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Determination of free amino acid enantiomers in rat brain and serum by high-performance liquid chromatography after derivatization with N-*tert.*-butyloxycarbonyl-L-cysteine and *o*-phthaldialdehyde

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ABSTRACT

The concurrent determination of free amino acid enantiomers and non-chiral amino acids in rat brain and serum was accomplished by high-performance liquid chromatography with fluorimetric detection after derivatization with N-*tert.*-butyloxycarbonyl-L-cysteine and *o*-phthaldialdehyde. The method revealed the presence of a large amount of free D-serine (0.22 μmol/g of tissue; D/D + L ratio = 0.25) in the brain whereas D-aspartate and D-alanine were established to be at trace levels. These results further support the presence of D-serine in adult brain tissues as demonstrated by recent work using gas chromatography.

INTRODUCTION

It has long been observed that proteins and free amino acid pools in mammals exclusively consist of L-amino acid isomers [1]. However, recent studies have provided evidence indicating that mammalian brain tissues contain appreciable amounts of D-amino acids in free form [2–6] or in metabolically stable proteins [7,8]. Although enzymatic, gas chromatographic (GC) and high-performance liquid chromatographic

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(HPLC) methods have been successfully employed to measure D-amino acids, these techniques have some disadvantages, which include low specificity (*e.g.*, enzymatic assay), time-consuming derivatization (*e.g.* GC analysis) or complicated extraction procedures [3,9]. Further, they do not allow the concurrent detection and determination of many amino acid enantiomers and some non-chiral amino acids such as glycine, *β*-alanine, O-phosphoethanolamine (O-PE), taurine and *γ*-aminobutyric acid (GABA) [9–15].

Recently, the simultaneous determination of a large number of chiral amino acids has been achieved by conventional reversed-phase HPLC following derivatization with *o*-phthaldialdehyde (OPA) and a variety of thiols including mercaptoethanol, N-acetyl-L-cysteine, N-acetyl-D-penicillamine and N-*tert.*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) [10–15]. The diastereoisomeric isoindolyl derivatives formed with Boc-L-Cys and OPA have been applied to the analysis of the amino acid composition and enantiomeric purity of peptides and proteins [11–13]. However, the determination of free DL-amino acids in biological samples has never been performed by this derivatization technique owing to the difficulties in the simultaneous separation of various amino acid enantiomers and non-chiral amino acids. We have now developed a method for the concurrent measurement of not only chiral but also non-chiral amino acids in the brain and serum using an HPLC method and fluorimetric detection with this derivatization procedure. The method is rapid for sample preparation, shows high resolution and sensitivity and has demonstrated that a considerable amount of free D-serine is present in the brain tissues of the mature rat.

EXPERIMENTAL

Reagents

All amino acids except L-homocysteic and (L-HCA), trichloroacetic acid (TCA), glacial acetic acid and OPA were obtained from Nacalai Tesque (Kyoto, Japan). L-HCA, Boc-L-Cys (optical purity >99.0%) and all solvents of HPLC grade were purchased from Sigma (St. Louis, MO, USA), Novabiochem (Läufelfingen, Switzerland) and Kanto Chemicals (Tokyo, Japan), respectively. Water was purified to ultra-pure quality by means of a Milli-Q system (Millipore, Tokyo, Japan).

Amino acid stock solutions were prepared by dissolving the individual amino acids in 0.01 *M* hydrochloric acid to a concentration of 0.1 μmol/ml with the exceptions of histidine, asparagine, glutamine and tryptophan, which were dissolved in 20% aqueous methanol. The stock solutions were used for calibration after appropriate dilution with 0.01 *M* hydrochloric acid. L-HCA was used as an internal standard according to the method of Patchett *et al.* [16].

Apparatus

The HPLC system consisted of a Yanaco L-4000W pump, a Yanaco SP-2000 solvent programmer (Yanaco, Tokyo, Japan), a KSP-600 autosampler (Kyowa Seimitsu, Tokyo, Japan), an 821-FP spectrofluorimeter with a 5-μl flow-cell (Jasco International, Tokyo, Japan), an ERC-3510 degasser (Erma Optical Works, Tokyo, Japan) and a 4-μm Nova-Pak C_{18} column (300 mm × 3.9 mm I.D.) (Waters, Tokyo, Japan) connected to a 20 mm × 4.6 mm I.D. precolumn (Eicom, Tokyo, Japan). Excitation and emission wavelengths were 344 and 443 nm, respectively. The column was operated at a constant flow-rate of 0.8 ml/min at 30°C. Data processing was performed using a SIC Chromatocorder 12 integrator (System Instruments, Tokyo, Japan).

An acetate buffer was prepared using 0.1 *M* glacial acetic acid adjusted to pH 6.0 with sodium hydroxide. Mobile phase A was the acetate buffer with 7% acetonitrile and 3% tetrahydrofuran (THF) and mobile phase B was the acetate buffer with 47% acetonitrile and 3% THF. The separation of amino acids was accomplished with a linear gradient from mobile phase A to B in 120 min.

Animals and tissue preparation

Male Wistar rats (Shizuoka Laboratory Animals and Clea Japan, Tokyo, Japan) weighing 215–235 g at the time of the experiments were

used. The animals were housed at 22.0 ± 0.5°C in a humidity-controlled room under a 12-h light–dark cycle (light on at 8 a.m.) and were allowed food and water *ad libitum*.

The perfusion of rat brain by 0.9% NaCl solution was carried out under pentobarbital anaesthesia (40 mg/kg, intraperitoneally) to eliminate contamination by amino acids in the blood. The perfused brain tissue, which contains telencephalon, diencephalon and midbrain, was dissected out on ice and stored at −80°C until used.

After the addition of L-HCA as an internal standard, the brain tissue was homogenized at 4°C in ten volumes of 5% TCA and the homogenate was centrifuged at 18 000 *g* for 30 min at 4°C. To remove TCA, the supernatant was extracted three times with water-saturated diethyl ether. The aqueous layer was then passed through a Millipore HV filter (0.45 μm) and stored at −80°C until derivatization.

Derivatization procedure

The Boc-L-Cys–OPA reactions with D- and L-amino acids were performed by previously described methods [11–13] with some modifications. The derivatization reagent was prepared daily by dissolving 50 mg of OPA and 50 mg of Boc-L-Cys in 5 ml of methanol. A borate buffer was made using 0.4 *M* boric acid adjusted to pH 9.0 with sodium hydroxide. A 200-μl volume of Boc-L-Cys–OPA reagent and 700 μl of the borate buffer were added to a vial containing 100 μl of the amino acid standard or sample. After derivatization for 2 min at ambient temperature, an aliquot (10 μl) of the reaction mixture was introduced into the HPLC system.

Calculations

The mean value of the ratios between the peak heights of the amino acid (AA) and the internal standard (I.S.) for the standard samples was used to calculate the AA concentration in the unknown samples according to the following equation:

AA (μmol/g wet weight) = (sample AA/I.S.) × (standard I.S./AA) × (μmol I.S. added/g wet weight)

RESULTS

Fig. 1 and Table I show a representative chromatogram and retention times of Boc-L-Cys-

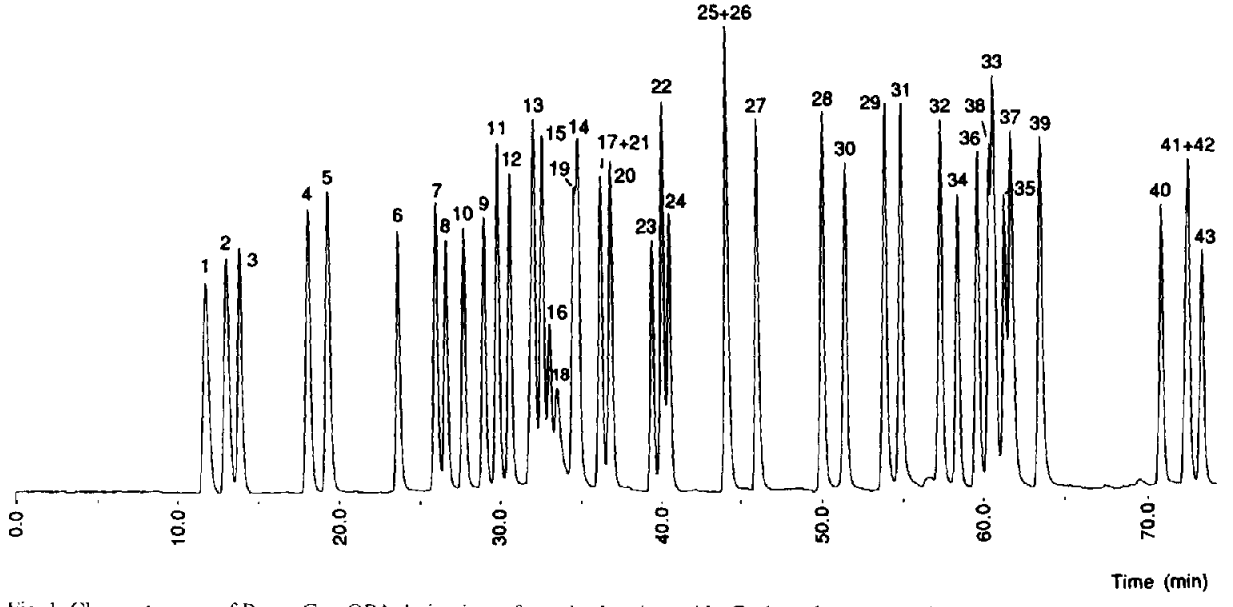

Fig. 1. Chromatogram of Boc-L-Cys–OPA derivatives of standard amino acids. Each peak corresponds to 25 pmol of free amino acid. For chromatographic conditions and peak numbers of amino acids, see Experimental and Table I, respectively.

TABLE I

RETENTION TIMES AND RESOLUTION FACTORS OF BOC-L-CYS–OPA DERIVATIVES OF STANDARD AMINO ACIDS

Chromatographic conditions as under Experimental. Abbreviations: HCA = L-homocysteic acid; O-PE = O-phosphoethanolamine; GABA = γ-aminobutyric acid.

Peak No.	Amino acid	Retention time (min)		Resolution factor
		L-Form	D-Form	
1, 2	Aspartate	11.7	13.0	4.3
3	HCA	13.8	–	–
4, 5	Glutamate	18.1	19.3	3.8
6, 7	Asparagine	23.6	25.9	5.5
8, 9	Serine	26.6	29.0	6.1
10, 11	Glutamine	27.7	29.8	5.3
12	Citrulline	30.6	–	–
13, 14	Threonine	32.1	34.8	6.6
15	Glycine	32.6		-
16, 17	Histidine	33.1	36.1	9.5
18	O-PE	33.5		–
19, 20	Arginine	34.6	36.7	5.4
21, 22	Alanine	36.1	40.0	9.6
23	β-Alanine	39.4		-
24	Taurine	40.5		–
25	GABA	44.1		–
26, 27	Tyrosine	44.1	46.0	4.6
28, 29	Valine	50.0	53.8	10.1
30, 31	Methionine	51.4	54.8	8.9
32, 33	Isoleucine	57.3	60.7	8.7
34, 35	Tryptophan	58.4	61.4	8.2
36, 37	Phenylalanine	59.6	61.8	5.9
38, 39	Leucine	60.4	63.5	8.1
40, 41	Ornithine	70.8	72.5	3.4
42, 43	Lysine	72.5	73.3	2.0

OPA derivatives of authentic amino acids. The Boc-L-Cys–OPA derivatives gave good resolutions for most of the amino acid enantiomers and non-chiral amino acids. The diastereomeric isoindolyl derivatives of DL-aspartate, DL-glutamate, DL-asparagine, DL-serine, DL-glutamine, L-citrulline, L-threonine, glycine, L-histidine, O-PE, D-arginine, D-alanine, β-alanine, taurine, DL-valine, DL-methionine, L-isoleucine, DL-tryptophan, DL-phenylalanine, D-leucine, L-ornithine and D-lysine were separated within 75 min. However, our chromatographic system failed to obtain good separations of the peaks corresponding to GABA, L-arginine, L-alanine, L-tyrosine, L-leucine, L-lysine, D-threonine, D-histidine and D-ornithine. Thus, all L-enantiomers and non-chiral amino acids were separated with the only exception being the overlap of GABA and L-tyrosine in this system, indicating that this HPLC system may be suitable not only for the separation of various amino acid enantiomers but also for that of amino acids which only consist of the L-enantiomer in the biological samples.

From a technical point of view, it seems noteworthy that a small amount of THF (3%) in the mobile phase improved the resolutions of L-serine–D-asparagine and taurine–D-alanine without altering those of the other amino acids. Further,

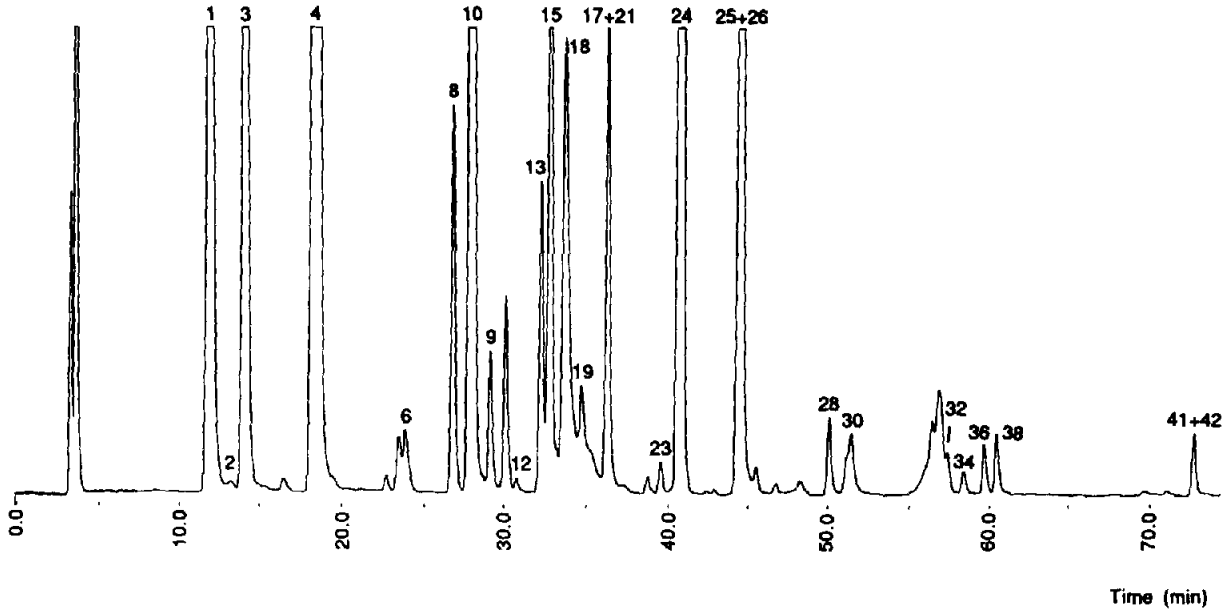

Fig. 2. Chromatogram of Boc-L-Cys–OPA derivatives of free amino acids in the brain. The concentration and the ratio (D/D + L) of free D-serine in rat brain were calculated to be 0.22 μmol/g of tissue and 0.25, respectively. For chromatographic conditions and peak numbers of amino acids, see Experimental and Table I, respectively.

a higher column temperature was detrimental to the resolutions of amino acid enantiomers (data not shown).

The fluorescence responses of the amino acid derivatives were linear over the range 2.5–500 pmol per injection (data not shown). The detection limits were in the lower picomole range for each of the amino acids, and are comparable to those obtained by HPLC with fluorimetric detection as reported in previous studies [15].

TABLE II

CONCENTRATIONS OF FREE D-SERINE AND THE RATIO OF FREE D-SERINE TO TOTAL SERINE IN RAT BRAIN AND SERUM

Values represent means with S.D. for five or six samples.

Amino acid	Brain (μmol/g wet weight)	Serum (nmol/ml)
D-Serine	0.22 ± 0.04	3.0 ± 0.4
L-Serine	0.66 ± 0.11	272.4 ± 37.0
Total serine	0.88 ± 0.14	275.4 ± 36.8
Ratio (%)	24.6 ± 0.9	1.1 ± 0.2

The HPLC method described here was applied to the determination of free amino acids in rat brain tissue and serum. As shown in Fig. 2, a peak with a substantial height co-eluted with the Boc-L-Cys–OPA derivative of authentic D-serine in the brain. The content of the D-amino acid was calculated to be 0.22 μmol/g of tissue, which was 24.6% of the total serine concentration (Table II). In contrast to the brain tissue, a very low level of D-serine was determined in rat serum (3 nmol/ml, D-serine/total serine = 1.1%) (Fig. 3). Moreover, trace amounts of D-aspartate and D-alanine were present in the brain (< 10 nmol/g of tissue) and serum, respectively, whereas no other D-amino acid was observed in the present HPLC analysis. Table III summarizes the concentrations of L- and non-chiral amino acids and total serine in the brain tissue and serum; these values are in agreement with previous results [17].

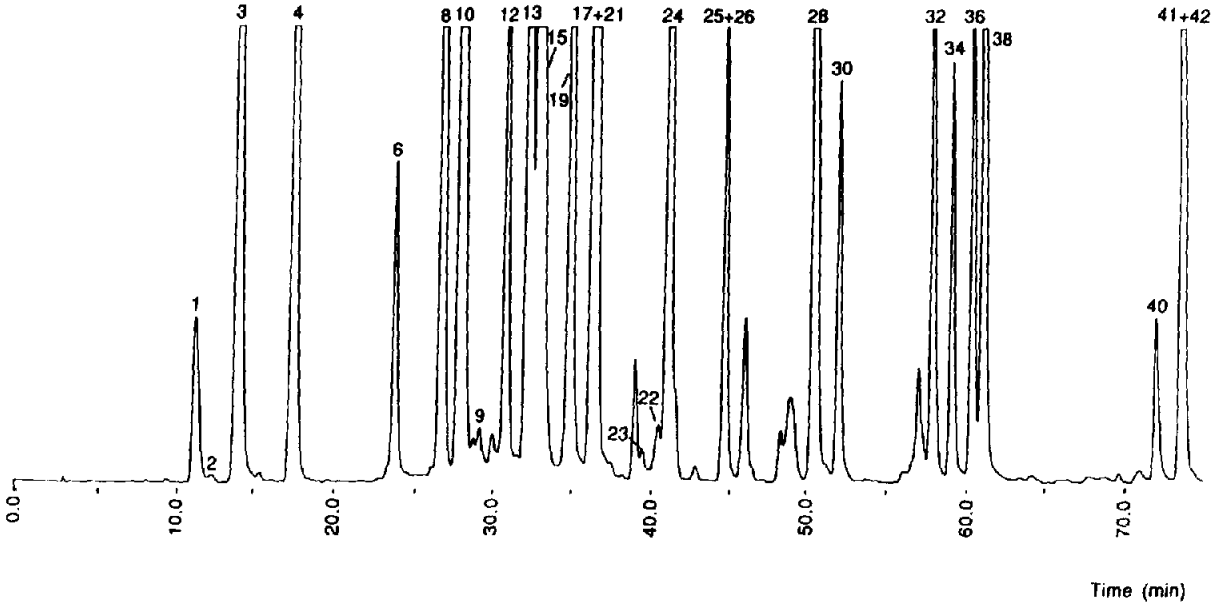

Fig. 3. Chromatogram of Boc-L-Cys-OPA derivatives of free amino acids in serum. The concentration and the ratio (D/D + L) of free D-serine in rat serum were calculated to be 3 nmol/ml and 0.01, respectively. For chromatographic conditions and peak numbers of amino acids, see Experimental and Table I, respectively.

DISCUSSION

The HPLC–fluorimetric assay described here provides a simple and sensitive method for the simultaneous determination of a large number of amino acid enantiomers and non-chiral amino acids in biological samples. This method offers several advantages over previously published methodologies: (1) it requires only minimum sample preparation in comparison with the more complicated time-consuming sample pretreatments (gas, ion-exchange or thin-layer chromatography); (2) despite its simplicity, the detection limits are comparable to those of other HPLC methods with fluorescence detection; (3) to our knowledge, no previously established method allows the concurrent detection of stereoisomers of aspartate, asparagine, serine, glutamate, glutamine, alanine, etc., and of non-chiral amino acids such as glycine, O-PE, β-alanine and taurine without interference; and (4) it has been shown to be suitable for the measurement of amino acid enantiomers in mammalian brain and serum. The reliability of the method is supported by the fact that the levels of L- and non-chiral amino acids in the rat brain tissue and serum in this study were consistent with those obtained previously using GC and HPLC techniques [6,17].

The present results have confirmed the existence of free D-serine in rat brain, which was re-

TABLE III

CONCENTRATIONS OF AMINO ACIDS IN RAT BRAIN AND SERUM

Values represent means with S.D. for five or six samples. Chromatographic conditions as under Experimental.

Amino acid	Brain (μmol/g wet weight)	Serum (nmol/ml)
L-Aspartate	2.69 ± 0.12	45.0 ± 9.1
L-Glutamate	11.48 ± 0.36	180.5 ± 23.1
L-Asparagine	0.06 ± 0.01	62.3 ± 5.8
Total serine	0.88 ± 0.14	275.4 ± 36.8
L-Glutamine	5.32 ± 0.15	725.6 ± 30.2
Glycine	0.81 ± 0.07	436.3 ± 74.8
L-Alanine + D-histidine	0.75 ± 0.07	631.7 ± 80.2
Taurine	7.24 ± 0.12	340.3 ± 77.4

cently discovered by GC–mass spectrometric analysis [6], and has demonstrated for the first time that the level of free D-serine is much higher in the brain than in the serum of the adult rat. D-Serine detected by our HPLC method could occur as a result of the possible racemization of L-serine during sample preparation. However, no racemization occurred in the authentic L-serine in this and similar assay procedures [14]. The very low ratio of D-serine to total serine in the serum appears to rule out the possibility that part of the D-serine in the biological samples may be formed by racemization of the L-form during sample preparation. Further, the observation that the level of brain D-serine determined by the present HPLC method is similar to that given by GC with a completely different derivatization and detection process [6] appears to support the accuracy of quantification by the HPLC–fluorescence detection technique.

The present HPLC and our previous GC analyses indicate that the concentration and D/D + L ratio of serine in the brain tissues are exceptionally high among those of the free D-amino acids determined in various tissues from young adult or aged mammals. Although the neonatal rat [2,5] and the mutant ddY mouse lacking D-amino acid oxidase [18] have been shown to have larger amounts or D/L ratios of D-aspartate, D-alanine and D-proline in the brain and peripheral tissues than the adult rat or normal ddY mouse, these levels are still lower than those of D-serine in adult rat brain.

The origin and metabolizing pathways of free D-serine in the brain tissues remain unclear. The transport across the blood brain barrier of neutral amino acids such as glycine, serine and alanine is slow [19,20] and prefers L- to D-amino acids [21]. The D-enantiomers of free neutral amino acids are good substrates for D-amino acid oxidase in the liver and kidney, and are excreted into the urine [22]. In addition, a trace amount of free D-serine in rat serum was detected in this study and the concentration of the amino acid in the brain of germ-free rat was identical with that of the specific pathogen-free rat used in the present experiments (unpublished data). These findings suggest that the free D-serine in rat brain may not result from the accumulation of D-serine derived from the diet and the intestinal microbial flora.

Several lines of evidence have accumulated showing that D-serine acts as a selective agonist at the strychnine-insensitive glycine binding site associated with the N-methyl-D-aspartate (NMDA)-type excitatory amino acid receptor complex [23–27]. The D-isoform of serine, which is equipotent to glycine itself, is about 100 times more potent than the L-isoform in displacing [^{3}H]glycine binding to the allosteric glycine site and in enhancing NMDA receptor-mediated responses [25]. The binding site of [^{3}H]-D-serine has been shown to share the same pharmacological profiles with the [^{3}H]glycine binding site [28]. Moreover, an intracerebroventricular application of D-serine potentiates the NMDA-induced seizure susceptibility [26] and reverses the behavioral effects of non-competitive antagonists of the NMDA receptor [27]. These data suggest that free D-serine might be a novel candidate for an endogenous ligand for the NMDA receptor-related glycine binding site in the brain. The present simple and reliable HPLC method for the detection of D-serine will make a contribution to the investigation of the physiological and pathophysiological roles of D-serine in mammalian tissues.

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