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Journal of Molecular Catalysis B: Enzymatic 12 (2001) 105–108

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Enantiomeric resolution of amino acids by thin-layer chromatography

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Received 23 July 1999; received in revised form 22 September 1999; accepted 25 September 1999

Abstract

A simple and rapid method of separating optical isomers of amino acids on a reversed-phase TLC plate, without using impregnated plates or a chiral mobile phase, is described. Amino acids derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide were spotted on a reversed phase pre-coated TLC plate. Enantiomers of glutamate and aspartate were separated most effectively with solvent consisting of 25% acetonitrile in triethylamine-phosphate buffer (50 mM, pH 5.5) (v/v). Separation of L- and D-serine was achieved with 30% of acetonitrile solvent. The enantiomers of threonine, proline and alanine were separated with 35% of acetonitrile solvent, and those of methionine, valine, phenylalanine and leucine with 40% of acetonitrile solvent. The possibility of using TLC for quantitative determination of amino acid enantiomers was shown by the quantitative recovery of D- and L-alanine from the TLC plate in the range of 0.56–4.48 nmol.

Keywords: Thin-layer chromatography; Amino acid; Enantiomer; Resolution; Analysis

1. Introduction

The occurrence of D-enantiomers of amino acids has been frequently reported in various tissues of various organisms. Even in mammals, more than 20% of endogenous free serine in the cerebrum was found to have the D-configuration [1,2], and serine racemase has been partially purified from rat brain tissues [3]. The endogenous brain D-serine is thought to be a neuromodulator which binds to the glycine site of *N*-methyl-D-aspartate receptor [4]. Amino acid residues in dietary proteins were reported [5] to have been significantly racemized, such as D-aspartate in heated toast and bacon, and D-aspartate and D-phenylalanine in soybean protein and zein that had been treated with diluted alkali. Proteins containing D-amino acids are less digestible than those without, and consequently less nutritious. Increased amounts of neutral free D-amino acids have been detected in human plasma from patients with renal diseases in proportion to the degree of renal disfunction [6]. For these reasons, a quick screening of multiple biological samples for D-amino acids is desirable. Optical isomers of amino acids are usually determined by high-performance liquid-chromatography (HPLC) or gas-chromatography. However, these methods require costly equipment and are time-consuming. On the other hand, thin-layer chromatography (TLC) is a simple and inexpensive technique that requires no sophisticated instruments. Enantiomeric resolution of amino acids has been achieved using chiral TLC plates [7], or by chromatography of dansylated or

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1381-1177/01/$ – see front matter
PII: S1381-1177(00)00209-5

dinitrophenylated amino acids on impregnated TLC plates [8–10].

In the present report, we describe a simple and rapid method of separating optical isomers of amino acids on a reversed-phase TLC plate, without using expensive impregnated plates or chiral mobile phase. Our method was developed from that of Marfey [11], which resolves amino acid enantiomers through high-performance liquid chromatography (HPLC). In Marfey's method, amino acids are derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) to form diastereomers of amino acids. The enantiomeric separation of FDAA amino acids is performed by HPLC, using a reversed-phase C_{18} column and eluting with a linear gradient from 10 to 40% acetonitrile in triethylamine-phosphate buffer (pH 3.5) over 45 min [11,12].

2. Materials and methods

Amino acids were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA or Marfey's reagent [11], Pierce, Rockford, IL, USA) according to Marfey's methods [11]. Briefly, 100 μg of a DL-amino acid in 20 μl H_2O and 8 μl 1 M $NaHCO_3$ was mixed with 400 μg FDAA in 40 μl acetone, and incubated at 40°C for 1 h with occasional shaking. The reaction was terminated by adding 4 μl 2 M HCl. The acetone, water and HCl were removed by evaporation under reduced pressure in a centrifugal evaporator. After evaporation, 20 μl methanol was added to dissolve the resultant FDAA amino acid. FDAA amino-acid solution (2 μl) thus prepared (0.5%, w/v) was spotted on a reversed phase pre-coated TLC plate (RP-18 F_{254S}, 5 cm × 10 cm, Merck, Darmstadt, Germany), and developed with acetonitrile/triethylamine-phosphate buffer (50 mM, pH 5.5) at 25/75 (v/v), 30/70, 35/65 or 40/60 in a pre-equilibrated glass chamber at 25°C. The FDAA amino acid spots were yellow and visible. When the ascending solvent front neared the top margin, the plate was removed from the chamber and dried with a hair-drier. The TLC was completed in 20 min at 25°C. A trial for quantitative analysis was made by applying several different amounts of DL-alanine to the plate. The yellow spots were scraped off the plate after the chromatography, and extracted with methanol/water (1/1, v/v). The absorbance of the extracts was measured at 340 nm with a spectrophotometer. Since FDAA is light sensitive, the FDAA amino acids were protected from exposure to light during all procedures.

All reagents used were of analytical or chromatographic grade, purchased from Nacalai (Kyoto, Japan) or Sigma (St. Louis, MO, USA).

3. Results and discussion

The R_F values for each L- and D-enantiomer of amino acids, and the ratio of the R_F value for each L-enantiomer to that of the corresponding D-enantiomer (the L/D ratio of R_F) are given in Table 1. Fig. 1 shows the chromatograms of FDAA amino acids whose enantiomeric resolution is the best of several trials with various concentrations of acetonitrile from 10 to 60% in solvent, except for serine (Fig. 1A). The spots of L-enantiomers move faster than those of the corresponding D-enantiomers, i.e. the FDAA D-enantiomers have greater affinity for the C_{18} silica gel than the corresponding FDAA L-enantiomers. The enantiomers of glutamate and aspartate were separated most effectively with a solvent consisting

Table 1
Separation of FDAA amino acids on a reversed phase TLC plate

Racemate	Solvent (acetonitrile %)	R_F value		
		L	D	L/D
Ser	25	0.201	0.165	1.220
Glu	25	0.150	0.114	1.319
Asp	25	0.176	0.133	1.377
Ser	30	0.367	0.320	1.147
Glu	30	0.317	0.271	1.149
Ser	35	0.437	0.394	1.109
Glu	35	0.417	0.375	1.112
Asp	35	0.438	0.403	1.087
Thr	35	0.395	0.328	1.204
Pro	35	0.276	0.224	1.232
Ala	35	0.270	0.203	1.330
Ser	40	0.475	0.449	1.058
Glu	40	0.450	0.420	1.071
Met	40	0.326	0.239	1.364
Val	40	0.294	0.200	1.470
Phe	40	0.235	0.143	1.643
Leu	40	0.216	0.144	1.500

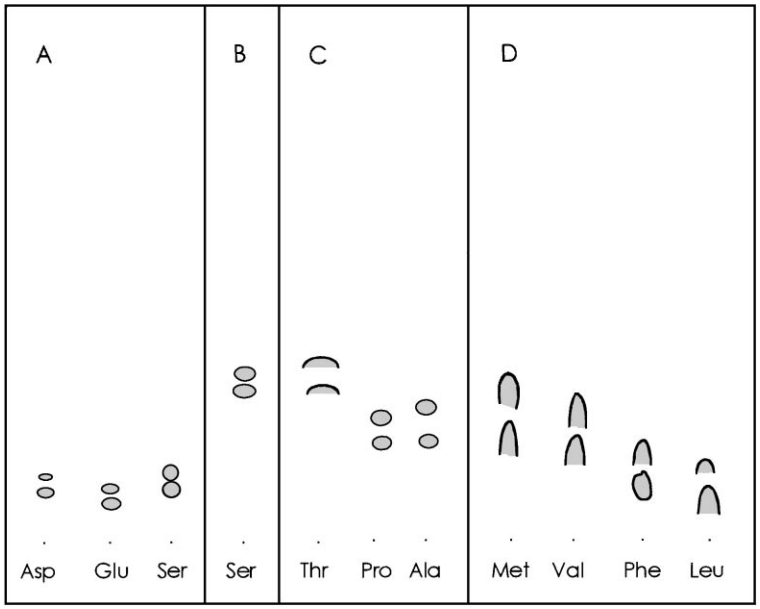

Fig. 1. Separation of optical isomers of FDAA amino acids on reversed phase TLC plates. Each racemic FDAA amino acid was spotted and developed in solvent, acetonitrile/50 mM triethylamine-phosphate buffer (pH 5.5): 25/75 (A); 30/70 (B); 35/65 (C) or 40/60, v/v (D) for 20 min at 25°C. The upper and the lower spots are L-and D-isomers, respectively, solvent front 75 mm.

of 25% acetonitrile in triethylamine-phosphate buffer (pH 5.5) (v/v) (Table 1, Fig. 1A). L- and D-serine were separated well with 30% of acetonitrile solvent (Table 1, Fig. 1B), the enantiomers of threonine, proline and alanine, with 35% of acetonitrile solvent (Table 1, Fig. 1C), and the enantiomers of methionine, valine, phenylalanine and leucine, with 40% of acetonitrile solvent (Table 1, Fig. 1D). As seen with serine and glutamate in Table 1, the R_F values of FDAA amino acids increased with increasing acetonitrile concentration in the elution solvent, while the L/D ratios of R_F values decreased. The L/D ratio of R_F value indicates the magnitude of difference of R_F values. The L/D ratio of serine was highest when eluted with 25% of acetonitrile solvent. However, the shape of the spots was different from that developed with 30% of acetonitrile solvent: the spots formed with 25% of acetonitrile solvent were slightly longer than those formed with 30% of acetonitrile solvent (Fig. 1A and B). Because of these factors, the best separation of serine enantiomers was achieved with 30% of acetonitrile solvent, indicating that the shape of the spot is important for a clear separation, in addition to the difference in R_F values between the L- and D-enantiomers. In spite of the elongation of the spots, sufficient separation of the optical isomers of methionine, valine, phenylalanine and leucine was achieved (Fig. 1D) because of the considerable difference in the R_F values. The width of the FDAA amino acid spots decreased with reduced application of sample, but their length remained unchanged. The separation of the enantiomers of FDAA-asparagine and -glutamine was less complete than that of the above amino acids. A better separation was obtained by repeating the chromatography, i.e. by putting the developed and dried plate back into the glass chamber for further development. The enantiomers were well-separated after the third development. The enantiomers of histidine were separated by two-dimensional TLC [12], using a plate coated with ordinary silica gel and developed in the first direction with 1-butanol/acetic acid/water and in the second direction by phenol/water.

All phenylthiohydantoin (PTH) amino acids are sensitive to light, and the PTH amino acids, which are optically active racemize easily [13]. The formation of FDAA amino acids, however, proceeds quantitatively and no racemization occurs during the reaction [12]. In this study, the amount of FDAA

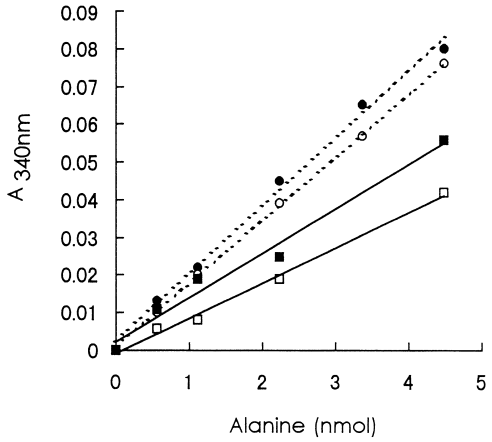

Fig. 2. Quantitative recovery of FDAA L-and D-alanine from reversed phase TLC plate. Various amounts of racemic FDAA alanine were spotted on the TLC plate and developed. After the separation of FDAA alanine into enantiomers on the plate, each spot was scraped from the plate, 1.0 ml water was added to the dried scrapings and the absorbance at 340 nm was measured using a spectrophotometer ((–□–), FDAA L-alanine; (–■ –), FDAA D-alanine). The same amount of either of FDAA L-alanine or FDAA D-alanine as applied to the TLC plate was added to 1.0 ml water, and the absorbance at 340 nm was measured ((. . . ○. . .) FDAA L-alanine; (. . . ●. . .) FDAA D-alanine).

alanine extracted from the plate was linearly related to that of FDAA alanine spotted onto the plate, within the range of 0.56–4.48 nmol applied (Fig. 2). Therefore, given a standard calibration curve, this method can be used for quantitative determination of the concentration of each enantiomer of any amino acid.

The enantiomeric determination of free amino acids in biological samples and foods may be carried out as follows: the free amino acids in a homogenate of the sample are separated from proteins by trichloroacetic-acid treatment, and are then purified by ion-exchange chromatography, and treated with FDAA. Each FDAA amino acid can be separated from the others by two-dimensional TLC [12]. Any FDAA amino acid can then be extracted from the plate with methanol/water (1/1, v/v), and subsequently analyzed by the method described in this paper to determine its enantiomeric configuration. In the presence of excess amounts of L-enantiomer over the corresponding D-enantiomer, treatment of the free amino acid sample with L-amino acid oxidase (EC 1.4.3.2) may be required before the formation of FDAA derivatives. This treatment prevents the tail end of a large L-enantiomer spot from overlapping with the small D-enantiomer spot on the TLC plate.

Szókán et al. [14] separated FDAA D- and L-amino acids by HPLC using a reversed phase column and methanol/Na-acetate buffer eluent system. Since a good separation of D- and L-serine was reported (the retention time was 5.6 and 10.0 min for L- and D-serine, respectively) [14], we tried to separate the optical isomers of serine by TLC using the reversed phase plate employed in the present method, and the same buffer as above, i.e. methanol/20 mM Na-acetate buffer (pH 4.0) (1/4, v/v). The enantiomers could not be separated with this system, however, all the spots on the TLC plate were connected to the point of sample application by long tails.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Cooperative Research (A) (No. 07308048) to YN from the Ministry of Education, Science and Culture of Japan.

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