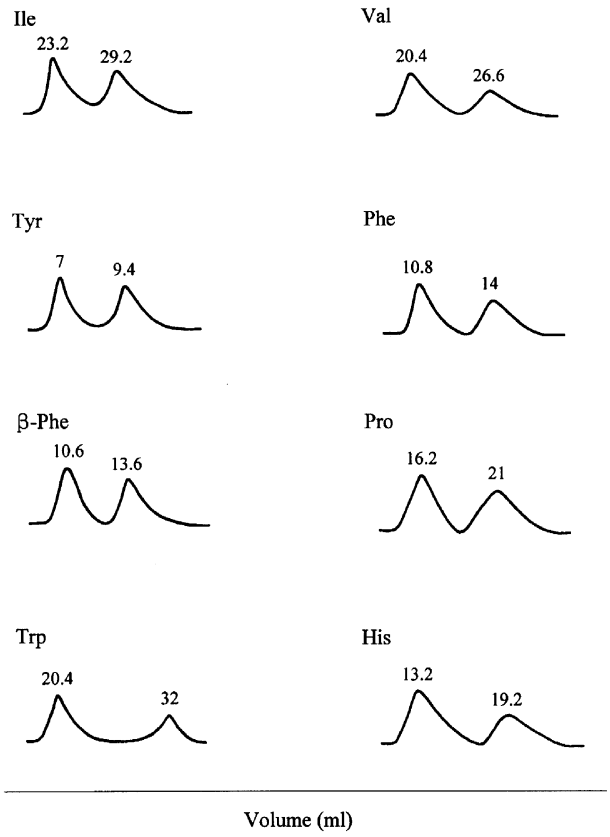

Figure 3. Separation of DL-amino acids. Column 4 mm i.d. × 250 mm, detector UV254, flow rate 0.2 mL/min, column temperature 30°C. Ile and Val: eluent 0.1 M $NH_4OH + 0.033$ mM $Cu(OAc)_2$/MeCN (70/30 V/V), Tyr, Phe, β-Phe, Trp, and Pro: eluent 0.3 M $NH_4OH + 0.1$ mM $Cu(OAc)_2$/MeCN (70/30 V/V), His: eluent 0.3 M $NH_4OH + 0.1$ mM $Cu(OAc)_2$. The retention volumes are marked on the peaks.

stabilization. The same phenomenon of retention volume increasing with solvent was reported and attributed to the dominance of polar and complex forming interactions under the conditions. (8)

In a review article, Davankov mentioned that ligand exchange took place faster in MeCN; the addition of MeCN and other polar modifiers might prove useful in enhancing the efficiency in chiral LEC. (9) In this contribution, we give an example that shows MeCN is useful in enhancing the selectivity.

Ammonia, Water, and Copper(II) Ion

The NH_3 effect is also observed in this work. It is the exchange of the analyte amino acid with the coordinated NH_3 in the Cu(II) complex that makes the chiral separation. There is an optimal NH_4OH concentration for this exchange. With the L-proline modified UF column 0.1 M NH_4OH+0.033 mM $Cu(OAc)_2$ is better than 0.3 M NH_4OH+0.1 mM $Cu(OAc)_2$. In the latter case, the retention volume and selectivity decreases. The same trend was reported and explained by competition of amino acid and ammonia on forming mixed adsorption complex. (8) It was reported, that on the L-proline modified low crosslinked polystyrene column, most of the DL-amino acids were resolved with 0.1 M and 0.3 M NH_4OH, but the basic amino acids with 1.5 M NH_4OH. (10) This indicates that the base matrix has an important role in determining the proper NH_4OH concentration of the eluent.

Without NH_3 the initial Cu(II) complex has 2 water molecules coordinated. It is the exchange of the analyte amino acid with the coordinated water that makes a chiral separation. This exchange does happen in some chiral mobile phases (11) and chiral stationary phase. (12) However, this is not our case. With the eluent 0.1 mM $Cu(OAc)_2$/MeCN (70/30, V/V), only a few DL-amino acids (Phe, β-Phe, and His) are resolved on our column and the retention volumes are large and changeable. The long retention of the amino acid solutes is caused by the fact that the amino acids easily replace water molecules from the Cu(II) coordination sites, but there are no species in the system that are able to bring the solutes back into the mobile phase.

It is difficult to keep the column stable without Cu(II) in the eluent, because copper is gradually eliminated from the column. It was reported that no separation of enantiomers was observed when Cu(II) was omitted from eluents under otherwise identical conditions. (8,13) In our experiment with the eluent H_2O/MeCN (70/30, V/V), all the amino acids injected are evenly adsorbed on the column.

Column Temperature

The column temperature also has an effect on the chiral separation. The eluent 0.1 M NH_4OH+0.033 mM Cu(OAc) resolves 8 DL-amino acids at 30°C, but 12 at 60°C. 7 of the 8 amino acids resolved at 30°C are also resolved at 60°C (Table 2). And for these 7 amino acids, in most cases the retention volume decreases but the enantioselectivity only has a little change. It seems that increasing column temperature makes the Cu(II) complexes with D- or L-amino acids less stable to the same extent.

Because of the low exchange rate, LEC usually has low column efficiency. The shape of the enantiomeric peaks is also poor. Increasing column temperature increases the ligand exchange rate and, thus, improves the column efficiency(9) and the peak shape. This can cause a better peak resolution, simulating an improved selectivity.

Elution Order

The elution order of the separated amino acid racemates is determined, except β-Phe. All the second peaks are L-amino acids except proline. The same elution order was already reported on silica, (14,15) polymethacrylate, (7) and polyacrylamide (16) columns, all having the epoxy spacer and L-proline or L-hydroxyproline selector. This is explained by Davankov, as a general rule for all the L-pro type stationary ligands having, in the N-substituent, an additional functional (particularly hydroxy) group, which is capable of coordinating in the axial position of the central Cu(II) ion. (17) The hydroxy group hinders the rotation of the R-group (around the Cα-Cβ axis) of the sorbed D-amino acids, thus diminishing the retention of the latter as compared with the L-enantiomers. The exceptional behavior of proline is explained in terms of the inability of its cyclic substituent R to rotate around the single bond to the α-carbon atom.

CONCLUSIONS

The L-proline via epichlorohydrin modified UF microspheres column has a good enantioselectivity for the chiral separation of amino acids. 18 DL-amino acids are resolved at one elution condition: 0.1 M NH_4OH + 0.033 mM $Cu(OAc)_2$ with 30 V% MeCN at 30°C. Increasing the MeCN content up to 30 V% and the column temperature up to 60°C increases the column selectivity. There is an optimal NH_4OH concentration (0.1 M) in the eluent for the LEC separation. Among the 17 DL-amino acids separated, 16 have the D-enantiomers eluted ahead of L-enantiomers, except proline.

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