Journal of Chromatographic Science 2012;50:393–395
doi:10.1093/chromsci/bmr004

Article

Simple and Low-Cost High-Performance Liquid Chromatographic Method for Determination of D- and L-Amino Acids

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Received 21 April 2010; accepted 10 August 2010

In this article, a simple and low-cost method for the analysis of amino acid enantiomers by using high–performance liquid chromatography (HPLC) is described. In this method, the amino acids are modified to diastereomers in order to be separated into enantiomers on a usual C_{18} reversed-phase column. Methanol instead of acetonitrile is used as an elution solvent; the results of HPLC with methanol elution are comparable with those of HPLC with acetonitrile elution. Sub-nanomolar sensitivity is attained by measuring the absorbance at 340 nm in analysis of 15 amino-acid enantiomers.

Introduction

The α-carbon atom of all the amino acids (except glycine) is chiral or asymmetric. The chiral amino acids exist as stereoisomers called enantiomers. Nowadays, the occurrence of D-enantiomers of amino acids in higher animals is being increasingly reported (1), owing to advancements in analytical techniques. These amino acids are present in free forms. The highly investigated D-amino acids are serine and aspartate in the context of the central nervous system and spermatogenesis in mammals, respectively. In a previous study, L- and D-amino acid contents were investigated, especially serine in silkworms in various growth stages, to elucidate the physiological functions of D-serine (2). To determine the enantiomers, high-performance liquid chromatography (HPLC) with a C_{18} reversed-phase column with acetonitrile as the elution solvent was used (3). However, since the last year, it became difficult and costly to obtain commercial acetonitrile because of a global decrease in its industrial production. Therefore, the usefulness of methanol as a substitute for acetonitrile was examined because the cost of methanol is only one-fifth that of acetonitrile.

Experimental

Chemicals and reagents

D- and L-enantiomers of serine, alanine, proline, asparagine, valine, leucine, threonine, and arginine were purchased from Wako (Osaka, Japan), and aspartic acid and phenylalanine were purchased from Nacalai Tesque (Kyoto, Japan). Further, glutamic acid, glutamine, histidine, and isoleucine were purchased from Katayama Chemical (Osaka, Japan), and glycine, from Sigma (St. Louis, MO). HPLC-grade acetonitrile and methanol were from Wako. 1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide [FDAA, Marfey's reagent (4)] was obtained from Sigma.

Determination of D- and L-amino acids

Amino acid enantiomers were determined according to the method described in a previous paper (3), except that methanol was used instead of acetonitrile. In brief, free amino acids were modified with FDAA, and the FDAA-amino acids (i.e., the diastereomers) were separated on a Silica Gel 60 plate (Merck, Darmstadt, Germany) by using one- or two-dimensional thin-layer chromatography (TLC), depending on the number of amino acids contained in the specimen. The diastereomers were extracted from the TLC plate by using 50% methanol.

Chromatographic conditions

The configurations of the FDAA-amino acids extracted from the TLC plate were analyzed using HPLC. The liquid chromatography systems comprised an 807-IT Integrator (Jasco, Tokyo, Japan), a PU-2089 Plus Quaternary Gradient Pump with a degasser (Jasco), and a UV-2075 Plus Intelligent UV/VS detector (Jasco) or a Hitachi L-4250 UV/VS detector. A Nova-Pak C_{18} reversed-phase column (150 x 3.9 mm i.d., 4 μm particle size, Waters, Milford, MA) was used. The mobile phase was a linear gradient from 0 to 100% B (100 to 0% A) in 40 min, at a flow-rate of 0.65 mL/min at 25°C. Solvent A was a 50 mM triethylamine − phosphate buffer (pH 3.5) containing 25% (v/v) methanol, and solvent B was the same buffer containing 70% methanol. Acetonitrile was also used in this study in order to compare the efficacy with that of methanol. Solvent A was 50 mM triethylamine − phosphate buffer (pH 3.5) containing 10% acetonitrile, and solvent B was the same buffer containing 40% acetonitrile; the mobile phase was a linear gradient from 0 to 100% B (100 to 0% A) in 40 min, and the flow-rate was 1.0 mL/min. The absorbance of the eluted fraction was monitored at 340 nm, and peak areas were automatically obtained by the integrator in the HPLC system.

Sample preparation

After the silkworm (*Bombyx mori* pupae or imagoes) were minced with a pair of scissors, they were homogenized in 4 volumes (v/w) of distilled water using a glass homogenizer (1,300 rpm for 5 min). The homogenates were centrifuged at 6,000 x g for 20 min at 4°C. Thereafter, the supernatants were treated with 5% trichloroacetic acid to precipitate and remove proteins by the subsequent centrifugation. The resultant supernatant was applied on a Dowex 50W×8 (H^+-form, Muromachi Chemicals, Tokyo) column; the column was washed with

distilled water, and amino acids were eluted with 2 M NH_4OH. The eluted fraction containing purified free amino acids were evaporated until dry in vacuo in a centrifugal evaporator (Tokyo-Rika, Tokyo) at a temperature below 40°C.

Quantification of samples

Various amounts of several authentic amino acids were added to the homogenates of the silkworms and treated by the same procedure described under sample preparation. The purified amino acids were modified with FDAA, and the resultant diastereomers were analyzed using HPLC, as described earlier. On the basis of the calibration lines obtained for each amino acid, the intrinsic amino acids in the silkworms were determined.

Results and Discussion

Amino acid enantiomers can be separated as diastereomers on the usual reversed-phase column by modifying these with FDAA. The chromatogram of alanine is presented in Figure 1. A greater difference between the retention times of L- and D-alanine was observed when methanol was used than when acetonitrile was used for elution. For some amino acids, slightly larger peak width and height were obtained with methanol elution than with acetonitrile elution.

The retention times of the L- and D-diastereomers obtained with methanol elution (Table I) were larger by 3–7 min and 0.4–5 min, respectively, than those with acetonitrile elution (3). For example, the retention times of L- and D-phenylalanine were 24.5 min and 32.4 min, respectively, with methanol elution and 31.3 min and 37.3 min, respectively, with acetonitrile elution (3). Therefore, the difference between the retention times of L- and D-diastereomers with methanol elution was equal to or greater than that with acetonitrile elution. Separation of D-asparagine from D-serine and that of D-alanine

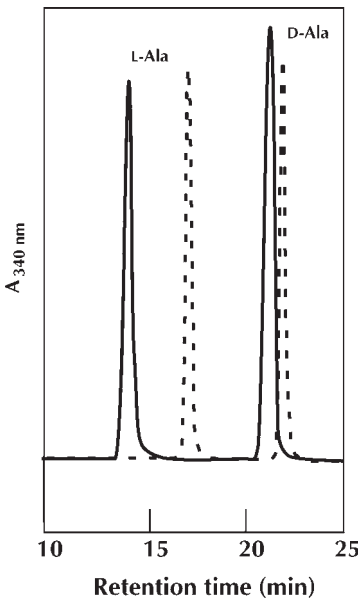

Figure 1. HPLC chromatogram of alanine. FDAA-D-alanine and FDAA-L-alanine were eluted from the C_{18} column with methanol (solid line) or acetonitrile (dotted line).

Table I
Retention Times of FDAA-Amino Acids Eluted With Methanol

	Retention time (min)	
Amino acid	L-Diastereomer	D-Diastereomer
Asparagine	8.8	11.0
Serine	9.0	11.0
Arginine	9.6	12.1
Threonine	10.0	16.6
Glutamine	10.3	12.9
Aspartate	10.8	14.8
Glutamate	12.7	17.9
Alanine	14.0	20.6
Proline	15.3	19.2
Methionine	19.9	28.3
Valine	20.6	29.6
Phenylalanine	24.5	32.4
Isoleucine	26.0	35.5
Leucine	27.3	36.1
Glycine	13.2	
FDAA, FDAA-OH	15.6	

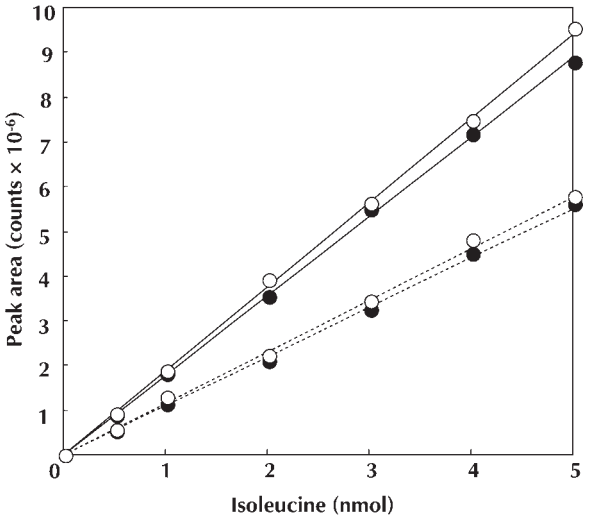

Figure 2. Counts of peak area as a function of the isoleucine amounts. Various amounts of D- (○) and L- (●) isoleucine were modified with FDAA and analyzed by HPLC with methanol (solid line) and acetonitrile (dotted line) as the eluents, as described in the Experimental section.

Table II
Peak Area of FDAA-Amino Acids in Reversed Phase HPLC*

	Peak area/pmol (counts × 10^{-3})	
Amino acid	L-Diastereomer	D-Diastereomer
Asparagine	0.928 ± 0.019	0.984 ± 0.039
Serine	0.770 ± 0.034	0.890 ± 0.039
Arginine	0.842 ± 0.020	0.813 ± 0.037
Threonine	0.583 ± 0.036	0.637 ± 0.057
Glutamine	1.158 ± 0.017	1.131 ± 0.021
Aspartate	0.975 ± 0.049	1.040 ± 0.033
Glutamate	0.627 ± 0.011	0.703 ± 0.009
Alanine	1.141 ± 0.067	1.467 ± 0.035
Proline	1.064 ± 0.023	1.360 ± 0.018
Methionine	1.488 ± 0.034	1.500 ± 0.056
Valine	1.485 ± 0.025	1.611 ± 0.028
Phenylalanine	1.705 ± 0.025	2.015 ± 0.078
Isoleucine	1.746 ± 0.019	1.757 ± 0.021
Leucine	1.191 ± 0.018	1.168 ± 0.016
Glycine	1.256 ± 0.053	

* Values are the mean ± S.D. of triplicate assays.

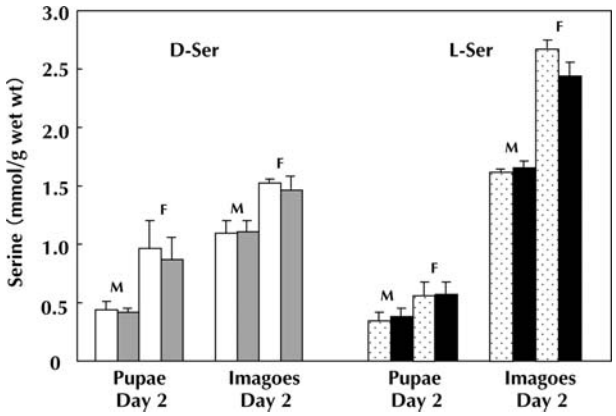

Figure 3. Intrinsic D- and L-serine concentrations in the homogenates of the silkworm *Bombyx mori* pupae and imagoes. M, male; F, female. The amounts of these enantiomers obtained with methanol (white and dotted bars) and acetonitrile (grey and black bars) as eluents are indicated. White and grey bars represent D-serine, and dotted and black bars represent L-serine. The values are mean ± S.D. for the triplicate assays.

from L-valine could not be achieved with this HPLC method. However, these amino acids and the excess free FDAA could be completely separated by the TLC that was performed before the HPLC. Although the differences between the retention times of the diastereomers (L-asparagine and L-serine, L-threonine and L-glutamine, and D-glutamine and L-glutamate) were small, they were completely separated from each other when simultaneously injected into the HPLC pump.

A known amount of each authentic amino acid was modified with FDAA, separated by TLC, and analyzed for its peak area by HPLC, with either methanol or acetonitrile as the elution solvent. The result with isoleucine is shown as an example of these analyses (Figure 2). The values for the peak area (counts x 10^{-3}) per picomols of each amino acid are listed in Table II. The peak areas obtained with methanol elution were usually greater than those obtained with acetonitrile elution.

Usage of methanol was confirmed by comparing the values for L- and D-serine contents in pupae and imagoes of the silkworm, determined by HPLC with methanol elution to those with acetonitrile elution. As revealed in Figure 3, the results show good coincidence between the values obtained with the different elution solvents.

Although there are several excellent HPLC methods for microanalysis of amino-acid enantiomers, those methods need equipments for post-column modification. The results presented here clearly showed that the elution with methanol yields a good result, comparable with that of acetonitrile. Methanol costs only one-fifth that of acetonitrile, and a conventional octadecylsilyl-bonded column, which has a much lower cost than a chiral column, was used. Therefore, this method is simple, low-cost and reliable in analysis of amino-acid enantiomers in sub-nanomolar amounts, and seems useful for analysis of many specimens.

Conclusion

A simple method for the enantiomeric analysis of amino acid enantiomers by using HPLC with a reversed-phase column and methanol was developed and verified. The amounts of D- and L-serine in the 4 homogenates of the silkworm sample obtained with methanol elution were almost equal to those obtained with acetonitrile elution; those results indicate that methanol is a reliable elution solvent and can be substituted for acetonitrile.

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