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# Short Communication

# Determination of D- and L-amino acids in mouse kidney by high-performance liquid chromatography

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ABSTRACT

A method for the enantiomeric analysis of amino acids of mammalian tissues is described. An excellent resolution of D- and L-enantiomers of common protein amino acids was achieved by employing a combination of thin-layer chromatography and high-performance liquid chromatography. D-Enantiomers and L-enantiomers of glutamate, aspartate, glutamine, asparagine, serine, threonine, alanine, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine and histidine, as well as glycine were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide. The amino acid diastereomers were separated by two-dimensional thin-layer chromatography. Each amino acid diastereomer was then analysed by reversed-phase high-performance liquid chromatography for the resolution of D- and L-enantiomers. Very sharp peaks were obtained using a conventional octadecylsilyl-bonded column, and the possibility of analysing these amino acids (except tyrosine and histidine) in subnanomole amounts was indicated. The method was used to demonstrate the presence of D-enantiomers of alanine, proline and serine in mouse kidney.

## INTRODUCTION

A facile method for the analysis of D-amino acids seems to be required in biological, biochemical, food chemical and pharmacological studies: very few methods for the analysis of D-amino acids have been reported. High-performance liquid chromatographic (HPLC) methods for the resolution of amino acid enantiomers that utilize a chiral stationary phase [1,2] or a chiral mobile phase [3,4] are expensive, and the high retention results in broad peaks. Hence, a third method, using a chiral reagent [5–8] that converts a sample into a diastereomeric derivative, seems to be useful. Marfey [9] developed a reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA), to produce diastereomers of amino acids, and a reversed-phase HPLC method for the determination of amino acid enantiomers using the reagent. The method needs neither post-column modifica-

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tion nor fluorimetric detection. In addition, the reagent is commercially available.

It is generally believed that mammals neither synthesize nor possess D-amino acids. However, we have found the presence of neutral free D-amino acids in mammalian tissues [10,11], using an enzymic method for the microdetermination of neutral D-amino acids [12]. A sensitive and convenient method, therefore, became necessary to determine each D-amino acid. Marfey reported the analysis of only six amino acids, alanine, aspartate, glutamate, methionine and phenylalanine [9] and cysteine [13]. Szőkán *et al.* [14] reported the application of Marfey's reagent to the resolution of twelve amino acids, using several solvent systems.

This paper describes the extension of this analysis to the most common amino acids, by employing two-dimensional thin-layer chromatography (TLC) to allow determination of amino acid enantiomers by subsequent HPLC. Subnanomolar sensitivity was attained in analysis of the D-enantiomers of alanine, proline and serine, and the presence of these three D-amino acids in mouse kidney was demonstrated.

## EXPERIMENTAL

### *Chemicals*

D-Amino acids and L-amino acids were purchased from Aldrich (Milwaukee, WI, USA) and Katayama (Osaka, Japan). FDAA (Marfey's reagent) was from Pierce (Rockford, IL, USA). Acetonitrile and methanol were of HPLC grade from Wako (Osaka, Japan). *n*-Butanol, acetic acid, phenol and hydrochloric acid were chromatography grade from Wako.

### *Derivatization of amino acids with FDAA*

This was done according to Marfey [9]. To 20 $\mu$l of a solution containing a combined total of 1.0 $\mu$mol of amino acids, 40 $\mu$l of 1% acetone solution of FDAA and 8 $\mu$l of 1 *M* $NaHCO_3$ were added. The molar ratio of FDAA to amino acids was 1.4:1.0. The mixture was incubated at 37–40°C in a reaction vial for 1 h. After cooling to room temperature, 4 $\mu$l of 2 *M* HCl were added to stop the reaction. The contents were brought to dryness using a centrifugal evaporator (VC-360, Taitec, Saitama, Japan) under vacuum. The residue was dissolved in 40 $\mu$l of methanol, and subjected to two-dimensional TLC.

### *Two-dimensional TLC*

This was performed on Whatman K6 plates (20 cm × 20 cm, Whatman, Clifton, NJ, USA) precoated with a 0.25-mm layer of silica gel. A 30-$\mu$l portion of the sample was applied to the TLC plate. The plates were developed in *n*-butanol–acetic acid–water (3:1:1, v/v/v) in the first dimension, and in phenol–water (3:1, v/v) in the second dimension, at 22°C. The yellow spots were scraped off the TLC plate and extracted with methanol–water (1:1, v/v). The extracts were analysed by HPLC, after being evaporated to dryness with the centrifugal evaporator. Identification of the spots on the TLC plate was performed, referring to FDAA-derivatized authentic amino acids and to the HPLC analysis described below.

### *Reversed-phase HPLC*

A Tosoh (Tokyo, Japan) gradient HPLC system, consisting of a Model PX-8010 controller, a Model CCPM pump, a sample injector (Rheodyne, Cotato, CA, USA), a Model MC-8010 mixer and a UV-8010 UV–visible detector, was used. A degasser (ERC-3312, Erma, Tokyo, Japan) was attached to the system. The absorbance was monitored on a Hitachi D-2500 Chromato-Integrator, which calculates the peak area as counts ($\mu$V × s). HPLC was carried out according to Marfey [9], with slight modifications. A reversed-phase column, Nova-Pak $C_{18}$ (150 mm × 3.9 mm I.D.) packed with 4-$\mu$m spherical silica (Waters, Milford, MA, USA) was used. The sample from the TLC plate was dissolved in methanol, and a 5-$\mu$l portion was injected into the HPLC system. Solvent A was 50 m*M* triethylamine–phosphate buffer (pH 3.5) and solvent B was acetonitrile. Derivatized amino acids were eluted from the column with a linear gradient from 10 to 40% B in A in 45 min, at a flow-rate of 1.0 ml/min at 22°C. The absorbance of the ef-

fluent was monitored at 340 nm, and peak areas were obtained integrated automatically. Peaks were identified by co-chromatography [15] with the FDAA-derivatized authentic amino acids.

*Extraction of free amino acids from mouse kidney*

After rinsing with phosphate-buffered saline (pH 7.4), the kidney was minced into small pieces, homogenized with four volumes of the saline in a glass homogenizer in an ice bucket, at 1000 rpm, and centrifuged at 160 000 *g* for 10 min at 4°C. To the supernatant extract, cold trichloroacetic acid solution was added to a final concentration of 5%. A 200-$\mu$l aliquot of the resultant supernatant fraction was applied to a Dowex 1–X8 (acetate form; Muromachi Chemicals, Tokyo, Japan) column (4.0 cm × 0.5 cm I.D.) to remove trichloroacetic acid and acidic amino acids. The effluent fraction was brought to dryness with the centrifugal evaporator under vacuum. The residue was dissolved in distilled water and derivatized with FDAA.

## RESULTS AND DISCUSSION

Fig. 1 represents the two-dimensional TLC pattern of amino acids derivatized with FDAA. The yellow diastereomers were visible on the TLC plate. Each amino acid was separated on the plate, except for the two spots that comprise tyrosine and valine (spot 1) and isoleucine, leucine and phenylalanine (spot 2). Only histidine was separated further into D- and L-diastereomers by TLC. The excess hydrolysed FDAA moved to the front of the second solvent, resulting in the complete separation of FDAA from the derivatized amino acids. This is of advantage for the HPLC analysis, because the elution time of hydrolysed FDAA is close to that of some FDAA-amino acids (see Table I), and a large FDAA peak would hide small peaks of FDAA-amino acids.

Fig. 2 shows HPLC of the extract of spot 2 (Fig. 1). A clear HPLC pattern with no peaks other than those intrinsic to the diastereomers could be obtained by employing TLC prior to HPLC. The diastereomers that comigrated in

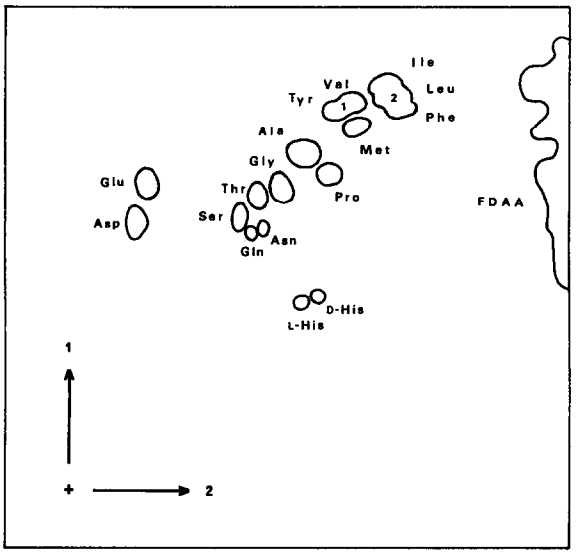


Fig. 1. Two-dimensional TLC pattern of amino acids derivatized with FDAA. The origin is marked with a cross; arrow 1, first dimension developed in *n*-butanol–acetic acid–water (3:1:1. v/v/v); arrow 2, second dimension developed in phenol–water (3:1, v/v).

TABLE I

ELUTION TIMES OF FDAA-AMINO ACIDS IN REVERSED-PHASE HPLC

| Amino acid | Elution time (min) | |
|---|---|---|
| | L-Diastereomer | D-Diastereomer |
| Histidine | 8.2 | 8.7 |
| Asparagine | 11.8 | 13.3 |
| Serine | 12.2 | 14.1 |
| Glutamine | 12.7 | 14.5 |
| Threonine | 13.6 | 19.6 |
| Aspartate | 13.9 | 16.6[a] |
| Glutamate | 15.5 | 19.0 |
| Alanine | 16.2 | 21.5[b] |
| Proline | 17.3 | 21.5[b] |
| Tyrosine | 23.6, 38.3 | 28.5, 44.1 |
| Methionine | 24.2 | 30.7 |
| Valine | 26.1 | 32.8 |
| Isoleucine | 28.0 | 35.8 |
| Leucine | 30.0 | 36.5 |
| Phenylalanine | 31.3 | 37.3 |
| Glycine | 16.6[a] | |
| FDAA, FDAA-OH | 22.3 | |

[a] D-Aspartate and glycine are co-chromatographed.
[b] D-Alanine and D-proline are co-chromatographed.

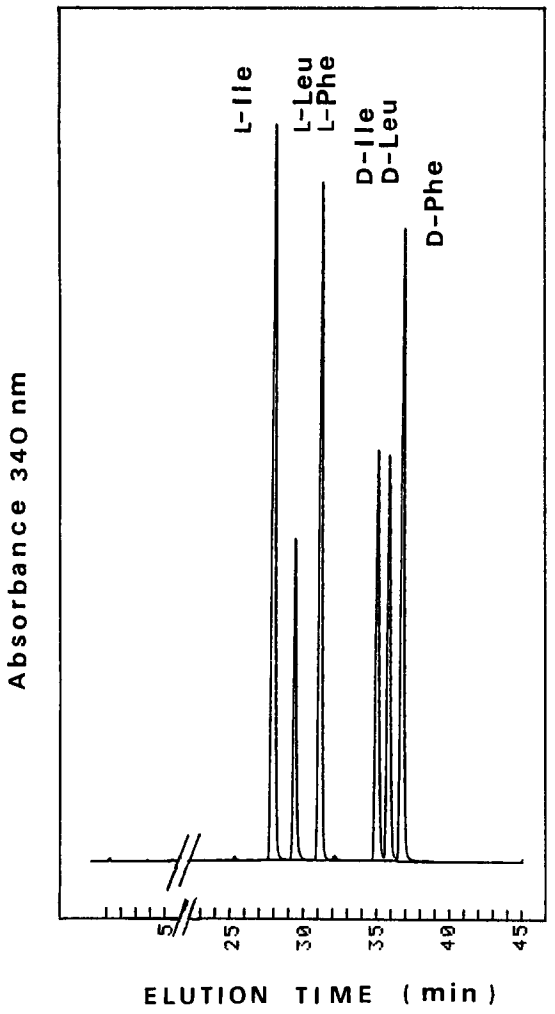


Fig. 2 HPLC profile of extract of spot 2 (Fig. 1). The extract comprises D- and L-enantiomers of isoleucine, leucine and phenylalanine derivatized with FDAA. HPLC conditions as in Experimental.

spot 1 were also well resolved by HPLC. Resolution of the D-diastereomer from the L-diastereomer was complete, even in the case of histidine, whose elution times are close each other. Table I indicates values for the elution times of the diastereomers. The elution times were the same for D-aspartate and glycine, and for D-alanine and D-proline, with the pH 3.5 elution buffer. Although these peaks were well separated when eluted with solvent A at pH 3.0 [9], we chose pH 3.5 to protect the column. The problem has been solved by separating these amino acids by TLC prior to HPLC. As to tyrosine, bis-tyrosine derivatives were formed. D-Diastereomers are always eluted from the column after L-diastereomers. The behaviour may be due to stronger intramolecular hydrogen bonding in D- than in L-diastereomers [9].

Peak areas were obtained for various amounts of amino acids derivatized with FDAA (Table II). A known amount (pmol or nmol) of amino acids was derivatized with FDAA, and separated by two-dimensional TLC. HPLC analyses without TLC gave the same values for peak areas as those with TLC, indicating complete recovery of the diastereomers from the TLC plate. The values seem low for amino acids that contain a hydroxy group, and high for hydrophobic amino acids. The difference in the values may be due to the difference in the rate of derivatization with FDAA, which may be a stereochemically controlled reaction. However, it is a matter for further study to elucidate the cause of this difference. Quantification of both enantiomers of histidine was impossible since values for peak areas of the diastereomers were very low. The condition for FDAA derivatization was not suitable for quantification of tyrosine diastereomers, either.

TABLE II

PEAK AREA PER PICOMOLE OF AMINO ACIDS DERIVATIZED WITH FDAA, IN REVERSED-PHASE HPLC

| Amino acid | Peak area/pmol (counts × $10^{-3}$) | |
|---|---|---|
| | L-Diastereomer | D-Diastereomer |
| Asparagine | 0.693 ± 0.009 | 0.808 ± 0.059 |
| Serine | 0.473 ± 0.057 | 0.400 ± 0.036 |
| Glutamine | 0.739 ± 0.008 | 0.752 ± 0.004 |
| Threonine | 0.406 ± 0.026 | 0.506 ± 0.042 |
| Aspartate | 0.608 ± 0.058 | 0.679 ± 0.078 |
| Glutamate | 0.456 ± 0.013 | 0.642 ± 0.005 |
| Alanine | 0.781 ± 0.023 | 0.991 ± 0.059 |
| Proline | 0.760 ± 0.039 | 0.900 ± 0.017 |
| Valine | 1.113 ± 0.051 | 1.296 ± 0.094 |
| Methionine | 1.010 ± 0.003 | 1.032 ± 0.061 |
| Isoleucine | 1.141 ± 0.062 | 1.145 ± 0.005 |
| Leucine | 0.921 ± 0.041 | 0.910 ± 0.002 |
| Phenylalanine | 1.143 ± 0.063 | 1.136 ± 0.029 |
| Glycine | 0.802 ± 0.046 | |

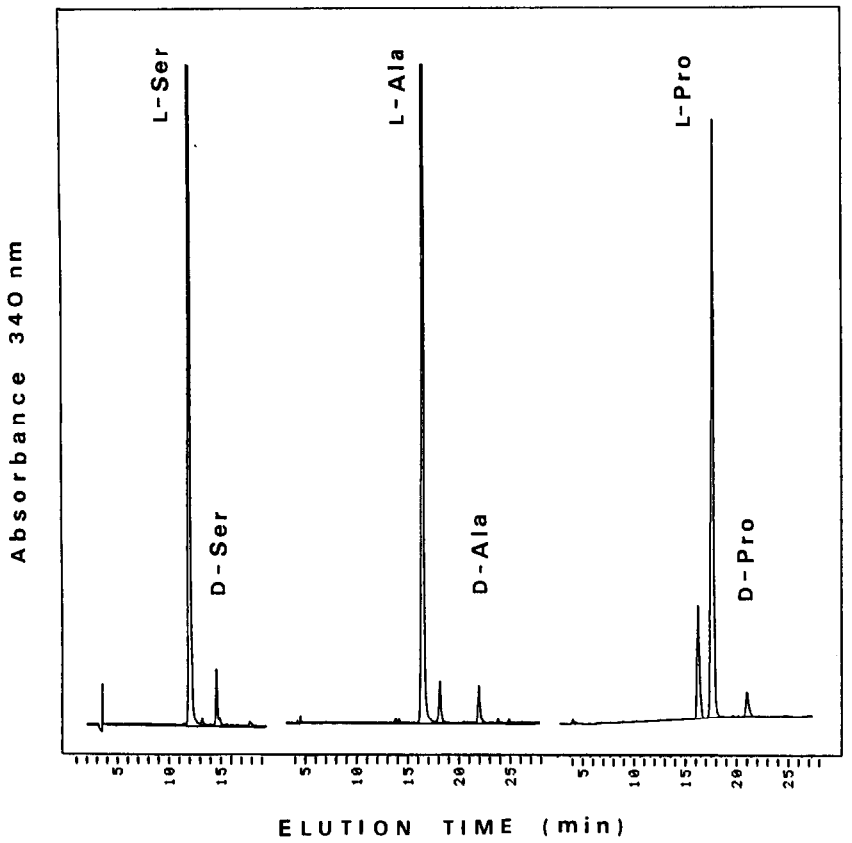


Fig. 3. HPLC profiles of free alanine, proline and serine obtained from mouse kidney. The free amino acids were extracted from the tissue homogenate as described in Experimental, derivatized with FDAA and separated by two-dimensional TLC. The diastereomers were recovered from the TLC plate and dissolved in 200 $\mu$l of methanol. A 5-$\mu$l portion was analysed by HPLC. HPLC conditions as in Experimental.

An amino acid is, thus, able to be quantified after derivatization with FDAA, referring to the value in Table II together with the value for the peak area given by the integrator in the HPLC system.

For the detection and quantification of D-amino acids in mammalian tissues, it is necessary to measure accurately small amounts of D-enantiomers in the presence of a large excess of L-enantiomers. Therefore, the analyses of D-alanine, D-proline and D-serine (Table II) were done especially at low concentrations in the presence of 50 nmol of L-enantiomers, since these three amino acids from mouse kidney were found to contain D-enantiomers. The detection limit was 20–50 pmol. No racemization occurred in D- and L-enantiomers of the three amino acids following derivatization with FDAA (data not shown).

Free amino acids extracted from the homogenate of mouse kidney were analysed by HPLC. As a result, the presence of D-enantiomers was demonstrated in only free alanine, proline and serine (Fig. 3). The D/L ratios of alanine, proline and serine were 0.035, 0.018 and 0.031, respectively, in a sample [16].

Analysis of a biological component in the presence of other components carries a risk of wrong determination when the analysis is performed by a single method. The present method appears to eliminate this risk, because it comprises two methods, TLC and HPLC.

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