Analysis of Amino Acids by High-Performance Liquid Chromatography with Circular Dichroism Detection

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Stereochemical detection and separation of the enantiomers of DL-amino acids were performed using a high-performance liquid chromatograph (HPLC) equipped with a chiral column and a circular dichroism spectrophotometer as a detector. Various racemic amino acids were separated by the chiral column and all L-amino acids and D-amino acids were detected as positive and negative circular dichroism peaks, respectively. Mixtures of two amino acids, DL-norleucine and DL-norvaline, DL-phenylglycine and DL-dopa, and glycine and DL-leucine were also analyzed.

Key words HPLC; CD; amino acid

Optically active amino acids are important chiral building blocks in organic synthesis.[1] The natural amino acids are of L-form, and their analysis has been used in the diagnosis of diseases.[2] However, D-amino acids also exist in the natural world, and synthetic peptides usually contain as impurities small amounts of D-amino acid. Therefore, a system for concurrent separation and determination of the absolute configuration of amino acids would be convenient.

Advances in high-performance liquid chromatography (HPLC) and the use of chiral columns have made feasible the stereochemical analysis of amino acids. Determination of the absolute configuration of the amino acids by usual ultraviolet (UV) or differential refractometric detectors, however requires the use of standard samples of each enantiomer. On the other hand, circular dichroism (CD) can determine absolute configuration by a comparison with known experimental data. The chirality even of molecules such as [2-^{2}H]glycine, which have an asymmetric center consisting of a proton and a deuterium, can be determined.[3] Several applications of CD spectrophotometers as HPLC detectors have been reported,[4-12] although no report has been published on their application to the analysis of amino acids. We describe here the stereochemical detection of various amino acids in HPLC analysis using a chiral column and a CD spectrophotometer as a detector.

Experimental

Materials All amino acids were purchased from Ajinomoto (Tokyo, Japan) or Wako Pure Chemicals (Tokyo). Perchloric acid was also purchased from Wako Pure Chemicals. The column for all HPLC analyses
was Crown Pak CR(−) (150 mm × 4 mm i.d.), obtained from Daicel (Tokyo).

Apparatus CD spectra were recorded on a JASCO Model J-720 CD spectrophotometer. All HPLC analyses were carried out on a JASCO 800 Series HPLC system (Tokyo), which consisted of an intelligent pump (JASCO Model 880-PU), a syringe-loading sample injector (Rheodyne Model 7125, Cotati, CA, U.S.A.) equipped with a 100 μl loop, a UV detector (JASCO Model 870-UV spectrophotometer), and a CD spectrophotometer (JASCO model J-720) with a flow-cell device (1 mm optical path, 19 μl volume). Both detectors were connected in series to give an HPLC-UV-CD system.

Preparation of Amino Acid Solutions Each amino acid was dissolved in the eluent for HPLC analysis, *i.e.*, pH 1.50 perchloric acid aqueous solution. The sample concentrations were 10 mg/l for measurement of CD spectra and 5 mmol/l for HPLC analysis.

Chromatographic Procedure HPLC was performed under the following conditions: The eluent was perchloric acid aqueous solution adjusted to pH 1.50 (*ca.* 0.2%, v/v). Flow rate and column temperature were appropriately adjusted to separate enantiomers at 0.4—1.2 ml/min and at 5—28 °C (Tables 2 and 3). The effluent was first monitored at 200 nm by the UV detector and subsequently at 200—227 nm (Tables 2 and 3) by the CD spectrophotometer.

Results and Discussion

The usual CD spectra of each amino acid were measured in order to select the most suitable monitoring wavelength and to know the peak polarity of each enantiomer for CD detection in HPLC analysis. The data are summarized in Table 1. L- And D-alanine showed a positive and negative CD peaks, respectively, and other L-amino acids also showed positive peak. The largest peak in the CD spectrum of each amino acid was chosen as the monitoring wavelength.

The separation of single racemic amino acids was accomplished by using a Crown Pak CR(−) column, and the separated enantiomers were detected by the UV detector and CD spectrophotometer connected in series. A chromatogram of DL-tyrosine is shown in Fig. 1. The use of CD detector showed that the first positive peak and the second negative peak were L- and D-tyrosine, respectively, whereas the UV detection simply showed two peaks. Note that the elution patterns are not corrected for the lag time between the two detectors. The results for other amino acids are summarized in Table 2. All L-amino acids were eluted first and detected as positive peaks with the CD spectrophotometer, and all D-amino acids were observed as the second, negative peaks.

Next, a mixed sample consisting of two amino acids was analyzed in this HPLC-CD system; the results and conditions are shown in Table 3. In the analysis of the mixture of DL-norleucine and DL-norvaline, although the elution order of D-norleucine and L-norvaline was reversed from that found in Table 2 because of different chromatographic conditions, the CD detector easily recognized this difference. A mixed sample of DL-phenylglycine and DL-dopa was also analyzed under different conditions from those in Table 2. With the CD detector four peaks were observed in the order positive, positive, negative and negative. Use of this detector made

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Table 1. CD Spectral Data on Various Amino Acids

Sample	Wavelength (nm) of peak	θ (mdeg)	$[\theta]$ (deg cm^2/dmol)	Sample	Wavelength (nm) of peak	θ (mdeg)	$[\theta]$ (deg cm^2/dmol)
D-Ala	205.5	−6.174	−5513	L-Pro	219.0	+2.364	+2722
L-Ala	209.0	+5.893	+5262	L-Ser	219.5	+2.304	+2421
L-Asn	219.0	+2.157	+2850	L-Thr	211.0	+4.597	+5476
L-Cys	219.5	+2.548	+3087	L-Try	225.5	+3.866	+7896
L-Gln	219.0	+1.533	+2241		264.5	+8.085	+16512
L-Iln	210.0	+5.420	+7110	L-Tyr	203.5	+2.876	+5211
L-Leu	209.5	+4.399	+5771		227.0	+5.750	+10419
L-Phe	218.5	+7.606	+12564	L-Val	210.5	+3.943	+4619

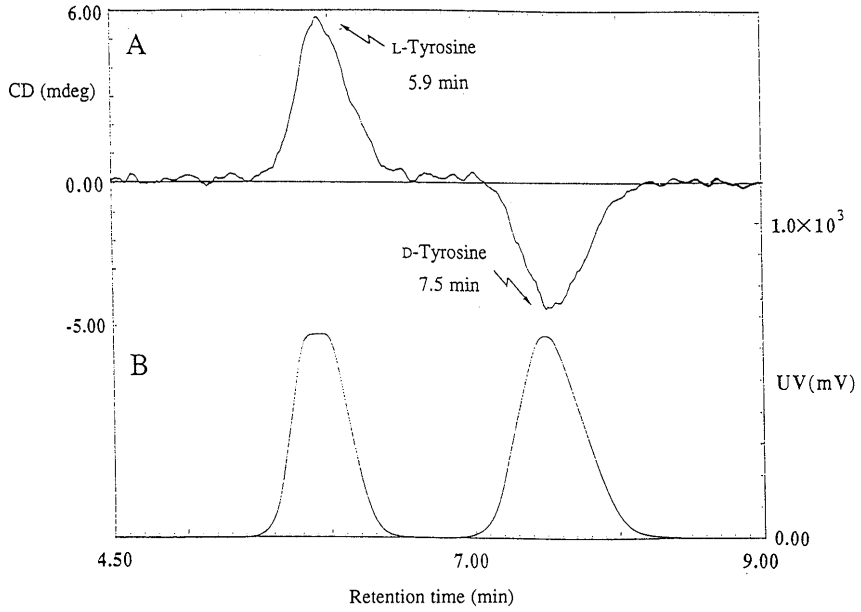

Fig. 1. HPLC-CD Chromatogram of DL-Tyrosine

(A) CD detection; (B) UV detection. Chromatographic conditions were as shown in Table 2.

Table 2. Chromatographic Conditions and Data on Various Amino Acids on HPLC-CD

Sample	Retention time (min)	CD polarity	Flow rate (ml/min)	CD detection wavelength (nm)	Temp. (°C)
DL-Alanine	L 5.2	+	0.4	205	23
	D 5.7	−			
DL-Leucine	L 4.5	+	0.8	210	23
	D 7.0	−			
DL-Norleucine	L 4.4	+	0.8	210	24
	D 6.6	−			
DL-Norvaline	L 5.9	+	0.8	200	26
	D 7.9	−			
DL-Phenylalanine	L 6.9	+	0.8	218	28
	D 8.5	−			
DL-Tryptophan	L 15.3	+	1.2	225	27
	D 18.3	−			
DL-Tyrosine	L 5.9	+	0.8	227	28
	D 7.5	−			

Table 3. Chromatographic Conditions and Data on the Mixed Sample of Two Amino Acids

Sample	Retention time[a] (min)	CD polarity	Flow rate (ml/min)	Detection wavelength (nm)		Temp. (°C)
				CD	UV	
DL-Norleucine	L 7.3	+	0.4	210	200	5
and	D 11.9	−				
DL-norvaline	L 13.8	+				
	D 22.6	−				
DL-Phenylglycine	L 5.3	+	0.8	210	200	5
and	D 32.1	−				
DL-dopa	L 12.5	+				
	D 16.1	−				
Glycine and	3.2[b]	Inactive	0.8	210	210	25
DL-leucine	L 7.0	+				
	D 10.1	−				

a) Detected by CD. *b*) Detected by UV.

it easy to recognize that D- and L-dopa were eluted between the two enantiomers of phenylglycine. The UV detector simply showed four peaks. A mixture of glycine as an achiral compound and DL-leucine was next examined. Three UV peaks were detected, but the CD detector showed only two, positive and negative, corresponding to L- and D-leucine.

Conclusion

Separation of DL-amino acids concomitantly with determination of the enantiomer configuration in comparison with corresponding CD data was achieved using an HPLC-CD system equipped with a chiral column. This system is also applicable to mixtures of DL-amino acids

such as DL-phenylglycine and DL-dopa. The CD spectrometer cannot detect achiral compounds like glycine. The use of UV detector in series with the CD spectrometer allowed the distinction of D- and L-leucine from glycine, without difficulty.

This HPLC-CD system can be used on-line to measure the CD spectrum of optically active compounds in eluates from a non-chiral column by the stopped flow method.[8] Thus, it can be employed to determine the absolute configuration of optically active compounds eluted from a non-chiral column by applying various empirical and nonempirical rules.[13-15] It should be applicable to many compounds.

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References

1) Coppola G. M., Schuster H. F., "Asymmetric Synthesis," John Wiley & Sons, New York, 1987.
2) Barbeau A., MacDowell F. H., "L-Dopa and Parkinsonism," F. D. Davis Co., Philadelphia, 1970.
3) Yamada H., Kurumaya K., Eguchi T., Kajiwara M., *J. Labelled Compd. Radiopharm.*, **24**, 562 (1987).
4) Drake A. F., Gould J. M., Mason S. F., *J. Chromatogr.*, **202**, 239 (1980).
5) Westwood S. A., Games D. E., Sheen L., *J. Chromatogr.*, **204**, 103 (1981).
6) Salvadori P., Rosini C., Bertucci C., *J. Org. Chem.*, **49**, 5050 (1984).
7) Rosini C., Altemura P., Pini D., Bertucci C., Zullino G., Salvadori P., *J. Chromatogr.*, **348**, 79 (1985).
8) Takakuwa T., Kurosu Y., Sakayanagi N., Kaneuchi F., Takeuchi N., Wada A., Senda M., *J. Liq. Chromatogr.*, **10**, 2759 (1987).
9) Kurosu Y., Sasaki T., Takakuwa T., Sakayanagi N., Hiba K., Senda M., *J. Chromatogr.*, **515**, 407 (1990).
10) Casarini D., Foresti E., Gasparrini F., Lunazzi L., Macciantelli D., Misiti D., Villani C., *J. Org. Chem.*, **58**, 5674 (1993).
11) Bertucci C., Salvadori P., Guimaraes L. F. L., *J. Chromatogr. A*, **666**, 535 (1994).
12) Zerbinati O., Aigotti R., Daniele P. G., *J. Chromatogr. A*, **671**, 281 (1994).
13) Harada N., Iwabuchi J., Yokota Y., Uda H., Nakanishi K., *J. Am. Chem. Soc.*, **103**, 5590 (1981).
14) Gonnella N. C., Nakanishi K., Martin V. S., Sharpless K. B., *J. Am. Chem. Soc.*, **104**, 3775 (1982).
15) Barrett G. C., "Techniques of Chemistry," Vol. IV, Bentley K. W., Kirby G. W., Eds., John Wiley & Sons, New York, 1972.