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Simultaneous determination of D-aspartic acid and D-glutamic acid in rat tissues and physiological fluids using a multi-loop two-dimensional HPLC procedure☆

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ABSTRACT

For a metabolomics study focusing on the analysis of aspartic and glutamic acid enantiomers, a fully automated two-dimensional HPLC system employing a microbore-ODS column and a narrowbore-enantioselective column was developed. By using this system, a detailed distribution of D-Asp and D-Glu besides L-Asp and L-Glu in mammals was elucidated. For the total analysis concept, the amino acids were first pre-column derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) to be sensitively and fluorometrically detected. For the non-stereoselective separation of the analytes in the first dimension a monolithic ODS column (750 mm × 0.53 mm i.d.) was adopted, and a self-packed narrowbore-Pirkle type enantioselective column (Sumichiral OA-2500S, 250 mm × 1.5 mm i.d.) was selected for the second dimension. In the rat plasma, RSD values for intra-day and inter-day precision were less than 6.8%, and the accuracy ranged between 96.1% and 105.8%. The values of LOQ of D-Asp and D-Glu were 5 fmol/injection (0.625 nmol/g tissue). The present method was successfully applied to the simultaneous determination of free aspartic acid and glutamic acid enantiomers in 7 brain areas, 11 peripheral tissues, plasma and urine of Wistar rats. Biologically significant D-Asp values were found in various tissue samples whereas for D-Glu the values were very low possibly indicating less significance.

1. Introduction

Except for glycine all proteinogenic α-amino acids are chiral and it was long believed that only the L-forms are present in higher animals such as mammals. However, since the 1980s, with the progress of modern analytical technologies, also the presence of naturally occurring D-amino acids in mammals has been revealed [1–3], such as D-serine (D-Ser) [4–6], D-aspartic acid (D-Asp) [7–13], D-alanine (D-Ala) [14–16], D-leucine (D-Leu) [17–19] and D-glutamic acid (D-Glu) [20–23]. Among these D-amino acids, D-Asp is found in various endocrine tissues of mammals (e.g., the pineal gland [8], testis [9], pituitary gland [10], and adrenal gland [11,12]) and is involved in hormonal synthesis and secretion. Both D-Asp and D-Glu have effects on regulating neuronal transmission [24–27], and these two D-amino acids are metabolized by the same enzyme, D-aspartic acid oxidase [28]. Therefore, for the metabolomics studies focusing on acidic amino acid enantiomers, the correlation between the intrinsic levels of D-Asp and D-Glu, as well as the correlations between their amounts and those of their L-enantiomers, are matters of interest.

The aim of the present study is to establish a sensitive and selective micro-2D-HPLC method for the simultaneous determination of Asp and Glu enantiomers in mammals, and to clarify their intrinsic distribution in the brain and peripheral tissues. Until now, various methods using GC [29,30], HPLC [17,20,21] and CE [22,23,31] have been reported for the simultaneous determination of free D-Asp and D-Glu, in biomatrices of rats, mice, and human beings. Using the GC method equipped with a Chirasil-L-Val chiral stationary phase (CSP), the distribution of D-Asp in the brain and peripheral tissues, and the amounts of D-Asp and D-Glu in the physiological fluids were determined (although in the forms of Asx and Glx) [29,30]. HPLC methods with chiral derivatization reagents such as *o*-phthaldialdehyde (OPA) plus chiral thiols were the most widely used ones for the determination of D-Asp and D-Glu [20,21]. Because of their simplicity and practically acceptable sensitivity, these HPLC methods were often applied to the determination of D-Asp and D-Glu in various mammalian tissues and physiological fluids. Recently, the CE [23,31]

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and MCE [22] methods were also reported for the determination of D-Asp and D-Glu in neuronal tissues, urine and cerebrospinal fluid.

By using these methods, several papers describe in detail the distribution of D-Asp in mammals; however, there are only few reports on the tissue distribution of intrinsic D-Glu in mammals. In addition, the reported values of D-Glu were different even in similar biological matrices [20,22,32,33]. Because the determination of small amounts of hydrophilic D-amino acids, such as D-Glu, is frequently interfered with unknown intrinsic substances of the complex biological matrices, it is necessary to investigate the distribution of D-Glu in mammals with a selective, accurate and precise method to evaluate the physiological importance and diagnostic value. Therefore, in the present investigation, a highly sensitive and selective 2D-HPLC system was established for the simultaneous determination of the enantiomer pairs of Asp and Glu in mammalian tissues and physiological fluids. This HPLC system employs a combination of a reversed-phase separation unit using a monolithic microbore-ODS column and an enantiomer separation unit using a self-packed narrowbore-enantioselective Pirkle-type column [16,34], and in the present study, the 2D-HPLC system was modified and optimized for the rapid metabolomics study focusing on Asp and Glu enantiomers. For chromatographic but also for sensitivity purposes, the amino acids get pre-column derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F, [35]). As biomatrices, 7 brain areas, 11 peripheral tissues, plasma and urine samples of rats were analyzed.

2. Experimental

2.1. Materials

D-Asp and L-Asp were obtained from Nacalai Tesque (Kyoto, Japan); D-Glu and L-Glu were obtained from Wako (Osaka, Japan). The derivatizing reagent, NBD-F was from Tokyo Kasei (Tokyo, Japan). Trifluoroacetic acid (TFA), citric acid monohydrate and boric acid were from Wako. Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were purchased from Wako and Nacalai Tesque, respectively. Water was purified using a Milli-Q gradient A 10 system (Millipore, Bedford, MA, USA). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Animals

Male Wistar rats (6 weeks of age, specific-pathogen-free) were purchased from Kyudo (Tosu, Japan), and they were housed in an animal center at Kyushu University, Graduate School of Pharmaceutical Sciences. All experiments were performed with the permission (A21-005-0) of the Animal Care and Use Committee of Kyushu University.

2.3. Sample preparation procedure

The rats were anesthetized with pentobarbital (0.125 mg/g body weight). The blood collected from the abdominal aorta was transferred to a heparinized tube (Nippon Becton Dickinson, Tokyo, Japan), and then centrifuged at 8000 × *g* and at 4 °C for 15 min to obtain the plasma. The urine was collected from the urinary bladder. The pineal gland, pituitary gland, cerebral cortex, hippocampus, hypothalamus, cerebellum, medulla oblongata, spinal cord, spleen, pancreas, adrenal gland, kidney, liver, testis, thymus, heart, lung and muscle were quickly excised and stored at −80 °C until their use after measuring their wet weight. To the plasma and urine (20 μL), 380 μL of MeOH was added and mixed for 2 min. The tissues were homogenized at 3500 rpm for 2 min in 20-fold volume of MeOH (pineal gland was homogenized in 200-fold volume of MeOH) using a micro-homogenizing system (Micro Smash™ MS-100R, Tomy, Tokyo, Japan) at 4 °C. The obtained MeOH mixtures or homogenates were centrifuged at 12,100 × *g* for 10 min, and the supernatants were collected and stored at −20 °C. An aliquot (10 μL) of the supernatant was evaporated to dryness under reduced pressure at 40 °C. To the residue, 10 μL of H_2O, 10 μL of 400 mM sodium–borate buffer (pH 8.0) and 10 μL of 20 mM NBD-F in anhydrous MeCN were added, then heated at 60 °C for 2 min. To the reaction mixture, 95 μL of 2 vol% TFA in H_2O was added, and 2 μL was subjected to the HPLC. In order to determine high amount of L-Asp and L-Glu, the reaction mixture was diluted with aqueous 2 vol% TFA depending on the intrinsic amount of L-amino acids, and 2 μL was re-subjected to the HPLC.

2.4. HPLC system for the determination of Asp and Glu enantiomers

The HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo, Japan) consisted of a type 3202 degasser, 3101 and 3201 pumps, a 3033 auto sampler, a 3004 column oven, two 3013 fluorescence detectors, a 3011 column-switching high pressure valve and a dual-loop valve (the volume of each loop is 150 μL). A data processing program, EzChrom Elite Client, was used to monitor the detector response and a column-switching valve and a dual-loop valve were controlled by a KSAA valve controlling system (Shiseido). The flow diagram of the HPLC system is shown in Fig. 1. The analytical column for the reversed-phase separation was a monolithic microbore-ODS column (750 mm × 0.53 mm i.d., prepared in a fused silica capillary, provided from Shiseido) maintained at 40 °C, and the mobile phase for reversed-phase separation was MeCN–TFA–water (8:0.05:92, v/v) with the flow rate of 20 μL/min. After the reversed-phase separation, the fraction of NBD-Asp (50 μL, 150 s) was immediately transferred to the enantioselective column via the column-switching valve (using loop 1 of the dual-loop valve), and the fraction of NBD-Glu was stored in loop 2 until the enantiomer separation of NBD-Asp was completed, then automatically introduced to the enantioselective column via the column-switching valve. For the separation of NBD-Asp and NBD-Glu enantiomers, a Sumichiral OA-2500S column having (S)-naphthylglycine as the chiral selector (250 mm × 1.5 mm i.d., self-packed, material was from Sumika Chemical Analysis Service, Osaka, Japan) was used at 25 °C. The mobile phase was 2 mM citric acid in MeOH–MeCN (50:50, v/v) with the flow rate of 200 μL/min. Fluorescence detection was carried out at 530 nm with excitation at 470 nm.

3. Results and discussion

3.1. Establishment of a micro-2D-HPLC system for the determination of Asp and Glu enantiomers as their NBD-derivatives

For the determination of Asp and Glu enantiomers in biological samples, a two-dimensional micro-HPLC system employing a microbore-reversed phase column and a narrowbore-enantioselective column was established as outlined in Fig. 1. By the present method the NBD derivatives of D- and L-Asp as well as of D- and L-Glu were non-stereoselectively separated in the first dimension on a microbore-ODS column. The fractions of NBD-Asp and NBD-Glu peaks were automatically transferred to a dual-loop valve and introduced onto a narrowbore-enatioselective column as a second dimension by which the enantiomer separations were carried out.

Several microbore-ODS columns including packed ODS columns and monolithic-ODS columns were tested for selecting the col-

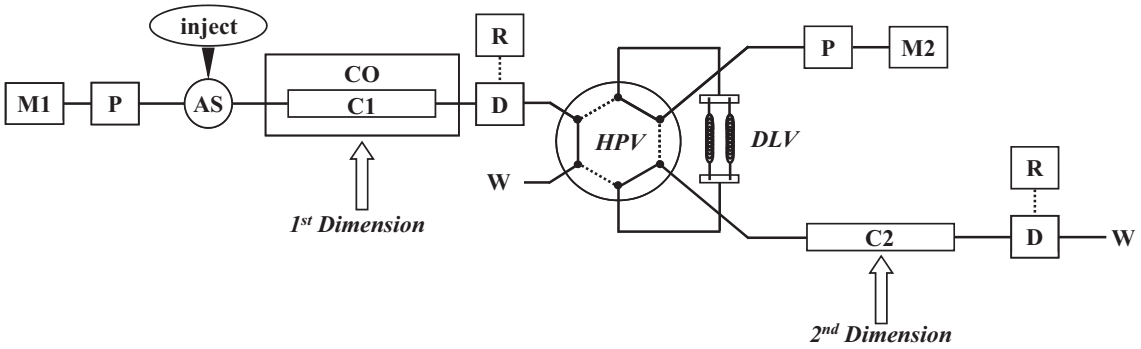

Fig. 1. Flow diagram of the 2D-HPLC system for the determination of NBD-Asp and NBD-Glu enantiomers. M, mobile phase; P, pump; AS, auto sampler; CO, column oven; C, column; D, detector; R, integrator; HPV, high pressure valve; DLV, dual-loop valve; W, waste.

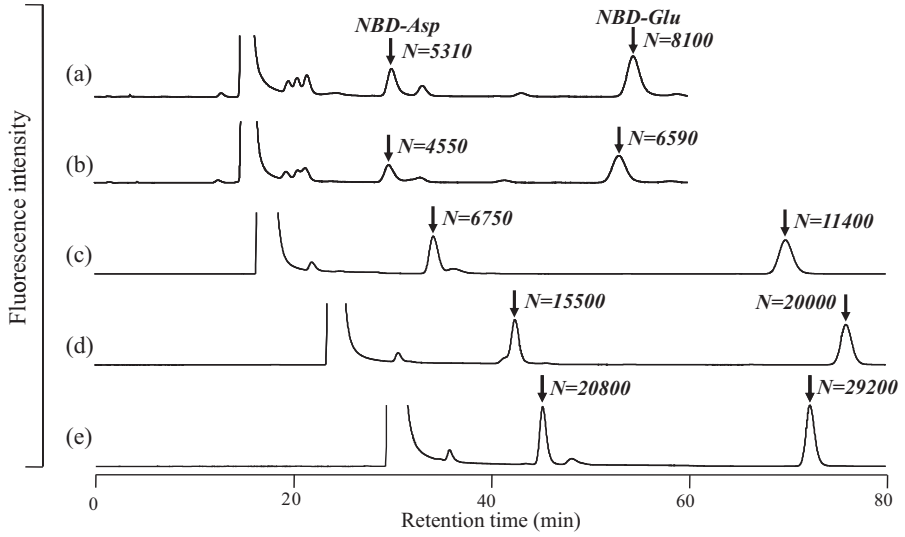

Fig. 2. Separation of NBD-Asp and NBD-Glu using various microbore-ODS columns. (a) CAPCELL PAK C18 MGII S3 (150 mm × 0.50 mm i.d., particle size 3 μm), (b) CAPCELL PAK C18 MGII S5 (150 mm × 0.50 mm i.d., particle size 5 μm), (c) monolithic ODS column (250 mm × 0.53 mm i.d.), (d) monolithic ODS column (500 mm × 0.53 mm i.d.), (e) monolithic ODS column (750 mm × 0.53 mm i.d.). Aqueous solutions containing 2–6 vol% MeCN and 0.05 vol% TFA were used as mobile phases (5% MeCN for CAPCELL PAK C18 MGII, 2% MeCN for monolithic ODS column of 250 mm, 4% MeCN for monolithic ODS column of 500 mm, 6% MeCN for monolithic ODS column of 750 mm).

umn for the first dimension. Fig. 2 shows the results obtained by the particle-packed columns of 150 mm length (a and b), and by the monolithic column of 250 mm (c), 500 mm (d) and 750 mm length (e). The mobile phase was selected to give the appropriate retention of NBD-Glu of around 60 min. The column pressure for the 3-μm particle-packed column was 15.2 MPa, and that for the 5-μm particle-packed column was 5.6 MPa. The monolithic column of 750 mm length gave the highest plate numbers ($N > 20{,}000$ for both NBD-Asp and NBD-Glu) and had a practically acceptable low column pressure of 8.5 MPa. According to these results, a monolithic-ODS column of 750 mm × 0.53 mm i.d. was selected. The mobile phase for the first dimension was investigated using 10 hydrophilic amino acids by changing the content of acetonitrile from 4% to 10%. By increasing the acetonitrile content, better separation of NBD-Glu from NBD-Thr was obtained. On the other hand, the separation of NBD-Asp and NBD-Gly was not sufficient when using acetonitrile content higher than 9%. Therefore, an aqueous solution containing 8% acetonitrile and 0.05% TFA in H_2O was selected. Under these conditions, NBD-Asp and NBD-Glu were eluted at 30 and 43 min, respectively, and completely separated from other proteinogenic amino acids (Fig. 3).

For the second dimension, various narrowbore-enantioselective columns were tested. As a result, Chiralpak QN-AX and QD-AX columns (150 mm × 1.5 mm i.d., Chiral Technologies Europe,

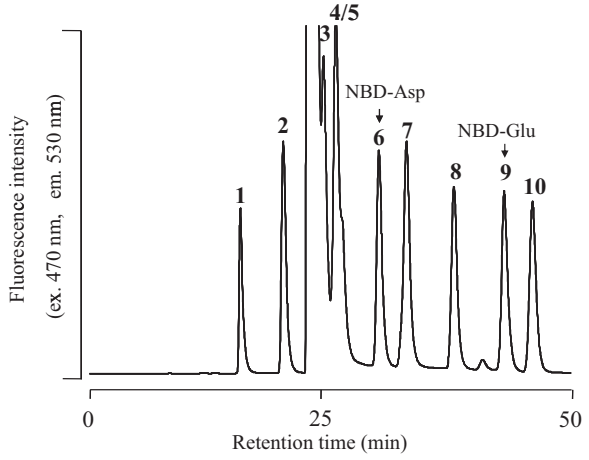

Fig. 3. Reversed-phase separation of 10 hydrophilic amino acids by using a microbore-monolithic ODS column. Peaks; 1, NBD-His; 2, NBD-Asn; 3, NBD-Ser; 4, NBD-Arg; 5, NBD-Gln; 6, NBD-Asp; 7, NBD-Gly; 8, NBD-*allo*-Thr; 9, NBD-Glu; 10, NBD-Thr. Column: monolithic ODS column (750 mm × 0.53 mm i.d., 40 °C). Mobile phase: MeCN–TFA–water (8:0.05:92, v/v) with the flow rate of 20 μL/min.

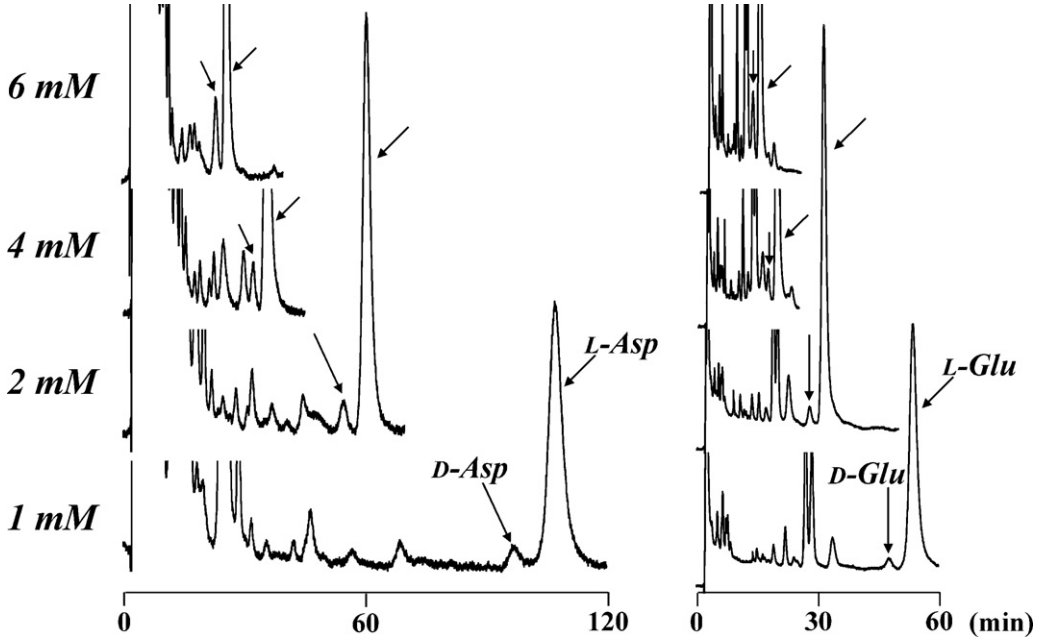

Fig. 4. Effect of the concentration of citric acid in the mobile phase used for the separation of NBD-Asp and NBD-Glu enantiomers in rat urine. Column: Sumichiral OA-2500S (250 mm × 1.5 mm i.d., 25 °C). Mobile phase: 1–6 mM citric acid in a mixed solution of MeOH–MeCN (50:50, v/v). Flow rate, 200 μL/min.

Illkirch, France) having quinine- and quinidine-modified moieties as the chiral selectors and a Sumichiral OA-2500S column (250 mm × 1.5 mm i.d.) having a naphthylglycine moiety as the chiral selector were found suitable for the separation of NBD-Asp and NBD-Glu enantiomers (Table 1). Regarding the elution order of the enantiomers, small amounts of D-forms are preferably eluted before the large amounts of L-forms. By using the Sumichiral OA-2500S column, the D-forms eluted faster for both Asp and Glu whereas on the Chiralpak QD-AX the NBD-Glu elutes from the column in a reversed elution order (see Table 1). Therefore, the Sumichiral OA-2500S column was selected for the second dimension in order to have consistently the minor peak in front of the major peak. For accurate determination of the Asp and Glu enantiomers in biological samples, the mobile phase of the second dimension was also optimized by changing the concentration of citric acid added (1–6 mM) and the compositions of organic solvents (MeOH–MeCN, from 90:10 to 40:60, v/v) in order to avoid the interference derived from matrix components. As shown in Fig. 4 many peaks derived from rat urine were observed in front of D-Asp and D-Glu, and changing the compositions of organic solvents did not affect the separation of D-Asp and D-Glu from these interfering substances. On the other hand, changing the concentration of citric acid was effective in separating small amounts of D-Asp and D-Glu from interfering substances in biological matrices. As a result, 2 mM citric acid in a mixed solution of MeOH–MeCN (50:50, v/v) was selected as a mobile phase to provide sufficient separations of NBD-D-Asp and NBD-D-Glu from their L-enantiomers (Asp; Rs = 1.41, α = 1.11, Glu; Rs = 1.58, α = 1.14) and also from the intrinsic substances.

According to these results, a monolithic ODS column (750 mm × 0.53 mm i.d.) and a Sumichiral OA-2500S column (250 mm × 1.5 mm i.d.) were connected via a program-controlled column-switching valve and a dual-loop valve, and a fully automated micro-2D-HPLC system was established. By using this system, the enantioselective 2D-HPLC determination of NBD-Asp and NBD-Glu was completed within 110 min; the obtained chromatograms are shown in Fig. 5.

3.2. Validation of the method in rat plasma

The present micro-2D-HPLC method was validated by establishing calibration lines, intra-day precision and inter-day precision using the standard amino acids and amino acids spiked into a rat plasma sample. Accuracy was also examined. As shown in Table 2, the calibration lines of the standard amino acids showed good linearity from 5 fmol to 500 fmol for the D-form and from 50 fmol to 5 pmol for the L-form, respectively, with correlation coefficients greater than 0.9998.

The intra-day precision (n = 4) and inter-day precision (4 days) were examined by determining the standard amino acids and also by determining amino acids spiked into a rat plasma sample. The obtained RSD values for the intra-day and inter-day preci-

Table 1
Enantiomer separation of Asp and Glu as their NBD derivatives using narrowbore-enantioselective columns.

Column	Aspartic acid				Glutamic acid			
	Retention time (min)				Retention time (min)			
	D	L	α	Rs	D	L	α	Rs
Sumichiral OA-2500S[a]	37.4	41.5	1.12	1.44	21.4	24.1	1.14	1.59
Chiralpak QN-1-AX[b]	52.9	43.2	1.23	1.28	20.9	23.2	1.12	0.71
Chiralpak QD-1-AX[b]	61.0	83.2	1.37	2.43	27.1	20.8	1.31	2.18

[a] Separation conditions for Sumichiral OA-2500S, 5 mM citric acid in a mixture of MeOH–MeCN (50:50, v/v) with a flow rate of 150 μL/min.
[b] Separation conditions for Chiralpak QN-1-AX and QD-1-AX, 10 mM citric acid in a mixture of MeOH–MeCN (50:50, v/v) with a flow rate of 200 μL/min.

Table 2
Validation results of the present 2D-HPLC method.

Amino acids	Calibration line[a]			Precision (RSD, %)												Accuracy (%)
	Standard amino acids			Standard amino acids[b]						Amino acids spiked into a rat plasma[b]						
	Calibration range (pmol)	Equation	Correlation coefficient	Intra-day			Inter-day			Intra-day			Inter-day			
				Low	Mid	High	Low	Mid	High	Low	Mid	High	Low	Mid	High	
D-Asp	0.005–0.5	$y = 6.12x - 0.01$	0.99991	3.17	4.36	0.99	5.86	2.15	5.50	3.38	5.15	2.67	6.28	5.01	3.67	96.1
L-Asp	0.05–5	$y = 4.95x + 0.04$	0.99993	1.40	4.62	1.07	5.46	2.38	5.75	3.75	5.29	2.64	2.09	6.18	3.47	100.8
D-Glu	0.005–0.5	$y = 8.87x - 0.02$	0.99988	5.52	2.17	3.69	3.29	3.16	6.20	4.82	6.38	2.94	1.45	3.19	0.69	105.8
L-Glu	0.05–5	$y = 7.34x + 0.02$	0.99988	0.97	2.53	3.76	7.05	2.40	6.60	4.05	6.78	2.90	2.42	6.17	1.03	97.3

[a] Equations were developed where x is the amount of amino acids added (pmol), and y is the fluorescence intensities (peak height, mV). Injected amounts of D-amino acids were 0.005, 0.015, 0.05, 0.15, 0.5 pmol and those of L-amino acids were 0.05, 0.15, 0.5, 1.5, 5 pmol (injected volume, 2 μL).
[b] The injected amounts or spiked amounts of D-amino acids: 0.005, 0.05, 0.5 pmol, L-amino acids: 0.05, 0.5, 5 pmol (injected volume, 2 μL).

sion for standard amino acids were 0.97–5.52% and 2.15–7.05%, respectively. The RSD values for the spiked amino acids into a rat plasma sample were 2.64–6.78% and 0.69–6.28%, respectively. Accuracy ranged between 96.1% and 105.8%. These results indicate that the present method is applicable to the quantification of Asp and Glu enantiomers in biological samples. Regarding the sensitivity using the established micro-2D-HPLC methods, the values of the lower limit of quantitation (LOQ) of D-Asp and D-Glu were 5 fmol/injection (0.625 nmol/g tissue or mL fluid).

Until now, various methods using GC, HPLC, CE and MEKC have been reported for the separation and determination of free D-Asp and D-Glu in biological samples. Concerning the sensitivity, using a GC method with pentafluoropropyl derivatization, the LOD value was 1 pmol for D-Asp and 2 pmol for D-Glu [36]. For the HPLC method, using various chiral derivatization reagents, the detection sensitivity depends on the reagents used. The *N*-α-(5-fluoro-2,4-dinitrophenyl)-(D or L)-valine amide (FDNP-Val-NH_2) derivatization gave an LOD of 5-10 pmol [37]. Using the OPA plus chiral thiols as the derivatization reagents, the LOD of D-Asp was reported to be 500 fmol [38] or 20 fmol [21], and the LOD of D-Glu was reported to be 10 fmol [21]. Using the NBD-F derivatization with an MS detector, the LOD values of D-Asp and D-Glu were around 500 fmol [39]. Using CE and MEKC methods with naphthalene-2,3-dicarboxaldehyde (NDA) and (+/−)-1-(9-anthryl)-2-propyl chloroformate (APOC) derivatization, the LOD values for D-Asp and D-Glu were reported to be at the amol level [23,31]. However, the injection volumes of these CE and MEKC methods were limited (nL level); therefore, the concentration sensitivity (nM level) is almost the same as those for the HPLC methods mentioned above. In the present method, the LOD values of D-Asp and D-Glu were around 2 fmol (0.25 nmol/g tissue or mL fluid) (*S/N* for D-Asp is 3.4 and *S/N* for D-Glu is 4.7). These LOD values were better than those of most of the already reported methods. Concerning the selectivity, D-Asp and D-Glu often co-eluted with some unidentified compounds in the tissues by one-dimensional chromatographic methods [23,30,37]. Although the already reported methods enable sensitive and rapid determination of D-Asp and D-Glu, a more selective method is required for the determination of trace amounts of the D-amino acids, especially D-Glu in mammalian tissues and physiological fluids to avoid the co-elution of various unknown intrinsic substances in the complex biological matrices. For this purpose, the 2D-HPLC method established in the present study is an effective tool. The NBD-Asp and NBD-Glu were separated from the other NBD-amino acids and from intrinsic interference materials in the first dimension by a microbore-monolithic ODS column. By employing this step, most of the interfering substances in biological matrices were removed before injection into the enantioselective column, and accurate determination of trace amounts of D-amino acids was facilitated in the second dimension.

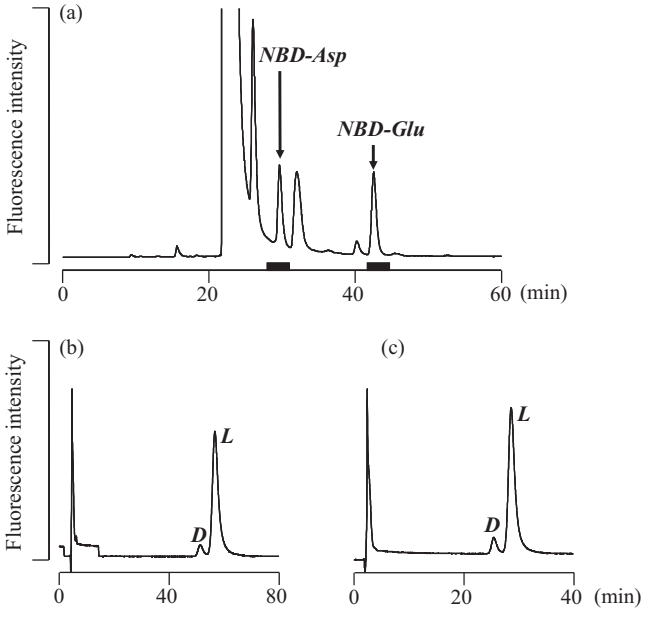

Fig. 5. Reversed-phase separation (a) and continuously interlinked enantiomer separation of NBD-Asp (b) and NBD-Glu (c). Injected amounts: 50 fmol for D-Asp and D-Glu, 500 fmol for L-Asp and L-Glu. The fractions indicated by closed bars were collected and injected into the enantioselective column.

3.3. Determination of free D-Asp and D-Glu in the brain and peripheral tissues of Wistar rats

Using the established 2D-HPLC system, the amounts of D- and L-Asp as well as D- and L-Glu were simultaneously determined in various biological samples listed in Table 3. D-Asp is mainly distributed in the endocrine tissues such as the pineal gland, pituitary gland, adrenal gland, testis and thymus (>80 nmol/g); the highest amount of D-Asp was observed in the pineal gland (2030 nmol/g). Concerning Glu, a small amount of the D-form is found in all the tested brain tissues and peripheral tissues, and the highest amount was observed in the thymus (9.0 nmol/g). The amounts of D-Asp and D-Glu in all the tissues were confirmed by changing the mobile phase compositions of the enantiomer separation, using 1.5 mM citric acid in a mixed solution of MeOH–MeCN (50:50, v/v), 0.5% formic acid in a mixed solution of MeOH–MeCN (25:75, v/v) in addition to the original mobile phase (2 mM citric acid in a mixed solution of MeOH–MeCN (50:50, v/v)). The amounts of D-Asp and D-Glu determined using the three different mobile phases were almost the same, which indicates the high selectivity and reliability of the present method for the deter-

Table 3
Amounts of Asp and Glu enantiomers in the rat tissues and physiological fluids.

	Asp			Glu		
	D	L	%D	D	L	%D
Brain tissues						
Cerebral cortex	12.8 ± 1.1	7047.4 ± 762.2	0.18	1.9 ± 0.1	4313.9 ± 371.7	0.04
Hippocampus	6.0 ± 0.6	3766.4 ± 309.0	0.16	3.9 ± 0.5	7382.5 ± 911.8	0.05
Hypothalamus	15.0 ± 1.9	4657.1 ± 620.1	0.32	3.2 ± 1.0	3652.1 ± 352.9	0.08
Cerebellum	28.4 ± 3.1	4866.9 ± 517.6	0.60	4.1 ± 0.3	2667.2 ± 73.3	0.15
Medulla oblongata	6.5 ± 0.8	7616.2 ± 193.2	0.09	2.3 ± 0.4	2255.3 ± 28.1	0.10
Pineal gland	2025.9 ± 147.8	6503.9 ± 475.6	23.9	7.2 ± 1.1	14128.0 ± 996.9	0.05
Pituitary gland	132.5 ± 32.3	3752.1 ± 486.6	3.34	2.6 ± 1.0	5268.1 ± 568.5	0.05
Peripheral tissues						
Spinal cord	6.7 ± 0.5	7898.7 ± 528.6	0.09	3.1 ± 0.4	4030.3 ± 256.0	0.08
Adrenal gland	574.3 ± 231.2	197.8 ± 21.4	70.5	0.7 ± 0.1	1424.2 ± 258.6	0.05
Testis	129.6 ± 14.1	667.0 ± 165.6	17.9	2.0 ± 0.4	6863.0 ± 650.2	0.03
Thymus	88.1 ± 0.5	7672.2 ± 596.1	1.15	9.0 ± 1.3	12533.7 ± 2931.7	0.09
Pancreas	19.3 ± 1.6	5136.1 ± 1019.8	0.41	1.8 ± 0.4	12710.4 ± 3351.1	0.01
Spleen	195.6 ± 17.3	5338.5 ± 775.0	3.61	4.4 ± 0.6	8019.5 ± 806.5	0.05
Lung	141.1 ± 13.6	3437.9 ± 340.5	3.99	1.6 ± 0.3	7162.3 ± 1150.4	0.02
Heart	20.2 ± 4.3	1914.9 ± 357.7	1.05	0.8 ± 0.1	12218.8 ± 2114.2	0.01
Kidney	34.1 ± 6.6	7781.9 ± 651.2	0.43	2.8 ± 0.5	18193.2 ± 2858.9	0.01
Liver	14.5 ± 3.0	1439.5 ± 202.7	0.98	2.3 ± 0.8	7706.7 ± 886.6	0.03
Muscle	3.5 ± 0.5	421.8 ± 191.5	1.13	1.9 ± 0.3	1778.8 ± 508.9	0.12
Physiological fluids						
Plasma	0.9 ± 0.2	30.0 ± 1.5	2.92	trace	135.8 ± 13.1	–
Urine	4.1 ± 0.9	37.1 ± 5.2	10.2	3.7 ± 1.0	108.4 ± 26.6	3.33

Values represent mean ± SE (nmol/g or mL) of 3 analyses. Rat; Wistar, male, 6 weeks of age. trace; between LOD and LOQ.

mination of D-Asp and D-Glu in biological samples. The typical chromatograms obtained for a rat testis sample are shown in Fig. 6.

In the present study, the highest amount of D-Asp (2030 nmol/g) and also a high %D value (23.9%) were observed in the pineal gland, and large amounts of D-Asp (around 80–580 nmol/g tissue) were observed in the pituitary gland, adrenal gland, testis, thymus, spleen and lung. The reported amount of D-Asp in the pineal gland is 1000–3500 nmol/g [1,17], and those in the other endocrine tissues are 40–220 nmol/g [1,40]. In other tissues, such as cerebral cortex, cerebellum, liver and kidney, the reported values of D-Asp were relatively low (below 70 nmol/g tissue) [1,40]. All of the values obtained in the present study are consistent with those reports. What is striking is the excess of D-Asp compared to L-Asp (70.5%:29.5%) in the adrenal gland, which may suggest a physiological significance of D-Asp in the tissue.

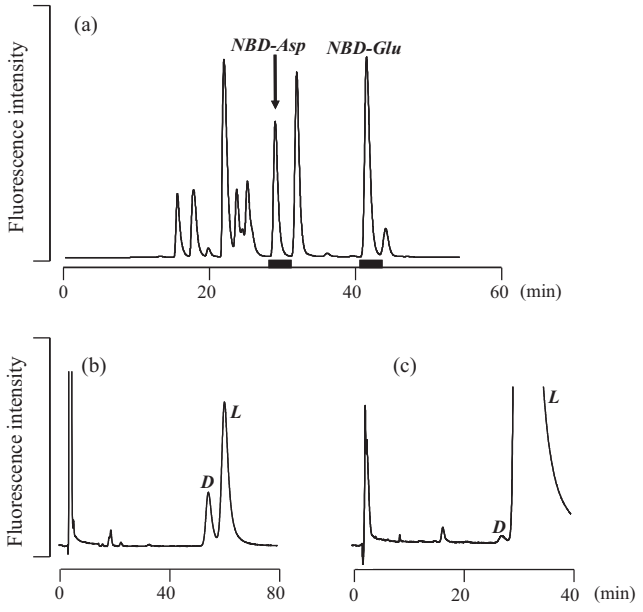

Fig. 6. Reversed-phase separation (a) and continuously interlinked enantiomer separation of NBD-Asp (b) and NBD-Glu (c) in the rat testis. The fractions indicated by closed bars were collected and injected into the enantioselective column.

Concerning D-Glu, only a few results have been reported for the determination of D-Glu in mammalian tissues [20–23,30]. The present study is the first report to show the detailed distribution of D-Glu in mammals. Until now, the amount of D-Glu in rat brain was determined by three different methods (an HPLC method with OPA plus *N*-acetyl-L-cysteine [20], an HPLC–MS/MS method with NBD-F derivatization [33] and an MCE method with fluorescein isothiocyanate derivatization [22]). The reported amounts of the D-Glu are 36.9 nmol/g, n.d., 6.1–12.0 nmol/g, respectively. Concerning the peripheral tissues, large amounts of D-Glu were reported [20] in the liver (133 nmol/g) and kidney (138 nmol/g), respectively. On the other hand, only small amounts of D-Glu (0.7–9.0 nmol/g) were observed both in the brain and peripheral tissues in the present study.

The inconsistency of those results compared to our results might be due to differences in the breeding conditions and/or the selectivity of the methodology used. Compared to the previously reported methods, a highly selective 2D-HPLC technique is adopted in the present study, and the analytical conditions were designed by a thorough investigation using tissue samples. The amounts of free D-Asp and D-Glu obtained in the present study were confirmed using two additional different mobile phases, which strongly proved the authenticity of the D-Asp and D-Glu values reported in the present study in rat tissues and physiological fluids. Further studies to elucidate the origin and physiological meaning of D-Asp and D-Glu in mammals are currently in progress.

4. Conclusion

In the present study, we have established a 2D-HPLC system for the simultaneous determination of the enantiomers of Asp and Glu in the form of their NBD-derivatives. The system was successfully validated, and the detailed distribution and correlation of D-Asp and D-Glu in mammals were clarified for the first time. Amazingly,

although large amounts of D-Asp were present in various endocrine tissues, the amounts of D-Glu were extremely small in all the tested tissues and physiological fluids. From these results, it can be concluded that D-Glu may not play a major physiological role compared to D-Asp which can occasionally be found in much higher values. The present 2D-HPLC system is an example for a powerful tool for the metabolomics study of acidic amino acid enantiomers, and further applications to clarify the physiological and diagnostic values of D-Asp and D-Glu in mammals can be appreciated in the future.

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