Journal of Chromatography A, 1217 (2010) 1056–1062

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Simultaneous determination of hydrophilic amino acid enantiomers in mammalian tissues and physiological fluids applying a fully automated micro-two-dimensional high-performance liquid chromatographic concept

Kenji Hamase[a,*], Yurika Miyoshi[a], Kyoko Ueno[a], Hai Han[a], Junzo Hirano[a], Akiko Morikawa[a], Masashi Mita[b], Tsuneaki Kaneko[b], Wolfgang Lindner[c], Kiyoshi Zaitsu[a]

[a] Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Innovative Science Research and Development Center, Shiseido Co., Ltd., 2-12-1 Fukuura, Kanazawa-ku, Yokohama 236-8643, Japan
[c] Institute of Analytical Chemistry and Food Chemistry, University of Vienna, Waehringerstrasse 38, A-1090 Vienna, Austria

ARTICLE INFO

Article history:
Available online 6 September 2009

Keywords:
Hydrophilic amino acid
Enantiomer separation
Two-dimensional HPLC
Fluorescence

ABSTRACT

A validated two-dimensional HPLC system combining a microbore-monolithic ODS column and a narrowbore-enantioselective column has been established for a sensitive and simultaneous analysis of hydrophilic amino acid enantiomers (His, Asn, Ser, Gln, Arg, Asp, *allo*-Thr, Glu and Thr) and the non-chiral amino acid, Gly, in biological samples. To accomplish this goal, the amino acids were first tagged with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) to the respective fluorescent NBD derivatives which were separated in the first dimension by a micro-reversed-phase column. The automatically collected fractions of the target peaks were then transferred to the second dimension consisting of a Pirkle type enantioselective column generating separation factors higher than 1.13 for all the enantiomeric target analytes. The system was validated using standard amino acids and a rat plasma sample, and analytically satisfactory calibration and precision results were obtained. The present 2D-HPLC system enables the fully automated determination of hydrophilic amino acid enantiomers in mammalian samples. The D-isomers of all the investigated 9 amino acids were found in rat urine but at various enantiomeric ratios.

1. Introduction

It had been long believed that only the L-isomers of proteinogenic amino acids were present in higher animals. However, several key reports showing the presence, function and origin of some D-amino acids in mammals were published during the last two decades [1–3], and these D-amino acids are now widely accepted as candidates of novel physiologically active substances and biomarkers in mammals. Especially D-aspartic acid (D-Asp) and D-serine (D-Ser) have been intensively investigated. For instance D-Asp is observed in various endocrine and neuroendocrine tissues, and regulates the hormonal synthesis and/or secretion in tissues [4,5]. D-Ser is localized to the frontal brain areas, and regulates *N*-methyl-D-aspartate (NMDA) receptor mediated neurotransmission [6–8]. In 2008, the biosynthesis of D-Ser by Ser racemase in mammalian brain was demonstrated using a Ser racemase knock-out mouse [9], and D-Ser is now recognized as an enzymatically synthesized neuroactive amino acid in mammals.

Several other D-amino acids have been reported to be also present in mammals [2,10–21]. These D-amino acids are expected to be intrinsic physiologically active molecules and/or biomarkers, and the simultaneous analysis of structurally related amino acid enantiomers thus became matters of interest also in the course of metabolomic studies. In this context the simultaneous determination of hydrophilic amino acid enantiomers including above mentioned D-Ser and D-Asp, and the structurally related analogs, threonine (Thr), *allo*-Thr, glutamic acid (Glu), asparagine (Asn) and glutamine (Gln) raised our interest. However, the expected amounts of most of the D-amino acids besides their L-congeners are extremely small, and their reliable determination in mammalian tissues and physiological fluids is difficult because of the lack of appropriate analytical methods.

The aim of the present paper is to establish a 2D-HPLC method for the simultaneous and sensitive determination of hydrophilic amino acid enantiomers, and to evaluate their amounts in mammalian tissues and physiological fluids. For the analysis of D-amino acids in mammals, a variety of methods including GC and HPLC have been reported [22,23]. Especially, chiral-GC methods using enantioselective columns such as Chirasil-L-Val [24,25] and Chirasil-γ-Dex [26] are useful for the enantioselective analysis of most of the proteinogenic amino acids. HPLC methods apply-

* Corresponding author. Tel.: +81 92 642 6598; fax: +81 92 642 6601.
E-mail address: hamase@phar.kyushu-u.ac.jp (K. Hamase).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.09.002

ing the diastereomer formation reagents such as *o*-phthalaldehyde (OPA) plus chiral thiols [11,27–30], (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC, [31]) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, [32]) are also suitable. These methods are powerful tools for the investigation of D-Ser and D-Asp in mammalian tissues which are present in relatively high amounts, e.g., D-Ser in the frontal brain tissues [6–8] and D-Asp in the endocrine tissues [4,5]. However, the determination of small amounts of D-amino acids (the amounts are around 1% or less of those of L-amino acids) is difficult in most cases, because of the potential interferences with a great number of peptides and amino compounds presented in the biological matrix. Therefore, a more selective method is needed, and the use of a two-dimensional chromatographic technique seems a straightforward approach for the analysis of small amounts of D-amino acids in mammalian tissues and physiological fluids.

Until now, several 2D-HPLC methods combining a microbore-reversed-phase column and an enantioselective column have been published for the determination of a single D-amino acid such as D-Ala [14], D-Ser [33], D-Leu [34] and D-Pro [16], and for the simultaneous determination of structurally related D-amino acids, such as branched aliphatic amino acids [17] and proline analogs [18]. However, a 2D-HPLC system for the simultaneous determination of hydrophilic amino acid enantiomers including all of the Ser analogs (Ser, Thr and *allo*-Thr) and Asp analogs (Asp, Glu, Asn and Gln) has not been reported. Because the physiological and diagnostic aspects of these D-isomers in mammals are of high interest, the proposed 2D-HPLC method turns out to be useful especially in the fields of neuroscience and endocrinology.

2. Experimental

2.1. Reagents and animals

The amino acid enantiomers were obtained from Sigma (St. Louis, MO, USA), Tokyo Kasei (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan) and Wako (Osaka, Japan). The fluorescence derivatizing reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), was from Tokyo Kasei. Methanol (MeOH), trifluoroacetic acid (TFA), citric acid monohydrate and boric acid were from Wako. Acetonitrile (MeCN) of HPLC grade was purchased from Nacalai Tesque. 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 used without further purification. Male Wistar rats (6–7 weeks of age, specific-pathogen free) were purchased from Kyudo (Kumamoto, Japan). All experiments were performed with the permission (A21-005-0) of the Animal Care and Use Committee of Kyushu University.

2.2. Sample preparation procedure of the tissues and physiological fluids

The rats were anesthetized with diethyl ether and euthanized by exsanguination from the abdominal aorta, and the blood was collected in a heparinized tube (Nippon Becton Dickinson, Tokyo, Japan). After centrifugation at 4500 × *g* and 4 °C for 15 min, the plasma was obtained. The cerebrum was quickly excised; the urine was collected from the urinary bladder and stored at −80 °C until use. For the physiological fluids, 200 μl of MeOH was added to 10 μl of the fluids, and mixed for 2 min. The tissues were homogenized at 3500 rpm for 2 min in MeOH (20× volume of the tissue wet weight) using a micro-homogenizing system (Micro Smash™ MS-100R, Tomy, Tokyo, Japan) at 4 °C. The obtained MeOH mixtures of the plasma and urine, and the MeOH homogenates of the tissues were centrifuged at 12,000 × *g* for 10 min to obtain the supernatants.

2.3. Pre-column fluorescence derivatization of amino acids with NBD-F

The supernatants (10 μl) obtained from the tissues and physiological fluids were evaporated to dryness under reduced pressure at 40 °C. To the residue, 20 μl of 200 mM sodium borate buffer (pH 8.0) and 5 μl of 40 mM NBD-F in dry MeCN were added and the samples were heated at 60 °C for 2 min. To the reaction mixture, 75 μl of 2% (v/v) TFA in water was added and 2 μl of the reaction mixture was then injected into the HPLC.

2.4. Two-dimensional HPLC system for the determination of hydrophilic amino acid enantiomers

The HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo, Japan) consisted of a type 3010 degasser, two 3201 pumps, a 3033 auto sampler, a 3004 column oven, two 3013 fluorescence detectors, a 3012 column-selection unit and a laboratory made multi-loop unit (set 9 loops (750 mm × 0.5 mm I.D., the volume of 1 loop is 150 μl) between dual 1- in 10-out valves (C5-2340EMTD, Valco instruments, Houston, TX, USA)). A data processing program, EzChrom Elite Client, was used to monitor the detector response, and the column-selection unit and the multi-loop unit were controlled by the KSAA valve controlling system (Shiseido). The flow diagram is shown in Fig. 1.

The analytical column for the reversed-phase separation was a microbore-monolithic ODS column (1000 mm × 0.53 mm I.D., prepared in a fused silica capillary, provided from Shiseido) maintained at 45 °C. The mobile phase was 6% (v/v) MeCN and 0.06% (v/v) TFA in water and the flow rate was 25 μl/min. For enantiomer separations, a narrowbore-Sumichiral OA-2500S enantioselective column (250 mm × 1.5 mm I.D., self-packed, material was from Sumika Chemical Analysis Service, Osaka, Japan) was used at 25 °C. The

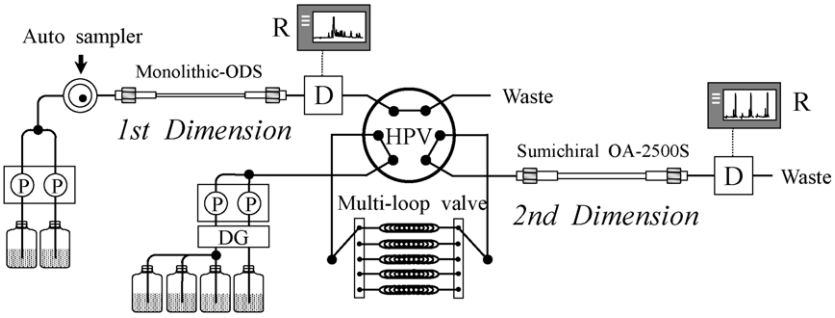

Fig. 1. Flow diagram of the 2D-HPLC system used in the present study for the determination of hydrophilic amino acids as their NBD derivatives. P, pump; D, detector; R, integrator; HPV, high pressure valve; DG, degasser.

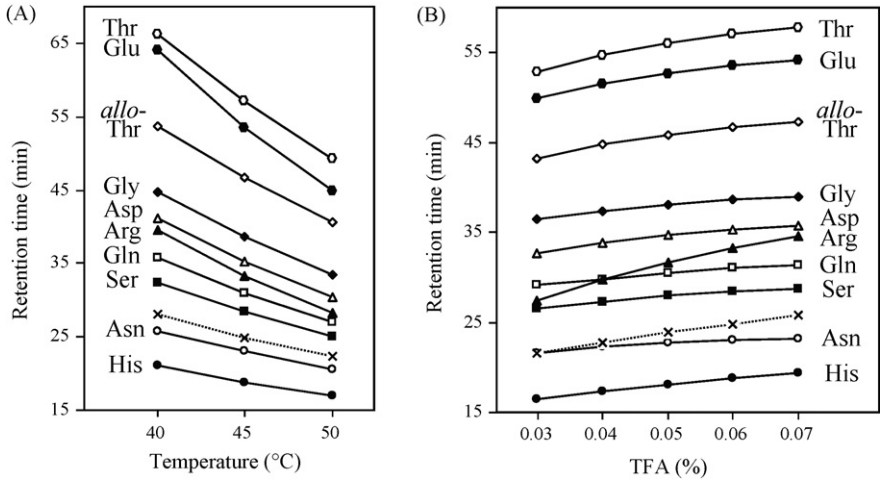

Fig. 2. Effects of column temperature and the TFA concentration on the retention time of the NBD-amino acids. (A) 0.06% TFA was used as the TFA concentration and (B) 45 °C was used as the column temperature. Dotted lines (symbol ×) indicate the retention time of the large peak derived from the reagent.

mobile phase for Asn, Ser, Gln, Gly, *allo*-Thr and Thr was 5 mM citric acid in the mixed solution of MeOH–MeCN (25:75, v/v) at the flow rate of 200 μl/min. The mobile phase for Glu and Asp was 2.5 mM citric acid in the mixed solution of MeOH–MeCN (25:75, v/v) at the flow rate of 200 μl/min (that for His was 0.5 mM citric acid in MeOH, 200 μl/min; that for Arg was pure MeOH, 75 μl/min). For confirmation of the quantitative data of the D- and L-amino acids as their NBD derivatives in the analyzed tissue samples, an enantioselective column containing the opposite chiral selector material (narrowbore-Sumichiral OA-2500(R), 250 mm × 1.5 mm I.D.) was used as it leads to an inversion of the elution order of the enantiomers. Fluorescence detection of the NBD-amino acids was carried out at 530 nm with excitation at 470 nm.

3. Results and discussion

3.1. Reversed-phase separation of hydrophilic amino acids as NBD derivatives

First the reversed-phase separation profiles of the 10 hydrophilic NBD-derivatized amino acids (His, Asn, Ser, Gln, Arg, Asp, Gly, *allo*-Thr, Glu and Thr) were investigated by varying the mobile phase compositions with respect to the MeCN and TFA content. To obtain the appropriate retention of the NBD-hydrophilic amino acids, the MeCN composition was selected at 6% (v/v), and the column temperature (40, 45 and 50 °C) and the composition of TFA (0.03–0.07%, v/v) were examined. Fig. 2(A) shows the effect of column temperature on the retention of the 10 target analytes. For all the tested TFA concentrations, the resolution of NBD-Glu and NBD-Thr increased with the increasing column temperature, and good separation was observed using 45 and 50 °C. On the other hand, NBD-Arg was not sufficiently separated from NBD-Asp and NBD-Gln at 40 and 50 °C. The effect of the TFA concentration was also examined at all column temperatures. The retention time of NBD-Arg increased with the increasing TFA concentration in the mobile phase, most probably due to an ion pairing effect, as shown in Fig. 2(B); it could not be separated from NBD-Ser, NBD-Gln and NBD-Asp using the mobile phases containing 0.03–0.05% and 0.07% TFA. In addition, NBD-Asn was not separated from the reagent peak using the TFA concentration lower than 0.05%. As a result of all these systematic investigations, an aqueous solution containing 6% (v/v) MeCN and 0.06% (v/v) TFA with the column temperature of 45 °C was selected as isocratic elution condition for the first LC dimension, and the complete separation of the 10 hydrophilic NBD-amino acids could be accomplished as shown in Fig. 3(A).

3.2. Enantiomer separation of hydrophilic amino acids as NBD derivatives

The enantiomer separations of 9 NBD-amino acids were investigated using a Pirkle type enantioselective column, Sumichiral OA-2500S. In order to perform the fully automated and highly sensitive analysis, a narrowbore-enantioselective column (250 mm × 1.5 mm I.D.) was packed and the separation profiles were examined using organic solvents (mixtures of MeOH and MeCN) containing citric acid as the mobile phases. By increasing the citric acid and MeCN concentrations, the elution of the NBD-amino acids became faster, and the appropriate retention was obtained using different concentrations of citric acid and MeCN depending on the characteristics of amino acids tested. As shown in Table 1, applying a mixed solution of MeOH and MeCN containing 5 mM citric acid, the enantiomers of NBD-Asn, NBD-Ser and NBD-*allo*-Thr were sufficiently separated, while those of NBD-Gln and NBD-Thr were separated using 4 mM citric acid. The enantiomers of NBD-Asp and NBD-Glu were resolved using 2.5 mM citric acid, and those of NBD-His and NBD-Arg were separated using 0.25 mM citric acid and pure MeOH, respectively. Concerning the elution order of the NBD-amino acid enantiomers, the D-enantiomers eluted faster than the L-enantiomers in all cases using the Sumichiral OA-2500S column. Under these conditions, the enantiomers of these NBD-amino acids were sufficiently separated with practically negligible interference from intrinsic compounds being present in the tissues and physiological fluids. Concerning NBD-Gly, appropriate retention was observed using a mixed solution of MeOH and MeCN (25:75, v/v) containing 5 mM citric acid as a mobile phase.

3.3. Establishment of a fully automated 2D-HPLC system and the method validation

Combining a microbore-monolithic ODS column and a narrowbore-enantioselective column via a high pressure valve (for column-switching) and a multi-loop valve (for fraction collection), a 2D-HPLC system for the simultaneous analysis of the enantiomers of the pre-selected hydrophilic amino acids in the form of their NBD derivatives was established. In the first dimension, the target analytes were separated as shown in Fig. 3(A) under the conditions described in Section 3.1, and the peak fractions (shown in gray bars) were collected in the multi-loop valve. The fractions were then

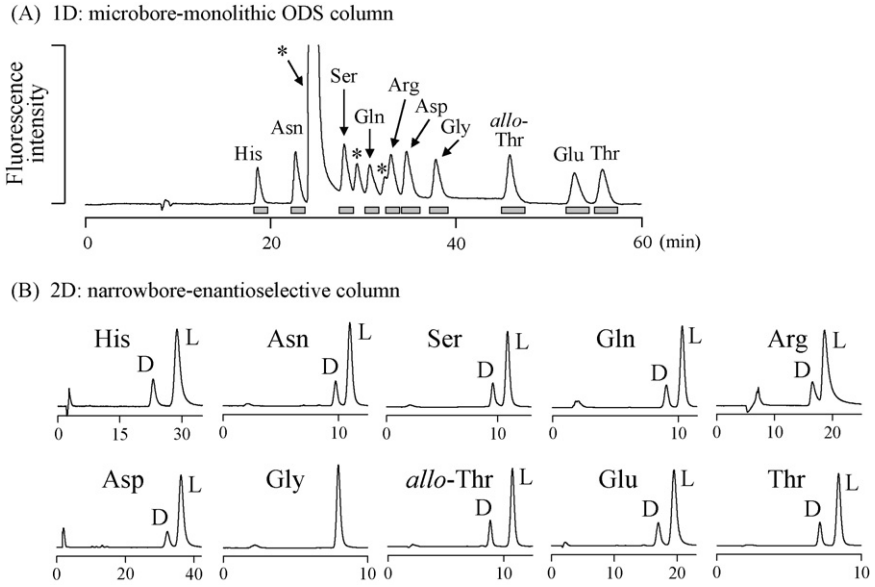

Fig. 3. Separation of hydrophilic amino acids as their NBD derivatives using a microbore-ODS column (A) and the continuously interlinked enantiomer separation using a narrowbore-Sumichiral OA-2500S column (B). The mixtures of NBD-derivatized D- and L-amino acids in a molar ratio of 1:4 were analyzed (injection amount of the D-form is 250 fmol, and that for the L-form is 1 pmol). The fractions indicated by gray bars are on-line collected to a multi-loop device and transferred to the enantioselective column by the second pumping system. Asterisks indicate the peaks derived from the reagent.

successively introduced to the narrowbore-enantioselective column representing the second dimension. By using the appropriate mobile phases shown in Table 1, all of the enantiomers were sufficiently separated, and the D- and L-amino acids were determined qualitatively and quantitatively by a fluorescence detector.

The present 2D-HPLC system was validated by calibration lines, within-day precision and day-to-day precision using the standard amino acid and the rat plasma samples. As shown in Table 2A, all of the calibration lines constructed using standard D- and L-amino acids were linear with correlation coefficients higher than 0.9999. Within-day precision and day-to-day precision were obtained for 3 different concentrations, and the RSD values of all the amino acids are around 5% or lower. Concerning the validation results obtained using the rat plasma sample (Table 2B), the calibration lines for the spiked amino acids were linear with correlation coefficients higher than 0.995, and the slopes were almost the same as those obtained for the standard amino acids. The RSD values of within-day and day-to-day precision were lower than 6.47%. These results indicate that the present 2D-HPLC system is practically acceptable for the simultaneous determination of hydrophilic amino acid enantiomers in biological samples.

For the rapid separation of almost all hydrophilic amino acid enantiomers, a chiral-GC method using Chirasil-L-Val as a stationary phase [23] has been reported. However, during the derivatization procedure of the amino acids, Asn and Gln were hydrolyzed to form Asp and Glu derivatives. HPLC methods using the diastereomer formation with chiral active reagents are also widely used for the simultaneous determination of hydrophilic amino acid enantiomers. Using FLEC, the enantiomers of 17 proteinogenic amino acids including 7 hydrophilic ones could be separated in about 70 min in a single run [31]. The use of OPA plus chiral thiol compounds, such as *N*-acetyl-L-Cys [28] and *N*-*tert*-butyloxycarbonyl-L-Cys [29,30], enables the diastereoselective separation of 6–8 hydrophilic amino acids by a reversed-phase column within 40 min. For the simultaneous determination of 41 amino compounds including the pairs of 17 proteinogenic amino acids, Brückner et al. reported the use of OPA in combination with *N*-isobutyryl-L-Cys [11,35]. By this method, all of the hydrophilic amino acid enantiomers except for *allo*-Thr were completely separated within 75 min. However, the determination of small amounts of D-amino acids in mammalian tissues and physiological fluids frequently requires a higher selectivity to eliminate the co-elution of unknown substances in the biological matrices. For that purpose, a multi-dimensional method using a chiral derivatization reagent, FDAA, in combination with preparative 2D-TLC followed by a reversed-phase HPLC determination of the enantiomers except for Arg and *allo*-Thr has been reported [13]. A 2D-HPLC setup for the enantiomer separation of 17 proteinogenic amino acids including

Table 1
Enantiomer separation of hydrophilic amino acids as their NBD derivatives using a Pirkle type column, Sumichiral OA-2500S.

	Retention time (min)		α	Rs	Mobile phase		Flow rate (μl/min)
	D-Form	L-Form			Citric acid (mM)	MeOH–MeCN	
His	22.64	28.33	1.28	2.57	0.25	96.25–3.75	200
Asn	9.32	10.46	1.15	1.98	5.0	25–75	250
Ser	9.23	10.47	1.17	2.14	5.0	25–75	250
Gln	8.87	10.09	1.17	1.97	4.0	40–60	250
Arg	16.54	18.54	1.19	1.12	–	100–0	75
Asp	30.12	33.76	1.13	1.67	2.5	25–75	250
allo-Thr	8.62	10.41	1.26	3.52	5.0	25–75	250
Glu	16.12	18.45	1.16	2.01	2.5	25–75	250
Thr	7.00	8.20	1.23	2.59	4.0	40–60	250

α: separation factor; Rs: resolution.

Table 2A
Calibration lines of standard amino acids and precision of the method.

Amino acids	Calibration line			Precision[a]					
	Calibration range (pmol)	Equation[b]	Correlation coefficient	Within-day			Day-to-day		
				Low	Middle	High	Low	Middle	High
His									
D	0.0025–2.5	$y = 4.62x - 0.03$	0.9999	4.01	4.17	3.06	5.15	6.23	6.95
L	0.01–10	$y = 3.49x - 0.22$	0.9999	3.78	4.23	3.30	5.79	6.78	7.46
Asn									
D	0.0005–2.5	$y = 49.4x - 0.03$	1.000	1.24	0.32	2.38	2.27	3.89	2.96
L	0.01–10	$y = 40.1x - 0.27$	1.000	1.06	0.24	2.10	1.85	4.04	2.91
Ser									
D	0.0005–2.5	$y = 72.7x - 0.12$	1.000	1.60	0.62	1.81	3.72	4.52	1.68
L	0.01–10	$y = 56.2x + 0.63$	1.000	2.98	0.73	2.86	2.96	4.85	1.91
Gln									
D	0.0005–2.5	$y = 42.5x + 0.00$	1.000	2.57	5.30	3.49	2.51	4.03	3.38
L	0.01–10	$y = 39.2x - 0.29$	1.000	2.51	5.60	2.10	0.98	3.15	3.42
Arg									
D	0.0005–2.5	$y = 15.5x - 0.10$	0.9999	3.08	4.67	2.60	5.83	2.16	6.03
L	0.01–10	$y = 13.2x - 0.83$	0.9999	5.69	5.46	3.18	5.10	1.90	6.12
Asp									
D	0.0025–2.5	$y = 12.0x + 0.01$	1.000	3.37	4.36	3.65	2.69	5.98	0.75
L	0.01–10	$y = 11.0x + 0.11$	1.000	3.39	0.96	3.59	4.79	5.43	0.97
Gly									
–	0.01–10	$y = 61.6x + 1.50$	1.000	2.24	2.32	1.95	4.13	5.31	2.54
allo-Thr									
D	0.0005–2.5	$y = 84.1x - 0.10$	1.000	1.52	0.96	1.27	3.48	4.51	2.68
L	0.01–10	$y = 63.8x - 0.89$	1.000	1.10	1.05	2.20	2.59	4.60	2.87
Glu									
D	0.0005–2.5	$y = 24.6x + 0.01$	1.000	1.78	2.58	4.07	4.91	5.46	1.67
L	0.01–10	$y = 20.0x + 0.08$	1.000	2.58	2.26	3.98	4.59	5.36	1.81
Thr									
D	0.0005–2.5	$y = 85.8x - 0.19$	1.000	1.17	1.77	3.04	2.85	3.71	1.97
L	0.01–10	$y = 67.8x - 1.21$	1.000	1.17	1.62	3.31	2.42	4.16	2.23

[a] Within-day precision and day-to-day precision ($n = 4$, RSD (%)) were determined using 25, 250 fmol and 2.5 pmol (injection amount) of D-amino acids and 100 fmol, 1 and 10 pmol of L-amino acids.

[b] Equations were made where x is the amount of amino acids added (injection amount, pmol), and y is the fluorescence intensities (peak height, mV). Injection amounts of D-amino acids are 0.0005, 0.0025, 0.025, 0.25, 2.5 and 5 pmol. Those for L-amino acids are 0.01, 0.1, 1 and 10 pmol.

all of the hydrophilic amino acids except for *allo*-Thr has also been reported combining a manual RP-HPLC fractionation of the NBD-derivatized amino acids and enantiomer separation of each amino acid fraction using a Pirkle type enantioselective column [36]. However, these multi-dimensional methods require the tedious manual collecting and concentration steps of the amino acid fractions containing the target amino acid derivative, and therefore a fully automated method with a high reliability was in focus for this work. Emphasis was also given to the analysis of *allo*-Thr should also be included because the conversion of the alpha position of Thr predominantly occurs in biological matrices.

The 2D-HPLC method established in the present study is a fully automated procedure enabling the complete enantioseparation of 10 hydrophilic amino acids including *allo*-Thr as NBD derivatives in a single two-dimensional run. As raised before, similar 2D-HPLC procedures for the enantiomer determination of a single amino acid [14,16,33,34], branched aliphatic amino acids [17] and Pro analogs [18] have already been demonstrated in our previous studies. However, hydrophilic amino acids being characterized by additional polar functional groups within their structures, such as hydroxyl, carboxyl and amide groups, make their simultaneous 2D-HPLC separation in a single run more difficult due to the variety of chemical property. In the present report, we have optimized the chromatographic conditions in both the first and second dimensions, and the comprehensive 2D-HPLC system, which could be applied to the determination of small amount of D-amino acids in mammalian samples, has been established for the first time. The present 2D-HPLC system has the same sensitivity as those of the previously reported 2D-HPLC procedures using the combination of a microbore-ODS column and a narrowbore-enantioselective column following the derivatization of amino acids with NBD-F [18,33]. The present method also has a sufficient selectivity to be applicable to biological samples.

3.4. Determination of hydrophilic amino acid enantiomers in rat tissues and physiological fluids

Using the 2D-HPLC system as described above, the amounts of hydrophilic amino acid enantiomers could be determined in rat cerebrum, plasma and urine. In the cerebrum, a large amount of D-Ser was observed, and small amounts of D-Asp, D-*allo*-Thr and D-Thr were also found. In the plasma, small amounts of D-Ser and D-Asp were observed. On the other hand, as shown in Fig. 4, all of the D-enantiomers of the hydrophilic amino acids were present in the urine. Especially, the amounts of D-Asn and D-Arg were high, and almost half of Asn and Arg were of the D-form. The amounts found for the hydrophilic amino acid enantiomers are summarized in Table 3; the values were confirmed by either using the opposite enantioselective column, Sumichiral OA-2500(R), or by changing the mobile phase compositions.

In the cerebrum, D-Ser is reported to be synthesized by Ser racemase [9], and the presence of D-Ser in the frontal brain areas

Table 2B
Calibration lines of amino acids spiked to a rat plasma sample and precision of the method.

Amino acids	Calibration line			Precision[a]	
	Spiked amount (pmol)	Equation[b]	Correlation coefficient	Within-day	Day-to-day
His					
D	0.0025–2.5	$y = 4.90x - 0.02$	1.000	2.38	4.13
L	0.1–10	$y = 3.80x + 1.57$	0.9992	2.68	4.18
Asn					
D	0.0025–2.5	$y = 52.1x + 0.11$	1.000	1.49	6.42
L	0.1–10	$y = 41.3x + 22.36$	0.9999	0.83	6.37
Ser					
D	0.0025–2.5	$y = 70.6x + 1.27$	1.000	1.55	2.01
L	0.1–10	$y = 55.1x + 103.32$	0.9997	1.15	3.49
Gln					
D	0.0025–2.5	$y = 43.0x + 0.80$	1.000	1.02	5.16
L	0.1–10	$y = 39.2x + 228.44$	0.9951	1.67	5.96
Arg					
D	0.0025–2.5	$y = 14.7x - 0.21$	0.9997	3.07	4.84
L	0.1–10	$y = 13.3x + 11.83$	0.9993	3.14	4.47
Asp					
D	0.0025–2.5	$y = 10.6x + 0.07$	1.000	5.40	6.47
L	0.1–10	$y = 9.89x + 0.76$	1.000	3.07	3.67
Gly					
–	0.1–10	$y = 61.5x + 215.60$	0.9985	1.08	4.24
allo-Thr					
D	0.0025–2.5	$y = 83.4x - 0.10$	1.000	1.38	3.52
L	0.1–10	$y = 64.0x - 0.86$	0.9987	1.48	3.81
Glu					
D	0.0025–2.5	$y = 22.0x - 0.14$	0.9999	1.73	3.62
L	0.1–10	$y = 17.8x + 14.67$	0.9998	2.76	3.73
Thr					
D	0.0025–2.5	$y = 83.7x - 0.15$	1.000	1.68	2.68
L	0.1–10	$y = 67.6x + 143.44$	0.9994	1.35	3.27

[a] Values represent RSD (%) of 4 analyses. Spiked amount; 250 fmol for D-amino acids and 1 pmol for L-amino acids.
[b] Equations were made where x is the amount of amino acids added (spiked amount, pmol), and y is the fluorescence intensities (peak height, mV). Spiked amounts of D-amino acids are 0.0025, 0.025, 0.25 and 2.5 pmol. Those for L-amino acids are 0.1, 1 and 10 pmol.

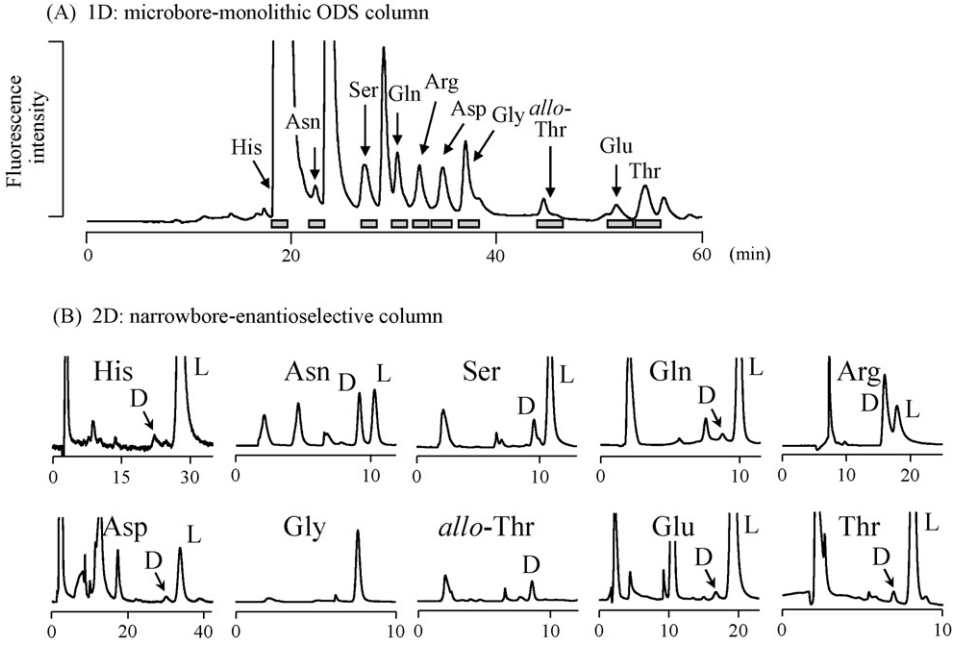

Fig. 4. Separation of NBD-derivatized hydrophilic amino acids in rat urine using the described 2D-HPLC system: a microbore-ODS column (A) and the continuously interlinked enantiomer separation using a narrowbore-Sumichiral OA-2500S column (B). The fractions indicated by gray bars are on-line collected to a multi-loop device and successively transferred to the enantioselective column.

Table 3
Amounts of hydrophilic amino acid enantiomers in the rat cerebrum, plasma and urine.

	Cerebrum		Plasma		Urine	
	D	L	D	L	D	L
His	n.d.	145.7 ± 1.5	n.d.	61.3 ± 2.9	1.7 ± 0.2	52.9 ± 7.3
Asn	n.d.	185.8 ± 8.7	n.d.	53.1 ± 1.8	28.9 ± 4.5	41.2 ± 5.1
Ser	257.6 ± 8.1	933.7 ± 42.4	1.1 ± 0.1	141.4 ± 9.4	7.3 ± 1.3	64.6 ± 7.3
Gln	n.d.	7618 ± 288	n.d.	710.2 ± 24.4	9.1 ± 1.4	314.8 ± 41.8
Arg	n.d.	178.5 ± 9.9	n.d.	185.8 ± 15.0	88.3 ± 26.4	75.1 ± 12.6
Asp	10.7 ± 1.0	5672 ± 577	0.5 ± 0.1	9.5 ± 1.1	0.9 ± 0.2	15.3 ± 2.1
Gly	–	2083 ± 13[a]	–	250.7 ± 20.4[a]	–	347.3 ± 30.7[a]
allo-Thr	7.6 ± 0.2	n.d.	n.d.	n.d.	8.3 ± 1.2	n.d.
Glu	n.d.	9702 ± 346	n.d.	87.3 ± 2.5	1.6 ± 0.1	70.0 ± 6.8
Thr	2.3 ± 0.1	488.5 ± 174.4	n.d.	198.8 ± 17.3	2.4 ± 0.6	96.3 ± 20.9

Values represent mean ± SE (nmol/g or ml) of 3 analyses. Rat: Wistar, male, 7 weeks of age. n.d.: not determined.
[a] Values for Gly are indicated in the columns for L-form.

has been described previously [8,30,36,37]. The reported amount of D-Ser in the cerebrum is around 300 nmol/g tissue, which is consistent with the values obtained in the present study. The presence of small amounts of D-Asp [38], D-Thr and D-*allo*-Thr [15] in the cerebrum was also reported, and the amounts were 13, 3.4 and 0.6 nmol/g, respectively. These values are also consistent with those obtained in the present study. In the plasma, small amounts of D-Ser and D-Asp were observed, and the values agreed with those reported in previous studies (D-Ser, 3.0 nmol/ml [30] and D-Asp, 2.2 nmol/ml blood [38]). In the urine, the presence of various D-amino acids has already been published [10–12,39–41], and concerning the hydrophilic D-amino acids, D-Ser [10,11,39–41], D-Thr [10,15,39–41], D-*allo*-Thr [15], D-Gln [11], D-Asx [10,40,41] and D-Glx [10,40,41] have been found using various species of mammals. In the present study, D-Ser, D-Thr and D-*allo*-Thr were also observed, and the amounts were consistent with the previously reported values. Concerning Asx and Glx, the present 2D-HPLC system is able to determine Asn besides Asp, and Gln besides Glu. In this context, relatively large amounts of D-Asn and D-Gln were found (see Table 3). In addition to these D-amino acids, a large amount of D-Arg and a small amount of D-His were observed. Especially, 41% of the urinary Asn and 54% of the urinary Arg were the D-enantiomers, and these results clearly indicate the necessity to separately determine amino acid enantiomers for the clinical analysis and diagnosis. The present study also demonstrated that various D-amino acids were present in mammals, and further studies to clarify their physiological meanings and diagnostic values are highly recommended.

4. Conclusion

In the present study, we have established a 2D-HPLC system for the simultaneous determination of hydrophilic amino acid enantiomers in the form of their NBD derivatives. The system was successfully validated using a rat plasma sample. The method should be a powerful tool for the metabolomic study of hydrophilic amino acid enantiomers in mammals. In rat urine, the D-enantiomers of all the 9 hydrophilic amino acid tested (His, Asn, Ser, Gln, Arg, Asp, *allo*-Thr, Glu and Thr) were found in various amounts. Further applications of the fully automated method to analyze diverse tissue samples and clinical samples, along with the studies to clarify the physiological and diagnostic meanings of the found D-enantiomers of target amino acids, are currently under investigation.

Acknowledgements

This work was supported in part by an Industrial Technology Research Grant Program in '05 from New Energy and Industrial Technology Development Organization (NEDO) of Japan. The authors appreciate Shiseido Co., Ltd. (Tokyo, Japan) for providing the HPLC system (NANOSPACE series) and the technical support.

References

[1] R. Konno, H. Brückner, A. D'Aniello, G. Fisher, N. Fujii, H. Homma (Eds.), D-Amino acids: A New Frontier in Amino Acids and Protein Research—Practical Methods and Protocols, Nova Science Publishers, New York, NY, 2007.
[2] K. Hamase, A. Morikawa, K. Zaitsu, J. Chromatogr. B 781 (2002) 73.
[3] N. Fujii, Orig. Life Evol. Biosph. 32 (2002) 103.
[4] T. Furuchi, H. Homma, Biol. Pharm. Bull. 28 (2005) 1566.
[5] A. D'Aniello, Brain Res. Rev. 53 (2007) 215.
[6] T. Nishikawa, Biol. Pharm. Bull. 28 (2005) 1561.
[7] S.H. Snyder, P.M. Kim, Neurochem. Res. 25 (2000) 553.
[8] A. Hashimoto, T. Nishikawa, T. Hayashi, N. Fujii, K. Harada, T. Oka, K. Takahashi, FEBS Lett. 296 (1992) 33.
[9] R. Inoue, K. Hashimoto, T. Harai, H. Mori, J. Neurosci. 28 (2008) 14486.
[10] H. Brückner, A. Schieber, Biomed. Chromatogr. 15 (2001) 166.
[11] H. Brückner, S. Haasmann, M. Langer, T. Westhauser, R. Wittner, H. Godel, J. Chromatogr. A 666 (1994) 259.
[12] D.W. Armstrong, M.P. Gasper, S.H. Lee, N. Ercal, J. Zukowski, Amino Acids 5 (1993) 299.
[13] Y. Nagata, K. Yamamoto, T. Shimojo, J. Chromatogr. 575 (1992) 147.
[14] A. Morikawa, K. Hamase, K. Zaitsu, Anal. Biochem. 312 (2003) 66.
[15] H. Zhao, K. Hamase, A. Morikawa, Z. Qiu, K. Zaitsu, J. Chromatogr. B 810 (2004) 245.
[16] K. Hamase, S. Takagi, A. Morikawa, R. Konno, A. Niwa, K. Zaitsu, Anal. Bioanal. Chem. 386 (2006) 705.
[17] K. Hamase, A. Morikawa, T. Ohgusu, W. Lindner, K. Zaitsu, J. Chromatogr. A 1143 (2007) 105.
[18] Y. Tojo, K. Hamase, M. Nakata, A. Morikawa, M. Mita, Y. Ashida, W. Lindner, K. Zaitsu, J. Chromatogr. B 875 (2008) 174.
[19] A. Morikawa, K. Hamase, T. Ohgusu, S. Etoh, H. Tanaka, I. Koshiishi, Y. Shoyama, K. Zaitsu, Biochem. Biophys. Res. Commun. 355 (2007) 872.
[20] S. Etoh, K. Hamase, A. Morikawa, T. Ohgusu, K. Zaitsu, Anal. Bioanal. Chem. 393 (2009) 217.
[21] K. Hamase, A. Morikawa, S. Etoh, Y. Tojo, Y. Miyoshi, K. Zaitsu, Anal. Sci. 25 (2009) 961.
[22] N.M. Maier, P. Franco, W. Lindner, J. Chromatogr. A 906 (2001) 3.
[23] V. Schurig, J. Chromatogr. A 906 (2001) 275.
[24] H. Frank, G.J. Nicholson, E. Bayer, J. Chromatogr. Sci. 15 (1977) 174.
[25] V. Schurig, M. Juza, M. Preschel, G.J. Nicholson, E. Bayer, Enantiomer 4 (1999) 297.
[26] W.A. König, R. Krebber, P. Mischnick, J. High Resolut. Chromatogr. 12 (1989) 732.
[27] D.W. Aswad, Anal. Biochem. 137 (1984) 405.
[28] N. Nimura, T. Kinoshita, J. Chromatogr. 352 (1986) 169.
[29] R.H. Buck, K. Krummen, J. Chromatogr. 315 (1984) 279.
[30] A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, T. Hayashi, J. Chromatogr. 582 (1992) 41.
[31] S. Einarsson, B. Josefsson, P. Möller, D. Sanchez, Anal. Chem. 59 (1987) 1191.
[32] H. Brückner, C. Gah, J. Chromatogr. 555 (1991) 81.
[33] Y. Miyoshi, K. Hamase, Y. Tojo, M. Mita, R. Konno, K. Zaitsu, J. Chromatogr. B 877 (2009) 2506.
[34] T. Inoue, K. Hamase, A. Morikawa, K. Zaitsu, J. Chromatogr. B 744 (2000) 213.
[35] H. Brückner, R. Wittner, H. Godel, Chromatographia 32 (1991) 383.
[36] K. Hamase, H. Homma, Y. Takigawa, T. Fukushima, T. Santa, K. Imai, Biochim. Biophys. Acta 1334 (1997) 214.
[37] Y. Nagata, K. Horiike, T. Maeda, Brain Res. 634 (1994) 291.
[38] D.S. Dunlop, A. Neidle, D. McHale, D.M. Dunlop, A. Lajtha, Biochem. Biophys. Res. Commun. 141 (1986) 27.
[39] H. Brückner, S. Haasmann, A. Friedrich, Amino Acids 6 (1994) 205.
[40] H. Brückner, A. Schieber, J. High Resolut. Chromatogr. 23 (2000) 576.
[41] R. Pätzold, A. Schieber, H. Brückner, Biomed. Chromatogr. 19 (2005) 466.