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OPTICAL RESOLUTION OF AMINO ACIDS, PEPTIDES AND HYDROXYCARBOXYLIC ACIDS USING A NEW CHIRAL COLUMN FOR LIGAND-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The enantiometric resolution of amino acids, amino acid derivatives, peptides and hydroxycarboxylic acids was investigated using a chiral column of the ligand-exchange type. It was found that this column separated all of these compounds satisfactorily. Columns containing either a D- or L-ligand gave almost identical chromatograms, except that the retention order for a pair of chiral isomers was reversed. These columns could be applied to resolve simultaneously ten different types of DL-amino acids. Optically active impurities present in D and L-amino acids could be determined at concentrations down to about 100 ppm.

INTRODUCTION

In recent years, increasing attention has been paid to the development of easier ways of resolving enantiomers, especially using high-performance liquid chromatographic (HPLC) methods. Some of the more important techniques are the use of a stationary phase carrying an optically active compound[1–5], the preparation of a derivative of a diastereomer before resolution[6–8] and the use of a mobile phase containing an optically active compound[9–12].

Each of these approaches has problems, but recently Shinbo *et al.*[5] developed a high-performance resolution column for DL-amino acids. In this column, a crown compound is adsorbed on an octadecylsilyl (ODS)-silica. Although it is silica-based, this column must depend on a strongly acidic eluent, such as perchloric acid. Also, it is ineffective for many DL-amino acids unless it is operated at low temperatures, such as 0°C. In addition, it is unsuitable for simultaneous resolution, because of the similar retention times of these amino acids. Nimura and co-workers[6–8] attempted simultaneous resolution using an ODS column. This approach follows a pre-column method, in which a compound such as 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) is used. It is suitable for simultaneous resolution, but has several disadvantages: some amino acids do not react to form derivatives, and it requires a time-consuming pretreatment process. Methods in which an optically active compound is

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used are unsuitable for routine analyses and preparations, because the compound is expensive for these purposes.

Using an MCI GEL CRS10W column (N,N-dioctyl-L-alanine-coated, ODS-silica), effective enantiometric resolution of DL-amino acids was obtained under mild operating conditions, and this column does not suffer from the above problems. One of its noteworthy features is that it eliminates pretreatment steps. In this study, we prepared a CRS10WD column, which was the same as the CRS10W column except that the ligand was N,N-dioctyl-D-alanine. We assessed its performance in detail. Optical impurities present in L- and D-amino acids were determined using both the CRS10W and CRS10WD columns. The enantiometric resolution of compounds other than amino acids, such as peptides, amino acid derivatives and hydroxycarboxylic acids, was also investigated. It was shown that the new column can resolve enantiomeric compounds other than amino acids.

EXPERIMENTAL

DL-Amino acids, D-amino acids, L-amino acids, amino acid derivatives, peptides and hydroxycarboxylic acids were supplied by Sigma (St. Louis, MO, U.S.A.) and Tokyo Kasei (Tokyo, Japan). All the other reagents were of special grade, supplied by Wako (Osaka, Japan).

The packed columns used were MCI GEL CRS10W and CRS10WD (a provisional name), both 50 mm × 4.6 mm I.D., supplied by Mitsubishi Kasei (Tokyo, Japan). CRS10WD is a newly developed column, differing from the former in its optical characteristics. The active ligand, N,N-dioctyl-D-alanine, is coated on ODS-silica (pore diameter 100 Å, particle size 3 μm).

The apparatus consists of a pump (Model LC-6A; Shimadzu, Kyoto, Japan), a sample injector (Model 7125; Rheodyne, Cotati, CA, U.S.A.) and a detector (Model SPD-6A; Shimadzu). Fluorimetric trace analysis was based on the post-column method using *o*-phthalaldehyde (OPA). A Model RF530 instrument (by Shimadzu) was used to detect fluorescence.

RESULTS AND DISCUSSION

Basic properties

The enantiometric resolution of DL-amino acids on MCI GEL CRS10W is based on the ligand-exchange reactions of a copper complex of an optically active ligand adsorbed on the column matrix with the amino acid to be separated. The resolution, therefore, is affected by various parameters, including eluent pH, copper (II) ion concentration and column temperature. The effects of these parameters have been described elsewhere[13]. Different copper (II) salts were screened as eluents with respect to the effects of the various anions on the resolution of amino acids. Fig. 1 shows the relationship among the anion species, retention time and resolution. Different amino acids were affected differently according to the anion species used. The retention time changed markedly with acidic amino acids, such as glutamic acid, whereas it changed little with neutral amino acids, such as valine. The resolution was also greatly affected by the anion species, which indicates the possibility of controlling the resolution by varying the anion.

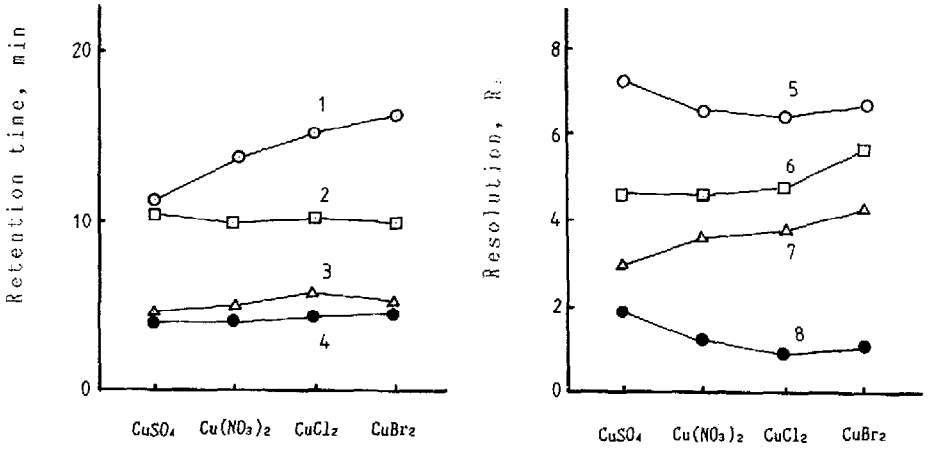

Fig. 1. Effect of salts on retention time and resolution. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* aqueous Cu salt solution; flow-rate, 1 ml/min; pressure, 110 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl. (1) L-Glu; (2) L-Leu; (3) L-Asp; (4) L-Val; (5) DL-Leu; (6) DL-Val; (7) DL-Glu; (8) DL-Asp.

Of the copper (II) salts screened, copper sulphate had the shortest retention time and was not deliquescent. Therefore, an aqueous solution of copper sulphate was used as the eluent in the measurements after the screening study. Fig. 2 shows the relationship between load and resolution for DL-valine, having a resolution, R_s, of about 6. Resolution was achieved, but without a considerable decrease in resolution, R_s, as long as the amount separated was 10 μg or less. For an amount of 0.1 mg separated, R_s was still fairly high (2.7). It is therefore possible to use the 50 mm × 4.6 mm I.D. column for preparative purposes.

MCI GEL CRS10W works effectively with an aqueous solution contained an organic solvent as the eluent. Acetonitrile and ethanol gave better chromatograms than methanol (Fig. 3). Fig. 4 shows the relationships among acetonitrile concentration, k' and R_s values for the resolution of DL-phenylalanine. An increase in acetonitrile concentration accelerated elution, sharpened the signals and reduced gradually

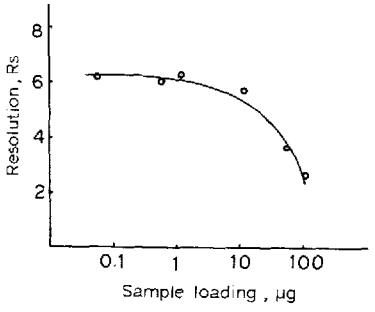

Fig. 2. Effect of sample throughput on resolution. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; pressure, 100 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl; sample, DL-valine.

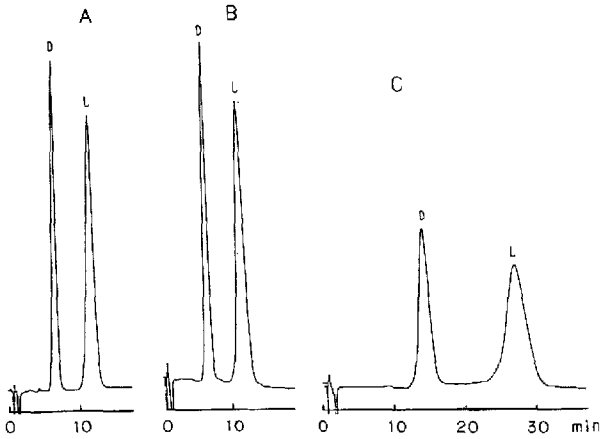

Fig. 3. Effect of addition of organic solvent to the eluent on glycyl-DL-leucine resolution. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* $CuSO_4$ in (A) 10% acetonitrile solution, (B) 10% ethanol solution and (C) 10% methanol solution; flow-rate, 1 ml/min; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl; sample, glycyl-DL-leucine (1 mg/ml).

R_s. Similar results were observed with other compounds. Adding an organic solvent, such as acetonitrile, greatly shortened the retention time of hydrophobic compounds, as discussed above, but its concentration should be limited to 15 vol.-%, because the optically active ligand may be eluted out in the presence of excessive amounts of organic solvent.

Determination of impurities in enantiomers

The analysis of a trace component that elutes after the main component is difficult[14], and a column of reversed activity that reverses the elution order is used to solve this problem. For DL-amino acids with low R_s, it is possible to determine a

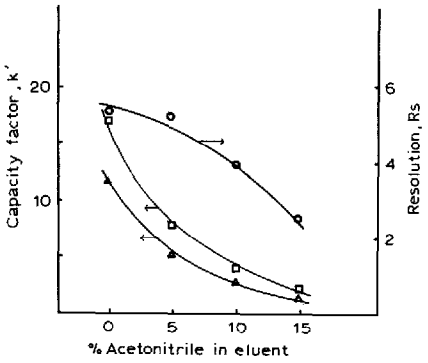

Fig. 4. Effect of acetonitrile content on capacity factor and resolution of DL-phenylalanine. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 2 m*M* $CuSO_4$–acetonitrile solution; flow-rate, 1 ml/min; pressure, 95 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl; sample, DL-phenylalanine (1 mg/ml).

TABLE I

OPERATING CONDITIONS FOR MCI GEL CRS10WD AND RESOLUTION OF AMINO ACIDS

Column temperature: room temperature.

Amino acid	*Eluent $CuSO_4$ concentration (mM)*	*Flow-rate (ml/min)*	*Retention time (min)*		*Separation factor, α*	*Resolution, R_s*
			L-isomer	*D-isomer*		
Lys	0.1	0.5	3.0	3.5	1.17	1.0
His	0.5	0.5	4.1	5.4	1.31	1.4
Ser	0.5	0.5	5.7	7.0	1.22	0.9
Thr	0.5	0.5	5.9	7.6	1.29	1.2
Ala	1.0	0.5	4.3	5.5	1.29	1.2
Cit	1.0	0.5	7.0	10.4	1.49	2.9
Pro	2.0	1.0	3.5	6.7	1.91	5.0
Val	2.0	1.0	4.3	8.0	1.85	5.8
Nval	2.0	1.0	5.3	10.2	1.92	5.7
Asp	2.0	1.0	7.0	8.4	1.20	1.7
Glu	2.0	1.0	15.0	22.7	1.55	4.6
Ileu	2.0	1.0	10.8	22.9	2.13	7.7
Ileu (*allo*)	2.0	1.0	8.4	16.4	1.95	6.6
Leu	2.0	1.0	10.0	19.1	1.91	7.1
Nleu	2.0	1.0	15.2	22.0	1.45	3.0
Met	2.0	1.0	8.7	13.9	1.59	5.0
Tyr	2.0	1.0	16.5	29.8	1.80	6.5
Eth	2.0	1.0	20.8	34.5	1.65	5.4
Phe	2.0	1.0	28.8	52.0	1.81	5.0

D-amino acid impurity present in L-amino acids using CRS10W, but the reverse is difficult using same column. Recently, the determination of D-isomer/L-isomer ratios and trace enantiomers has attracted attention because of their potential for dating[15] and analysis of food production processes. The authors have developed and assessed a packed column that uses an optically active ligand with the reverse optical activity for these purposes.

Table I shows the analytical conditions under which the CRS10WD column

TABLE II

DETERMINATION OF IMPURITIES IN OPTICAL ENANTIOMERS

Amino acid	*Column*	*Manufacturer*	*Concentration of optical impurity (ppm)*
L-Alanine	CRS10W	A	160
L-Alanine	CRS10W	B	80
L-Alanine	CRS10W	C	350
D-Alanine	CRS10WD	C	910
L-Aspartic acid	CRS10W	C	5700
D-Aspartic acid	CRS10WD	C	4750
L-Phenylalanine	CRS10W	C	650
D-Phenylalanine	CRS10WD	C	800

was operated and the resolution results for each amino acid. These columns were used to determine enantiomer impurities present in some L- and D-amino acids. The measurements were based on OPA fluorimetry (post-column method).

The impurities were determined by the standard addition method. The results are given in Table II. The L-alanine samples from different suppliers contained different amounts of impurities. Further, different amino acids had widely varying impurities. Because of this, great care must be taken when these amino acid products are used for analysis. The detection limit will correspond to that of OPA fluorimetry for a compound having a high resolution, and will reach about 100 ppm for a DL-amino acid having a resolution of 1.5–2.

Resolution of DL-*amino acid mixtures*

CRS10WD gives widely varying retention times, depending on the type of amino acid being separated, as shown in Table I. Hence it is possible to effect the resolution of a number of amino acids simultaneously using a single CRS10WD column. Figs. 5 and 6 illustrate examples of simultaneous resolution, in each instance ten different DL-amino acids being separated with a single 50-mm column by isocratic

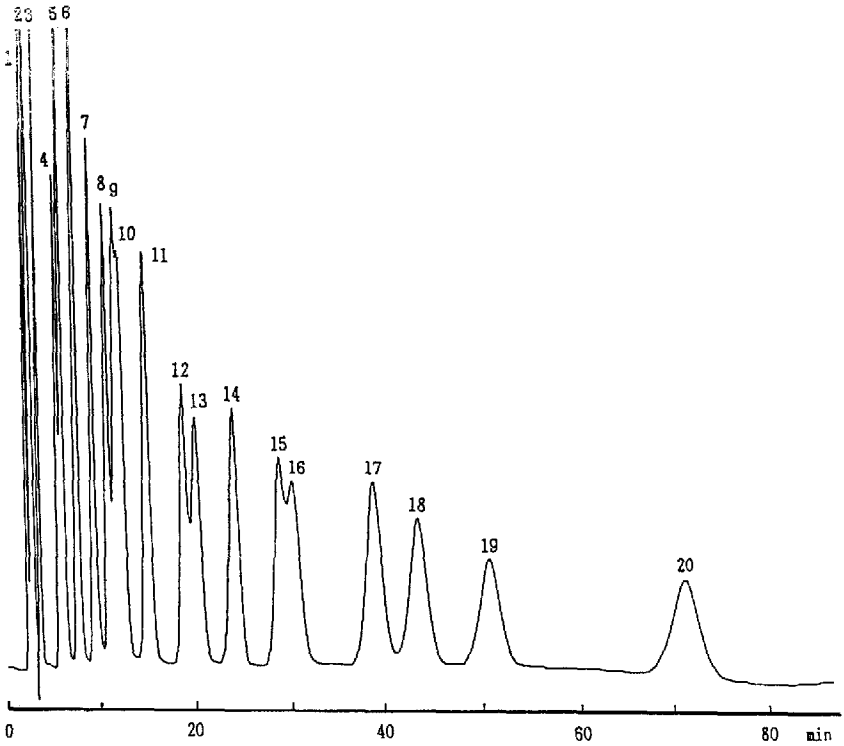

Fig. 5. Resolution of ten amino acid racemates using an MCI GEL CRS10WD column. Column, MCI GEL CRS10WD (50 mm × 4.6 mm I.D.); eluent, 1 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; pressure, 110 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl. Sample: 1, L-Ser; 2, D-Ser; 3, L-Cit; 4, D-Cit; 5, L-Val; 6, L-Nval; 7, L-Asp; 8, D-Asp; 9, D-Val; 10, L-Met; 11, D-Nval; 12, L-Glu; 13, D-Met; 14, L-Tyr; 15, D-Glu; 16, L-Eth; 17, L-Phe; 18, D-Tyr; 19, D-Eth; 20, D-Phe.

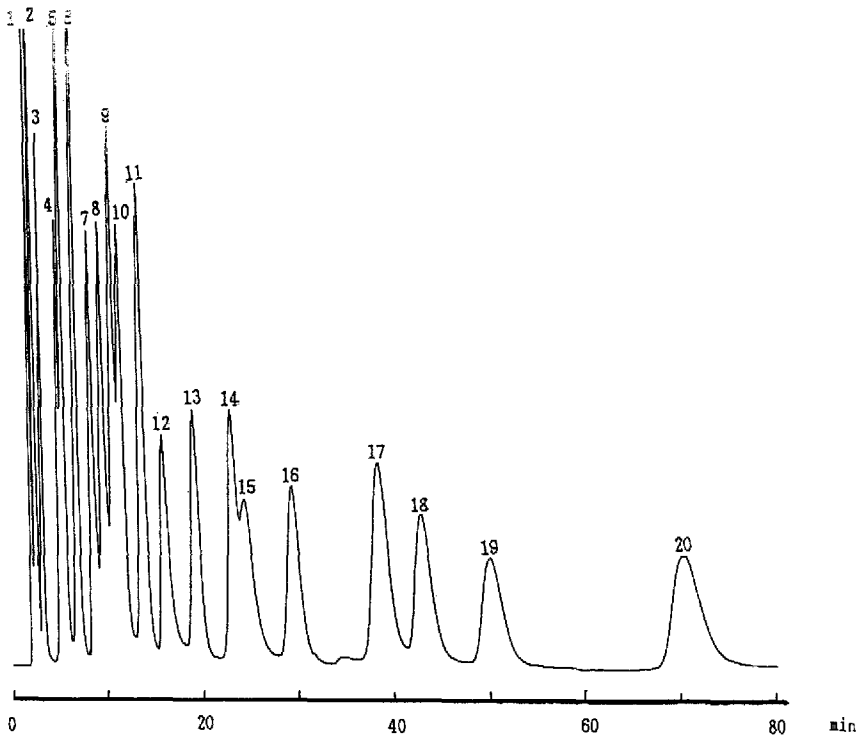

Fig. 6. Resolution of ten amino acid racemates using an MCI GEL CRS10W column. Column: MCI GEL CRS10W (50 mm × 4.6 mm I.D.); eluent, 1 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; pressure, 113 kg/cm^2; temperature, room temperature; detection, UV (254 nm); injection volume, 20 μl. Sample: 1, D-Ser; 2, L-Ser, 3, D-Cit; 4, L-Cit; 5, D-Val; 6, D-Nval; 7, D-Asp; 8, L-Asp; 9, L-Val; 10, D-Met; 11, L-Nval; 12, D-Glu; 13, L-Met; 14, D-Tyr; 15, L-Glu; 16, D-Eth; 17, D-Phe; 18, L-Tyr; 19, L-Eth; 20, L-Phe.

elution. CRS10W gave similar chromatograms except that the elution order of the D- and L-isomers was reversed.

Applicability of CRS10W to enantiomers other than amino acids

The applicability of CRS10W to enantiomers other than amino acids was also investigated. The results are shown in Table III. Of amino acid derivatives, N-acetyl derivatives, such as N-acetyl-DL-alanine, DL-leucine,-DL-tryptophan and DL-valine were well separated (Fig. 7). N-carbobenzoxy (CBZ) derivatives, on the other hand, were difficult to elute, even with the aid of an eluent to which an organic solvent had been added. This may have resulted from strong interactions between the CBZ group and hydrophobic groups of the matrix.

Dipeptides, such as glycyl-DL-leucine and DL-leucyl-DL-phenylalanine, were separated well when an organic solvent was added to the eluent, as shown in Fig. 8. Similar results were obtained for tri-peptides, such as DL-alanylglycylglycine and DL-leucylglycyl-DL-phenylalanine. Hence it can be concluded that CRS10W provides a good method for resolving peptide enantiomers.

TABLE III

RESOLUTION OF AMINO ACIDS DERIVATIVES, PEPTIDES AND HYDROXY CARBOXYLIC ACIDS ON MCI GEL CRS10W

Column temperature: room temperature.

Compound	*Optimum conditions*		*Capacity factor*		*Resolution, R_s*
	Eluent $CuSO_4$ concentration (mM)	*Flow-rate (ml/min)*	k'_D	k'_L	
N-Acetyl-DL-alanine	2.0	1.0	7.0	8.4	1.71
N-Acetyl-DL-leucine	2.0[a]	1.0	38.0	54.1	2.90
DL-Ala–Gly–Gly	0.5	0.5	2.3	3.0	0.58
DL-Leu–Gly–Gly	2.0	1.0	10.3	16.4	2.99
Gly–DL–Leu	2.0[a]	1.0	20.9	11.7	2.93
DL-Leu–L-Tyr	2.0[a]	1.0	12.5	15.6	0.96
DL-Leu–DL-Phe	2.0[a]	1.0	16.7	20.6	1.21
Glyceric acid	0.5	1.0	11.0	18.9	2.68
Malic acid	0.5[a]	1.3	44.2	36.4	1.13
Lactic acid	2.0	1.0	12.5	15.9	2.44
Pantothenic acid	2.0	1.0	40.0	45.2	0.41
Mandelic acid	2.0[a]	1.0	52.5	72.3	3.98
2-Hydroxy-*n*-butyric acid	2.0[a]	1.0	9.8	14.2	3.40
Tartaric acid	2.0[a]	1.0	49.5	32.5	0.90
DL-α-Amino-ε-caprolactam	1.0	0.5	1.7	2.4	1.36

[a] In 10% aqueous acetonitrile solution.

Of hydroxycarboxylic acids, DL-mandelic acid, DL-lactic acid, DL-malic acid and DL-tartaric acid were separated well. However, the resolution for DL-pantothenic acid was insufficient, as shown in Fig. 9. For DL-malic acid and DL-tartaric acid, the elution order was reversed.

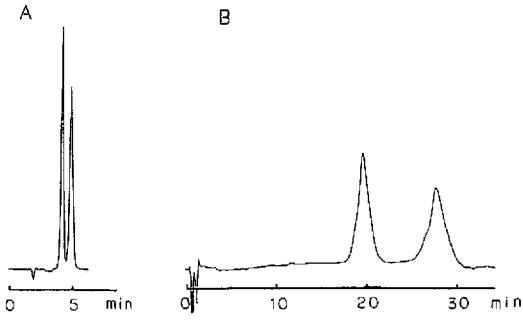

Fig. 7. Resolution of amino acid derivatives. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); flow-rate, 1 ml/min; temperature, room temperature; detector, UV (254 nm). (A) Eluent, 2 m*M* $CuSO_4$ aqueous solution; sample, N-acetyl-DL-alanine (2 mg/ml); injection volume, 15 μl. (B) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; sample, N-acetyl-DL-leucine (0.2 mg/ml); injection volume, 20 μl.

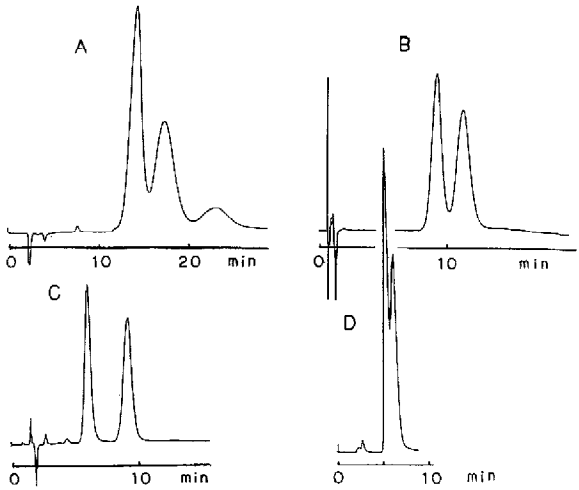

Fig. 8. Resolution of peptides. Column, MCI GEL CRS10W (50 mm × 4.6 mm I.D.); temperature, room temperature; detection, UV (254 nm). (A) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 0.3 ml/min; sample, DL-leucyl-L-tyrosine (2 mg/ml); injection volume, 5 μl. (B) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 1 ml/min; sample, DL-leucyl-DL-phenylalanine (1 mg/ml); injection volume, 20 μl. (C) Eluent, 2 *M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; sample, DL-leucylglycylglycine (1 mg/ml); injection volume, 10 μl, (D) Eluent, 0.1 m*M* $CuSO_4$ aqueous solution; flow-rate, 0.3 ml/min; sample, DL-alanylglycylglycine (2 mg/ml); injection volume, 5 μl.

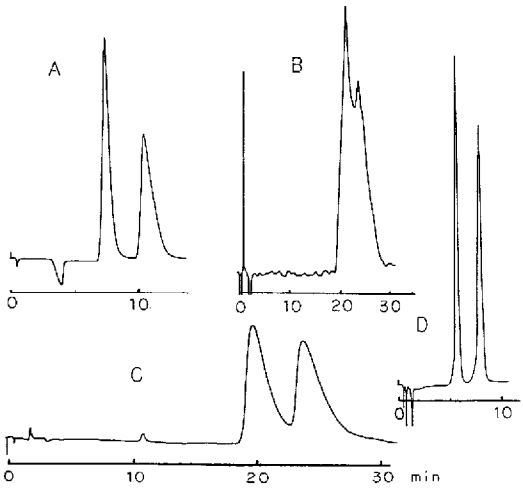

Fig. 9. Resolution of hydroxycarboxylic acids. Column: MCI GEL CRS10W (50 mm × 4.6 mm I.D.); temperature, room temperature; detection, UV (254 nm). (A) Eluent, 0.5 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; sample, DL-glyceric acid, Ca salt (4 mg/ml); injection volume, 20 μl. (B) Eluent, 2 m*M* $CuSO_4$ aqueous solution; flow-rate, 1 ml/min; sample, DL-pantothenic acid, Ca salt (4 mg/ml); injection volume, 20 μl. (C) Eluent, 0.5 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 1.3 ml/min; sample, DL-malic acid (2 mg/ml); injection volume, 2 μl. (D) Eluent, 2 m*M* $CuSO_4$–10% acetonitrile aqueous solution; flow-rate, 1 ml/min; sample, DL-2-hydroxy-*n*-butyric acid, Li salt (2 mg/ml); injection volume, 5 μl.

Of amines, DL-α-amino-ε-caprolactam was separated well, but 1-phenylethylamine was eluted without interacting with the column.

CONCLUSION

When the elution conditions are carefully controlled, MCI GEL CRS10W and CRS10WD can resolve most DL-amino acids. They retain other compounds, including amino acid derivatives, peptides and hydroxycarboxylic acids.

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