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Determination of D- and L-enantiomers of threonine and allo-threonine in mammals using two-step high-performance liquid chromatography

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Abstract

A sensitive and selective method for the determination of four threonine (Thr) isomers (L-Thr, D-Thr, L-allo-Thr and D-allo-Thr) in mammalian tissues has been established using two-step high-performance liquid chromatography. This method includes the precolumn fluorescence derivatization of amino acids with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and the separation using a combination of a reversed-phase column and a chiral column. The calibration ranges of D-Thr, D-allo-Thr and L-allo-Thr spiked in the rat cerebellum sample are 2.5 fmol–5 pmol per injection, and that of L-Thr is 50 fmol–50 pmol. Within-day and day-to-day precisions of the determination of the four Thr isomers are approximately 5% in the rat cerebellum. By using this method, the tissue distributions of D-Thr, D-allo-Thr and L-allo-Thr in mammals have been demonstrated for the first time in rats, and found that significant amounts of D-Thr and D-allo-Thr are present in the frontal brain areas and urine. Among the 12 tissues tested, the highest amounts of D-Thr (0.85 ± 0.05 nmol/g wet tissue) and D-allo-Thr (5.01 ± 0.32 nmol/g wet tissue) were found in the corpus striatum. L-allo-Thr was not present in any of the tested tissues and physiological fluids.

Keywords: Chiral separation; Amino acid; HPLC; Threonine; allo-Threonine

1. Introduction

The presence of free D-amino acids has recently been shown in various living organisms including mammals, and their functions and origins have gradually been determined [1,2]. In mammalian tissues, relatively large amount of D-serine (D-Ser) is present in the frontal brain areas and is thought to be an endogenous agonist of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptors [3–5]. D-Aspartic acid (D-Asp) is reported to play important roles in the hormonal regulations of various endocrine glands [6–9]. In addition to D-Ser and D-Asp, small amounts of D-amino acids such as D-alanine (D-Ala) [10,11], D-leucine (D-Leu) [12,13] and D-proline (D-Pro) [14] are found in a variety of mammalian species, and their tissue distributions and regulation mechanisms have been partially investigated [15,16]. Although their physiological functions have not yet been clarified, these D-amino acids of small amounts are thought to be novel physiologically active substances and their detailed investigations are expected.

D-Threonine (D-Thr) is one of these rare D-amino acids, and it has been reported that a small amount of D-Thr is present in the urine of mammals [17–19]. However, the tissue distribution of D-Thr in the mammalian body has never been reported. In addition, Thr has two chiral centers as shown in Fig. 1, and four isomers (L-Thr, D-Thr, L-allo-Thr and D-allo-Thr) are present. Concerning the epimerization of L-Thr, the reversal of the chiral center at the α-carbon, which usually occurred for other amino acids [20,21], produces D-allo-Thr. The conversion of the chirality at the β-carbon of L-Thr produces L-allo-Thr. D-Thr is produced by the reversal of the optical forms on both of the two chiral carbons. Therefore, in the research of the Thr epimers, not only D- and L-Thr, but

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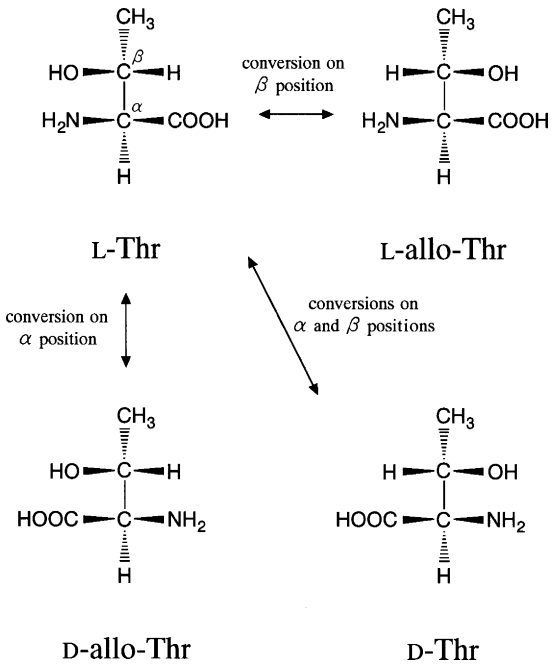

Fig. 1. Structures of L-Thr, D-Thr, L-allo-Thr and D-allo-Thr.

also the enantiomers of allo-Thr should be considered, and the clarification of these amounts in mammals is helpful for the study on the metabolisms of amino acids having two chiral centers. In addition, D-Thr and D-allo-Thr have structures similar to that of the widely observed D-Ser (Thr has a methyl group instead of a hydrogen at the β-carbon), therefore, a detailed investigation of the Thr isomers could also provide an important insight into the biochemistry of D-amino acids. However, the analytical method that enables the determination of both Thr and all-Thr enantiomers in the complicated tissue samples has never been reported, and there has been no report showing the amounts in mammals.

In the present report, a highly sensitive method for the determination of the four isomers of Thr in mammalian tissues has been established using a two-step HPLC system. This method includes the pre-column derivatization of amino acids with the highly fluorescent derivatizing reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [22], and the NBD-derivatives are separated by a combination of a micro-ODS column and a chiral column. Using this method, the presence of D-Thr, L-Thr, D-allo-Thr and L-allo-Thr in the rat tissues and physiological fluids has clearly been demonstrated.

2. Experimental

2.1. Chemicals

D-Thr and L-Thr were purchased from Wako (Osaka, Japan), and D-allo-Thr and L-allo-Thr were purchased from Tokyo Kasei (Tokyo, Japan). The derivatizing reagent NBD-F was a product of Tokyo Kasei. Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were purchased from Nacalai Tesque (Kyoto, Japan). Trifluoroacetic acid (TFA), citric acid monohydrate, and boric acid were obtained from Wako. Water was purified using a Milli-Q gradient A 10 system (Millipore, Bedford, MA, USA).

2.2. Animals

Male Wistar rats (8 weeks of age, specific-pathogen free) were purchased from Seac Yoshitomi (Fukuoka, Japan) and were housed at an animal center of Kyushu University, Graduate School of Pharmaceutical Sciences, under a 12-h light/12-h dark cycle for one week (lights on at 07:00 a.m.). They had free access to diet (type NMF, Oriental Yeast, Tokyo, Japan) and water.

2.3. Sample preparation procedure

The rats were anesthetized with diethyl ether and killed by exsanguination from the abdominal aorta, and then specific tissues (cerebrum, cerebellum, corpus striatum, hypothalamus, adrenal gland, kidney, liver, lung, pancreas, spleen, testis, and heart) were excised; these tissues were weighed and stored at −80 °C until use. The tissues were homogenized in 20× volumes MeOH on ice, and the homogenates were centrifuged at 4500 × *g* for 5 min to obtain the supernatants. The blood collected from the abdominal aorta was centrifuged at 4500 × *g* at 4 °C for 15 min, and to the obtained serum, 20× volumes of MeOH was added and mixed for 2 min. The urine was collected from the urinary bladder, and 20× volumes of MeOH was added and mixed for 2 min. These mixtures were centrifuged at 4500 × *g* for 5 min, and the supernatants were collected. 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 10 μl of 20 mM NBD-F in dry MeCN were added, and heated at 60 °C for 2 min. To the reaction mixture, 20 μl of 5% (v/v) TFA in water was added and the mixture was filtered through a 0.2-μm membrane filter (W-3-2, Tosoh, Tokyo, Japan), and 5 μl of the filtrate was then injected into the HPLC.

2.4. HPLC system for the determination of Thr isomers

The HPLC system consisted of a DG 980-50 degasser (JASCO, Tokyo, Japan), an LG-980-02 gradient unit (JASCO), a PU-980 pump (JASCO), a 7725 injector (Rheodyne, Cotati, CA, USA), a CO-965 column oven (JASCO), an FP-920 fluorescence detector and an 807-IT integrator (JASCO). The analytical column for the reversed-phase separation was a Capcell pak C18-AQ (250 mm × 2.0 mm i.d., Shiseido, Tokyo, Japan) maintained at 35 °C. The mobile phase was MeCN-TFA-water (12/0.08/88, v/v) and the flow rate was 0.2 ml/min. The column was washed using a mobile

phase with a high concentration of MeCN (80%) for 5 min after analyzing the tissue samples.

The analytical column for enantioseparation was a Sumichiral OA-2500S (250 mm × 4.6 mm i.d., Sumika Analytical Center, Osaka, Japan) maintained at 35 °C. The mobile phase was 1 mM citric acid in the mixed solution of MeOH–MeCN (60/40, v/v), and the flow-rate was 1 ml/min. For the confirmation of the determined values in the tissue samples, a Sumichiral OA-2500R (250 mm × 4.6 mm i.d., Sumika Analytical Center) was also used with a mobile phase of 3 mM citric acid in MeOH. Fluorescence detection of NBD-Thr and NBD-allo-Thr was carried out at 530 nm with excitation at 470 nm.

3. Results and discussion

3.1. Two-step HPLC separation of the enantiomers of Thr and allo-Thr

For the determination of the D- and L-enantiomers of Thr and allo-Thr in biological samples, a two-step HPLC system has been developed. Because the amounts of D-Thr, D-allo-Thr and L-allo-Thr in biological samples are extremely small, the determination of these Thr isomers is difficult due to the interference of large amounts of L-amino acids and a large number of biological substances such as peptides and amines. A two-step HPLC system is a powerful tool for the separation and determination of small amounts of substances in biological samples, and in the present research, a combination of a C18-column and a Pirkle-type chiral column was adopted. For the sensitive determination, these amino acids are derivatized with a fluorescence derivatizing reagent, NBD-F. By using this system, NBD-Thr and NBD-allo-Thr can be separated as D and L mixtures using a C18-column; the fractions of these NBD-amino acids are collected for 5 min (the volumes of both fractions are 1 ml). Portions (100 μl) of these fractions are then subject to a chiral column, and the enantiomers of Thr and allo-Thr are determined.

For the reversed-phase separation of NBD-Thr and NBD-allo-Thr from the other amino acids and interfering substances such as excessive reagent, the concentration of MeCN and TFA added to the mobile phase was investigated. The concentration of MeCN was investigated in order to obtain good separation of the NBD-amino acids, and 12% (v/v) was selected. The concentration of TFA strongly affected the retention times of the reagent peaks, and as shown in Fig. 2, their retention times became drastically shorter by increasing the concentration of TFA. As a result, satisfactory separation of NBD-Thr and NBD-allo-Thr from the large reagent peak was obtained using MeCN-TFA-water (12/0.08/88, v/v) as the mobile phase. Under these separation conditions, NBD-Thr and NBD-allo-Thr are eluted in about 60 min with minimized interference by excess reagents and other amino acids.

The conditions for the enantioseparation were also investigated. As described in previous reports [13,23], the enantiomers of the NBD-amino acids could be clearly separated using a Pirkle-type chiral column, Sumichiral OA-2500S with MeOH containing citric acid as the mobile phase. In the present investigation, the enantiomers of NBD-Thr, the enantiomers of NBD-allo-Thr and NBD-Gly should be separated, because the high concentration of Gly in biological samples always results in the contamination of a non-negligible amount of Gly into the fraction of NBD-allo-Thr. Thus, the separation conditions were investigated using a mixed solution of these five amino acids. The enantiomers of NBD-Thr and NBD-allo-Thr were clearly separated using MeOH containing citric acid, however, the retention time of NBD-Gly is almost the same as that of NBD-D-allo-Thr. Therefore, we investigated the addition of MeCN to the mobile phase. Fig. 3 shows the effect of MeCN added to the mobile phase (mixed solution of MeOH and MeCN, the concentration of citric acid is 2 mM) on the retention times of NBD-Thr, NBD-allo-Thr and NBD-Gly. With an increase in the MeCN concentration in the mobile phase, the retention time of NBD-Gly decreased more than those of NBD-Thr and NBD-allo-Thr, and a good resolution was obtained using the mixed solution of MeOH–MeCN (60/40, v/v) containing citric acid. The citric acid concentration was selected in order to obtain sufficient separation of the five NBD-amino acids, and 1 mM citric acid in MeOH–MeCN (60/40, v/v) was used as the mobile phase of the chiral separation. The typical chromatograms obtained by a micro-ODS-column and a chiral column are shown in Fig. 4.

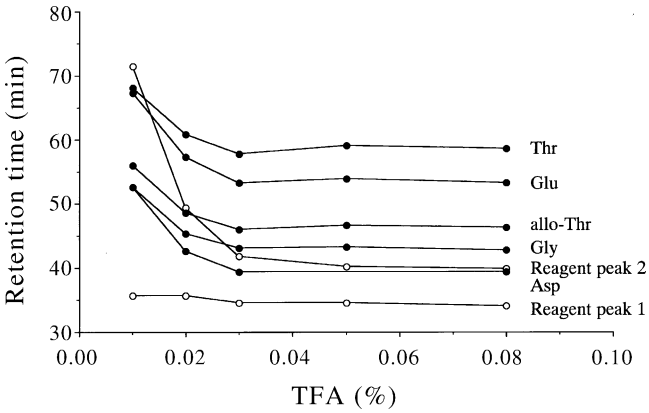

Fig. 2. Effect of the concentration of TFA added to the mobile phase used for the reversed-phase separation on the retention times of the NBD-amino acids and the reagent peaks. Filled circles denote the retention times of the NBD-amino acids, and the open circles denote those of the reagent peaks. The HPLC conditions are as follows: column, Capcell Pak C18 AQ (250 mm × 2.0 mm i.d., 35 °C, Shiseido); mobile phase, mixed solutions of MeCN–TFA–water (concentration of MeCN is 12%, v/v); flow rate, 0.2 ml/min.

3.2. Validation of the method

For the validation of this method, calibration lines, within-day precisions and day-to-day precisions were investigated using the rat cerebellum. Because the cerebellum is well known to have a high D-amino acid oxidase activity, which

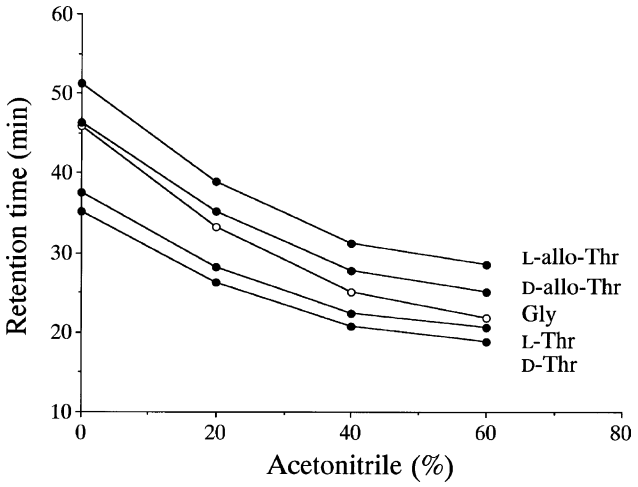

Fig. 3. Effect of MeCN added to the mobile phase used for chiral separation on the retention times of NBD-D,L-Thr, NBD-D,L-allo-Thr and NBD-Gly. The HPLC conditions are as follows: column, Sumichiral OA-2500S (250 mm × 4.6 mm i.d., 35 °C, Sumika Analytical Center); mobile phase, 2 mM citric acid in the mixed solution of MeOH–MeCN; flow rate, 1.0 ml/min.

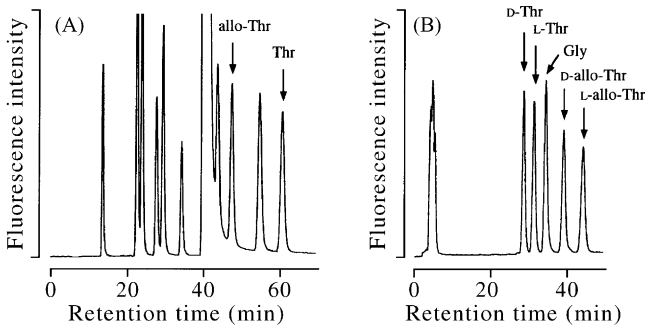

Fig. 4. Chromatograms of NBD-derivatives of standard amino acids. (A) Standard allo-Thr and 20 amino acids constructing proteins were derivatized and injected into the reversed phase column (injection amount of each amino acid, 10 pmol). The HPLC conditions are as follows: column, Capcell Pak C18 AQ (250 mm × 2.0 mm i.d., 35 °C, Shiseido); mobile phase, a mixed solution of MeCN–TFA–water (12/0.08/88, v/v); flow rate, 0.2 ml/min. Hydrophobic amino acids eluted later than NBD-Thr are not shown. (B) Separation of NBD-D-Thr, NBD-L-Thr, NBD-D-allo-Thr, NBD-L-allo-Thr and NBD-Gly using a Sumichiral OA-2500S chiral column (injection amount, 250 fmol each). The HPLC conditions are as follows: column, Sumichiral OA-2500S (250 mm × 4.6 mm i.d., 35 °C, Sumika Analytical Center); mobile phase, 1 mM citric acid in MeOH–MeCN (60/40, v/v); flow rate, 1.0 ml/min. The other conditions are described in the text.

catalyzes the oxidation of various neutral D-amino acids, this tissue usually contains extremely low levels of neutral D-amino acids [1,13]. For the calibration lines of D-Thr, D-allo-Thr and L-allo-Thr, 0.25, 0.5, 2, 10, 100 and 500 pmol of D-Thr, D-allo-Thr and L-allo-Thr (injection amounts of these amino acids on the chiral column are 0.0025, 0.005, 0.02, 0.1, 1 and 5 pmol, respectively) were added to a supernatant (10 μl) of a cerebellum sample. In mammalian tissues, large amounts of L-amino acids are present, therefore, the calibration line of L-Thr was constructed by adding 5, 20, 100, 1000 and 5000 pmol of L-Thr (injection amounts are 0.05, 0.2, 1, 10 and 50 pmol) to the supernatant (10 μl) of a cerebellum sample. As shown in Table 1, all of these calibration lines were linear with good correlation coefficients. In contrast to the abundant L-Thr in the tissue samples, the amounts of D-Thr, D-allo-Thr and L-allo-Thr are extremely small, therefore, scaled calibration lines have also been constructed (injection amount on the chiral column is 2.5–100 fmol). The equations of these scaled calibration lines are $y = 98.4x + 0.078$ ($r = 0.9998$) for D-Thr, $y = 86.6x - 0.001$ ($r = 0.9997$) for D-allo-Thr, and $y = 72.2x + 0.602$ ($r = 0.9986$) for L-allo-Thr; the slopes are almost equal to those described in Table 1. Within-day and day-to-day precisions were investigated using a rat cerebellum sample spiked with 10 pmol of D-Thr, D-allo-Thr and L-allo-Thr in 10 μl of the supernatant (injection amounts of these spiked amino acids are 100 fmol on the chiral column). Concerning L-Thr, the intrinsic amount was used for the precision tests. As shown in Table 1, the within-day and day-to-day precisions as described by the R.S.D. values of five analyses are 1.99–6.76%.

For the determination of the Thr and allo-Thr isomers, pre-column derivatization reversed-phase HPLC methods using *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanineamide (FDAA) [24], or the combination of *o*-phthaldialdehyde (OPA) and *N*-isobutyryl-L-cysteine (IBLC) [25] have been reported. These methods have been established with the view of the simultaneous determination of various amino acid enantiomers in a single separation, and the lower limits of detection were reported to be low picomoles per injection.

Table 1
Calibration lines and the precisions of the method

Amino acid	Calibration line[a]			Precision (R.S.D., %)[c]	
	Calibration range (pmol)	Equation[b]	Correlation coefficient	Within-day ($n = 5$)	Day-to-day (5 days)
D-Thr	0.0025–5	$y = 101x - 0.193$	1.000	5.29	4.54
L-Thr	0.05–50	$y = 98.2x + 166$	1.000	5.40	6.76
D-allo-Thr	0.0025–5	$y = 82.4x + 0.426$	1.000	4.17	1.99
L-allo-Thr	0.0025–5	$y = 70.2x + 0.829$	1.000	3.93	2.42

[a] Calibration lines were constructed by adding known amounts of standard amino acids to a rat cerebellum sample.
[b] Equations were made where x is the amount of amino acids added (injection amount on the chiral column, pmol), and y is the fluorescence intensities (peak height, mV).
[c] Within-day and day-to-day precisions were determined using a rat cerebellum sample spiked 100 fmol (injection amount on the chiral column) of D-Thr, D-allo-Thr and L-allo-Thr.

Table 2
Distribution of D-Thr, D-allo-Thr, L-Thr and L-allo-Thr in the rat tissues and physiological fluids

Tissue	D-Thr	D-allo-Thr	L-Thr	L-allo-Thr
Brain tissues				
Cerebrum	0.56±0.05	3.39±0.22	311 ± 17.7	n.d.
Cerebellum	n.d.	n.d.	319 ± 15.3	n.d.
Corpus striatum	0.85±0.05	5.01±0.32	515 ± 32.3	n.d.
Hypothalamus	n.d.[a]	2.00±0.09	460 ± 28.7	n.d.
Peripheral tissues				
Kidney	n.d.	n.d.	383 ± 18.3	n.d.
Liver	n.d.	n.d.	222 ± 4.7	n.d.
Heart	n.d.	n.d.	202 ± 21.2	n.d.
Lung	n.d.	n.d.	584 ± 42.2	n.d.
Pancreas	n.d.	n.d.	485 ± 42.5	n.d.
Spleen	n.d.	n.d.	348 ± 10.9	n.d.
Adrenal gland	n.d.	n.d.	377 ± 19.3	n.d.
Testis	n.d.	n.d.	145 ± 8.8	n.d.
Physiological fluids				
Serum	n.d.	n.d.	197 ± 10.7	n.d.
Urine	1.16±0.12	5.40±0.85	47.2 ± 8.1	n.d.

Values represent means ± S.E.M. (nmol/g wet tissue) of five rats. Values for serum and urine are means ± S.E.M. (nmol/ml) of three rats; n.d.: not determined (smaller than the lower limit of determination); lower limit of determination: 0.5 nmol/g wet tissue or ml.

[a] Trace amount of D-Thr was observed.

However, the resolution and the sensitivity are not sufficient for the determination of small amounts of D-Thr, D-allo-Thr and L-allo-Thr in the tissue samples. In the present investigation, all of the four isomers of Thr could be clearly separated from other amino acids and the interfering substances in the tissues using the two-step HPLC, and the lower quantitation limit is 2.5 fmol per injection. In addition, the present method has sufficient R.S.D. values for the within-day and day-to-day precisions. These results indicate that the present method is suitable for the determination of D-Thr, L-Thr, D-allo-Thr and L-allo-Thr in mammalian tissues.

3.3. Tissue distribution of the four Thr isomers in the rat

The amounts of the four Thr isomers were determined in 12 tissues including the brain and periphery using the two-step HPLC system described above. The amounts of the Thr isomers in the serum and urine were also investigated. The results are presented in Table 2. In all the tested tissues, a high concentration of L-Thr (over 100 nmol/g wet tissue) was present, while the concentration of L-allo-Thr was smaller than the lower limit of determination. Concerning the D-isomers, low but significant amounts of D-Thr and D-allo-Thr are present in the frontal brain areas and urine. Compared to the concentrations of D-Thr, the concentrations of D-allo-Thr are higher in all the tested tissues. In the urine, the highest concentrations of D-Thr and D-allo-Thr were observed; their typical chromatograms are shown in Fig. 5. As a confirmation of the results, the amounts of D-Thr and D-allo-Thr in the urine were also determined using a different chiral column having an opposite chiral center (Sumichiral OA-2500R). The obtained values were 1.38 ± 0.10 and 5.43 ± 0.81 nmol/ml urine for D-Thr and D-allo-Thr, respectively; these values are almost equal to those described in Table 2. The authenticity of D-Thr and D-allo-Thr is confirmed in all tissues using Sumichiral OA-2500R, as well as carefully spiked checks using two different separation conditions, indicating that the peaks observed at the retention times of D-Thr and D-allo-Thr are actually these compounds.

Until now, D-Thr was determined in the serum and urine of various animals including humans [17–19], and the amounts of D-Thr in the urine were reported to be lower than 11.4 nmol/ml. However, in the serum, D-Thr was not observed in all species. In the present investigation, D-Thr was not observed in the serum of the rat, and the value for the urine was 1.16 nmol/ml; these results are consistent with those previously reported for other animals. In addition to the urine and serum, the tissue distribution of D-Thr was investigated for the first time, and it was found that considerable amount of D-Thr is present in the frontal brain areas (cerebrum and striatum). The distribution of D-allo-Thr and L-allo-Thr in mammals were also investigated for the first time, showing that L-allo-Thr are not present in mammalian tissues and physiological fluids, even though it is an L-amino acid. On the contrary, D-allo-Thr is present in the frontal brain areas (cerebrum, striatum and hypothalamus) with higher amounts than those of D-Thr. In mammalian tissues and physiological fluids, a large amount of L-Thr is present, and the reversal of the chiral center at the α-carbon of L-Thr produces D-allo-Thr. Both of the chiral centers should be changed to form D-Thr. Therefore, it is likely that D-allo-Thr is a major component compared to D-Thr.

Concerning the distribution of D-Ser, it is widely known to be present in the frontal brain areas at an extremely high concentration (about 200 nmol/g wet tissue) [1,3]. Because

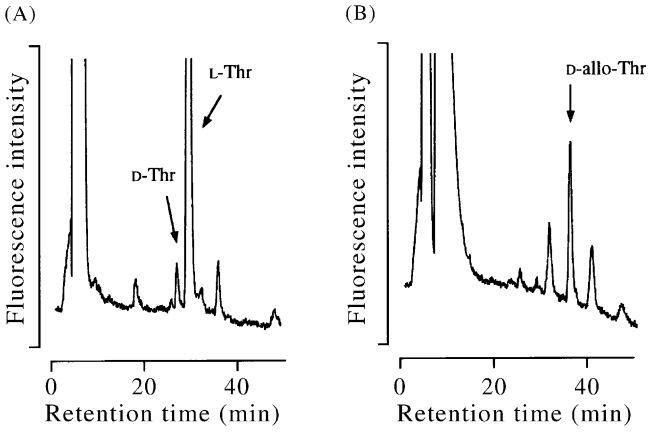

Fig. 5. Chromatograms of NBD-D-Thr, NBD-L-Thr and NBD-D-allo-Thr in rat urine obtained using a Sumichiral OA-2500S chiral column. Fractions of (A) NBD-Thr and (B) NBD-allo-Thr collected by a micro-ODS-column were injected and the enantiomers were separated. The HPLC conditions are as follows: column, Sumichiral OA-2500S (250 mm × 4.6 mm i.d., 35 °C, Sumika Analytical Center); mobile phase, 1 mM citric acid in MeOH–MeCN (60/40, v/v); flow rate, 1.0 ml/min. The other conditions are described in the text.

D-Thr and D-allo-Thr have structures similar to that of D-Ser, it is not unlikely that the amounts of these two D-amino acids are higher in the frontal brain areas than those in other tissues.

4. Conclusion

In the present investigation, a sensitive and selective two-step HPLC method has been established and validated for the determination of the enantiomers of Thr and allo-Thr in mammalian tissues. By using this method, the tissue distribution of D-Thr and D-allo-Thr in mammals has been demonstrated for the first time, showing that considerable amounts of these two D-amino acids are present in the frontal brain areas, while L-allo-Thr was not observed in all of the tested tissues. The amounts of D-allo-Thr are higher than those of D-Thr and L-allo-Thr, suggesting that the epimerization of the chiral center of the α-carbon predominantly occurs, compared with the epimerization of the β-carbon or racemization. The established method and the obtained findings should be fundamental contributions for the study of D-amino acids, the candidates of new biologically active substances.

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References

[1] K. Hamase, A. Morikawa, K. Zaitsu, J. Chromatogr. B 781 (2002) 73.
[2] N. Fujii, Orig. Life Evol. Biosph. 32 (2002) 103.
[3] A. Hashimoto, T. Oka, Prog. Neurobiol. 52 (1997) 325.
[4] A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, J. Neurochem. 60 (1993) 783.
[5] S.H. Snyder, P.M. Kim, Neurochem. Res. 25 (2000) 553.
[6] H. Wolosker, A. D'Aniello, S.H. Snyder, Neuroscience 100 (2000) 183.
[7] Y. Takigawa, H. Homma, J.A. Lee, T. Fukushima, T. Santa, T. Iwatsubo, K. Imai, Biochem. Biophys. Res. Commun. 248 (1998) 641.
[8] Y. Nagata, H. Homma, J.A. Lee, K. Imai, FEBS Lett. 444 (1999) 160.
[9] A. D'Aniello, M.M.D. Fiore, G.H. Fisher, A. Milone, A. Seleni, S. D'Aniello, A.F. Perna, D. Ingrosso, FASEB J. 14 (2000) 699.
[10] A. Morikawa, K. Hamase, K. Zaitsu, Anal. Biochem. 312 (2003) 66.
[11] A. Morikawa, K. Hamase, T. Inoue, R. Konno, A. Niwa, K. Zaitsu, J. Chromatogr. B 757 (2001) 119.
[12] T. Inoue, K. Hamase, A. Morikawa, K. Zaitsu, J. Chromatogr. B 744 (2000) 213.
[13] K. Hamase, H. Homma, Y. Takigawa, T. Fukushima, T. Santa, K. Imai, Biochim. Biophys. Acta 1334 (1997) 214.
[14] K. Hamase, T. Inoue, A. Morikawa, R. Konno, K. Zaitsu, Anal. Biochem. 298 (2001) 253.
[15] R. Konno, T. Oowada, A. Ozaki, T. Iida, A. Niwa, Y. Yasumura, T. Mizutani, Am. J. Physiol. 265 (1993) G699.
[16] R. Konno, A. Niwa, Y. Yasumura, Biochem. J. 268 (1990) 263.
[17] H. Bruckner, A. Schieber, Biomed. Chromatogr. 15 (2001) 166.
[18] H. Bruckner, A. Schieber, J. High Resolut. Chromatogr. 23 (2000) 576.
[19] H. Bruckner, S. Haasmann, A. Friedrich, Amino Acids 6 (1994) 205.
[20] T. Yoshimura, N. Esaki, J. Biosci. Bioeng. 96 (2003) 103.
[21] H. Wolosker, S. Blackshaw, S.H. Snyder, Proc. Natl. Acad. Sci. USA 96 (1999) 13409.
[22] K. Imai, Y. Watanabe, Anal. Chim. Acta 130 (1981) 377.
[23] T. Fukushima, M. Kato, T. Santa, K. Imai, Biomed. Chromatogr. 9 (1995) 10.
[24] S. Hess, K.R. Gustafson, D.J. Milanowski, E. Alvira, M.A. Lipton, L.K. Pannell, J. Chromatogr. A 1035 (2004) 211.
[25] H.P. Fitznar, J.M. Lobbes, G. Kattner, J. Chromatogr. A 832 (1999) 123.