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Short communication

Determination of minute amounts of D-leucine in various brain regions of rat and mouse using column-switching high-performance liquid chromatography

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Abstract

A highly sensitive method for the determination of minute amounts of D-Leu in biological samples was developed. For accurate and sensitive determination, a column-switching system using a micro ODS column and a chiral column was adopted. After pre-column derivatization of D- and L-Leu with NBD-F, the derivatives of the enantiomers were purified on a micro ODS column as a DL mixture. The eluted DL-Leu was then introduced to the chiral column, and each enantiomer was determined. The calibration curve for D-Leu, which was constructed by adding known amounts of D-Leu to a rat hippocampus, was linear from 1 to 1000 fmol ($r>0.999$), and the detection limit of added D-Leu was 1 fmol ($S/N=5$). Within-day and day-to-day precisions of D-Leu determination using the same homogenate of rat hippocampus were 5.11 and 5.25% (RSD), respectively. The content of D-Leu in rat hippocampus was 0.69 nmol/g wet tissue (the percentage of D-enantiomer for total Leu was 0.97%), which was consistent with the reported value. The distribution of D-Leu in mouse brain was also investigated, and the presence of D-Leu in various regions of the mammalian brain was first observed.

Keywords: Leucine; Amino acids

1. Introduction

During the last decade, an increasing amount of evidence was reported for the presence of free D-amino acids in higher animals, and physiological roles and the origins of D-amino acids were partially clarified. Particularly, relatively large amounts of D-Asp and D-Ser were observed in the brain and periphery, and these two D-amino acids were well investigated [1–3]. D-Asp is widely observed in neuroendocrine and endocrine organs such as testis [4,5], pineal gland [6,7] and adrenal gland [5,8], and the amino acid is closely related to the synthesis of testosterone [4,9] and the secretion of melatonin [6]. Concerning D-Ser, the biosynthesis of D-Ser has also been demonstrated in rat brain [10,11] and was

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thought to be the modulator of the *N*-methyl-D-aspartate subtype of the glutamate receptor [1,2,12]. In addition to D-Asp and D-Ser, several D-amino acids such as D-Ala [2,13–15], D-Leu [2,7,16] and D-Pro [2,15,16] were found in a variety of mammalian species. However, the intrinsic amounts of these D-amino acids were small, and their determination was often interfered with by the co-existence of an excess amount of L-amino acids (major components of amino acids in organisms) and abundant endogenous substances. Therefore, for the determination of minute amounts of D-amino acids, high selectivity and high sensitivity were required. In our previous report, D-Leu, a rare D-amino acid, was found in the hippocampus and pineal gland of the rat brain [7] using high-performance liquid chromatography (HPLC) with two different columns. D-Leucine was also found in the urine of rats and mice by Armstrong et al. [16]. In both investigations, a reversed-phase column was used to isolate and quantify Leu as a DL mixture, and a chiral separation column was used to determine the enantiomeric ratio of isolated Leu. However, for this method the possibility exists of the appearance of unexpected interfering peaks at the retention times of D- and L-Leu both on reversed-phase and chiral columns. In addition, only a part of D-Leu separated on a reversed-phase column could be introduced to the chiral column. Thus, the method has disadvantages in accuracy and sensitivity. For an accurate and sensitive analysis, a column-switching system was used [17]. Therefore, we tried to establish the system for the determination of minute amount of D-Leu with a micro ODS column and a chiral column. Using a micro ODS column for the purification of Leu in biological samples, the volume of separated DL-Leu fraction would be reduced to about 100 μl. Therefore, a large part of the DL-Leu fraction was introduced to the next chiral column, and D-Leu could be determined directly without loss of accuracy and sensitivity. In this paper, we describe the first determination of D-Leu in the rat hippocampus, the method was validated using a rat hippocampus sample. Since the distribution of D-Leu in the mammalian brain has not been investigated in detail, the D-Leu content in various regions of the mouse brain was also examined.

2. Experimental

2.1. Materials

D- and L-Leu, methanol (MeOH), acetonitrile (MeCN) and tetrahydrofuran (THF) of HPLC grade were purchased from Nacalai Tesque (Kyoto, Japan). Trifluoroacetic acid (TFA), citric acid monohydrate and boric acid were obtained from Wako (Osaka, Japan). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was a product of Tokyo Kasei (Tokyo, Japan). D-Amino acid oxidase (DAAO) was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Water was purified by a Milli-QII system (Millipore, Bedford, MA, USA). Other reagents and solvents were of reagent grade.

2.2. Animals

Male Wistar rats (6 weeks of age, SPF) and male ddY mice (8 weeks of age, SPF) were purchased from Seac Yoshitomi (Fukuoka, Japan). The animals were housed under a 12-h light/12-h dark cycle (light on at 06:00 a.m.) and were fed food and water freely.

2.3. Sample preparation

Rats were anesthetized with diethyl ether and sacrificed by exsanguination from the abdominal aorta; immediately the hippocampus was excised, weighed and stored at −80°C until analysis. To the tissue, MeOH (×20 volumes of the tissue mass) was added and homogenized on ice (1000 rpm, 20 times) and centrifuged at 4500 *g* for 5 min. The supernatant (20 μl) was evaporated to dryness under reduced pressure at 40°C. To the residue, 20 μl of 200 m*M* Na–borate buffer (pH 8.0), 10 μl of water and 30 μl of 20 m*M* NBD-F in MeCN were added and heated at 60°C for 2 min. To the reaction mixture, 190 μl of aqueous 2% TFA was added and filtered through a 0.2-μm membrane filter, and 5 μl of the filtrate was subjected to HPLC as described in Section 2.4.

Mice were anesthetized with diethyl ether and decapitated. Cerebrum, cerebellum, hippocampus, hypothalamus, medulla oblongata, pituitary gland and pineal gland were quickly excised, weighed and

stored at −80°C until analysis. The other regions such as pons or midbrain were excised together and analyzed as ''others''. The tissues were homogenized in 20 volumes of MeOH and centrifuged at 4500 *g* for 5 min. In the case of the hypothalamus, pituitary gland and pineal gland, each tissue was homogenized in 500 μl of MeOH. The supernatant (200 μl for pineal gland, 100 μl for pituitary gland, 50 μl for hypothalamus, 20 μl for other regions) was evaporated to dryness, then derivatized and analyzed in the same way as the rat samples.

2.4. Column-switching HPLC system

The HPLC system consisted of a DG-980-50 degasser (Jasco, Tokyo, Japan), two PU-980 pumps (Jasco), a 7725 injector (Rheodyne, Cotati, CA, USA), a CO-965 column oven (Jasco), two FP-920 fluorescence detectors (Jasco), two 807-IT integrators (Jasco) and an HV-992-01 column selection unit (Jasco). The analytical column of the micro RP-HPLC system was a Mightysil RP-18 GP (100×1.0 mm I.D., Kanto, Tokyo, Japan) maintained at 35°C. Mobile phase was THF–TFA–MeCN–water (1:0.02:32.5:66.5, v/v), and the flow-rate was 50 μl/min. The analytical column for enantioseparation was a Sumichiral OA-2500S (250×4.6 mm I.D., Sumika Analytical Center, Osaka, Japan) with a guard column of TSK-gel ODS 120T (15×3.2 mm I.D., Tosoh, Tokyo, Japan) maintained at 35°C. Mobile phase was 2 m*M* citric acid–MeOH, and the flow-rate was 0.8 ml/min. Fluorescence detection of NBD-Leu was carried out at 530 nm with excitation at 470 nm.

2.5. Enzymatic degradation of D-Leu

The supernatant (10 μl) of each tissue (described in Section 2.3) was evaporated to dryness and dissolved in 20 μl of 200 m*M* Na–borate buffer (pH 8.0). D-Amino acid oxidase ($7.5 \cdot 10^{-3}$ U) in 10 μl of water was added and incubated at 37°C for 120 min. To this, 30 μl of 20 m*M* NBD-F in MeCN was added and heated at 60°C for 2 min. To the reaction mixture, 190 μl of aqueous 2% TFA was added, filtered through a 0.2-μm membrane filter, and 5 μl was subjected to HPLC as described in Section 2.4.

3. Results and discussion

3.1. Column-switching system for the separation of D- and L-leucine

For the selective determination of minute amounts of D-Leu, we attempted to establish a column-switching system using a micro ODS column and a chiral column. Pre-column derivatization of D- and L-Leu was carried out with NBD-F, which is a superior fluorescence derivatizing reagent, and the products have strong fluorescence in a wide pH range. The flow diagram of the HPLC system is shown in Fig. 1. D- and L-Leu derivatized with NBD-F were separated on a micro ODS column as a DL mixture and introduced to a chiral column using a loop transfer system. In this system, each enantiomer of Leu was separated on a chiral column and directly determined by a fluorescence detector. Separation conditions of NBD-Leu on a micro ODS-column were examined considering the peak height, fraction volume of

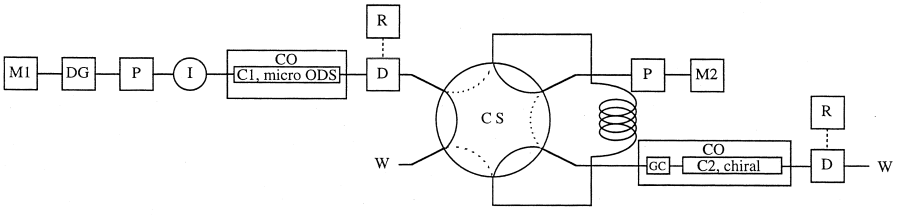

Fig. 1. Column-switching HPLC system for the determination of D-Leu. C1, Micro ODS column (Mightysil RP-18GP, 150×1.0 mm I.D.); C2, chiral column (Sumichiral OA-2500S, 250×4.6 mm I.D.); GC, guard column; M, mobile phase; DG, degasser; P, pump; I, injector; CO, column oven; D, detector; R, integrator; CS, column selection unit; W, waste.

NBD-Leu and the resolution of NBD-Leu from an interfering peak due to the reagent, NBD-F. By the increase in the MeCN concentration of the mobile phase, the retention time of NBD-Leu became shorter and the fraction volume decreased. However, when the mobile phase contained more than 35% MeCN, NBD-Leu was not separated from the reagent peak. Therefore, the mobile phase containing 32.5% MeCN was selected. Concerning the flow-rate of the mobile phase, the peak height of NBD-D-Leu became higher, and the retention time become shorter by the increase in the flow-rate of the mobile phase. However, the column pressure became higher by the increase of the mobile phase, and the pressure was about 100 kg/cm^2 at 50 μl/min. Therefore, the flow-rate of 50 μl/min was selected. The chromatogram of NBD-Leu on a micro ODS column using these conditions is shown in Fig. 2. The NBD-Leu fraction isolated on a micro ODS column was introduced to a loop (400 μl) for 150 s and subjected to a chiral column. Using these column-switching conditions, 85% of the NBD-Leu injected on a micro ODS column was introduced to the chiral column.

Separation conditions of the chiral column were also investigated, because a large interfering peak caused by the mobile phase of a micro ODS column co-injected with NBD-Leu was observed. As a result, a better resolution was observed at a lower concentration of citric acid, and 2 m*M* citric acid–MeOH was selected for the mobile phase. The flow-rate of the mobile phase was also examined for the rapid separation of NBD-D-Leu, and 0.8 ml/min was selected. Using these conditions, NBD-D-Leu was eluted within 30 min and completely separated from NBD-L-Leu and the interfering peak caused by the mobile phase of a micro ODS column (Fig. 3). For an application to biological samples, a minute amount of D-Leu in the rat hippocampus was determined, and a calibration curve, within-day precision and day-to-day precision, were investigated using the rat hippocampus sample.

3.2. Determination of D-Leu in rat hippocampus

The concentration of the borate buffer used for the derivatization of D-Leu was investigated considering the recovery of D-Leu in biological samples, and 200 m*M* was selected. Under this condition, the recovery of D-Leu was about 100% for the rat hippocampus. A calibration curve of D-Leu was constructed by the addition of known amounts of D-Leu in the supernatant obtained from a rat hippocampus homogenate.

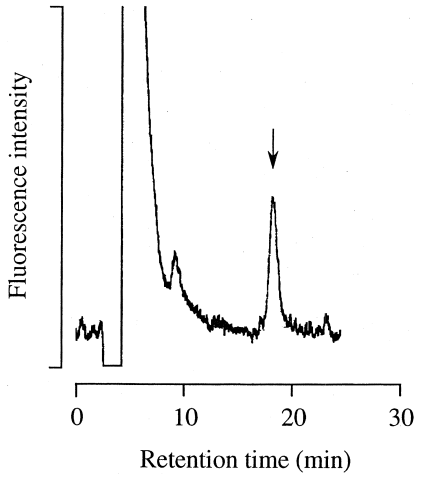

Fig. 2. Chromatogram of derivative for 1 fmol Leu (as a DL mixture) on a micro ODS column. DL-Leu was derivatized with NBD-F and subjected to HPLC as described in the text. The peak of NBD-Leu is indicated with an arrow.

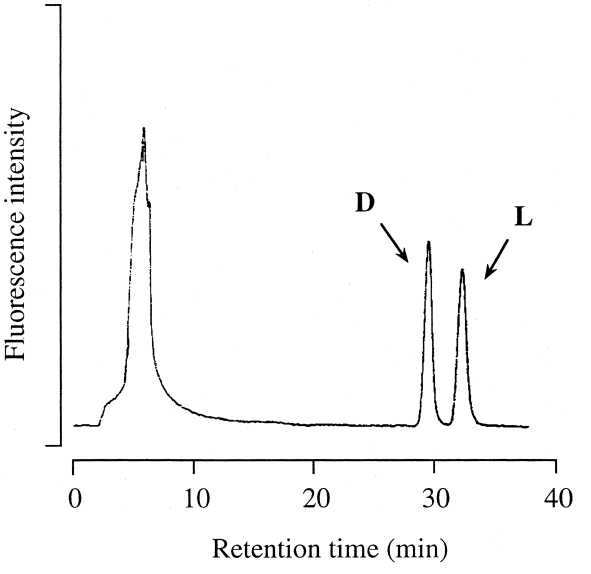

Fig. 3. Chromatogram of derivatives for 100 fmol D- and L-Leu on the Sumichiral OA-2500S column using column-switching system. Peaks: D, NBD-D-Leu; L, NBD-L-Leu. HPLC conditions as described in the text.

The rat hippocampus sample was prepared as described in Section 2.3, and D-Leu (0.05, 0.5, 5, 50 pmol) was added to 20 μl of the supernatant, then derivatized and analyzed as described in Experimental. As a result, the calibration curve was linear from 1 fmol to 1 pmol (injection amount) with the correlation coefficient higher than 0.999. The detection limit for spiked D-Leu was 1 fmol (injection amount, $S/N=5$). Using the same rat hippocampus sample (without addition of D-Leu), within-day precision and day-to-day precision of the method were examined. Within-day precision was determined by derivatizing five portions within a day, and the obtained relative standard deviation (RSD) was 5.11%. Day-to-day precision was determined by derivatizing the same sample on each day (5 days), and the obtained RSD was 5.25%. Fig. 4 shows the chromatograms for D- and L-Leu in the rat hippocampus on Sumichiral OA-2500S, with (A) or without the use of the column-switching system (B). Without the use of the column-switching system, many interfering peaks due to the endogenous substances in the rat hippocampus were observed. While using the column-switching system, most of the interfering compounds were removed, and selective determination of D- and L-Leu in the rat hippocampus could be carried out. The recovery of spiked L-Leu was almost the same as that of D-Leu. With this system, the amount of D-Leu determined in the rat hippocampus was 0.69 nmol/g wet tissue, and the % *D* (the percentage of D-Leu for total Leu) was 0.97%; these were consistent with the reported values [7]. These results indicated that a minute amount of D-Leu in the rat hippocampus samples could be selectively determined using the present method, which should be a powerful tool for various biological samples.

3.3. Distribution of D-Leu in mouse brain

The intrinsic amount of D-Leu in mammalian tissues is too small, and its distribution in the mammalian brain has not been investigated in detail. Therefore, we determined D- and L-Leu in eight brain regions of mice, a major experimental animal of mammals, using the analytical method described in Section 3.2. Table 1 shows the D-Leu content in each brain region (cerebrum, cerebellum, hippocampus, hypothalamus, medulla oblongata, pituitary gland, pineal gland and others) determined for three mice. In the hippocampus, hypothalamus, pituitary gland and pineal gland, D-Leu was detected for all mice. However, in the cerebellum and medulla oblongata,

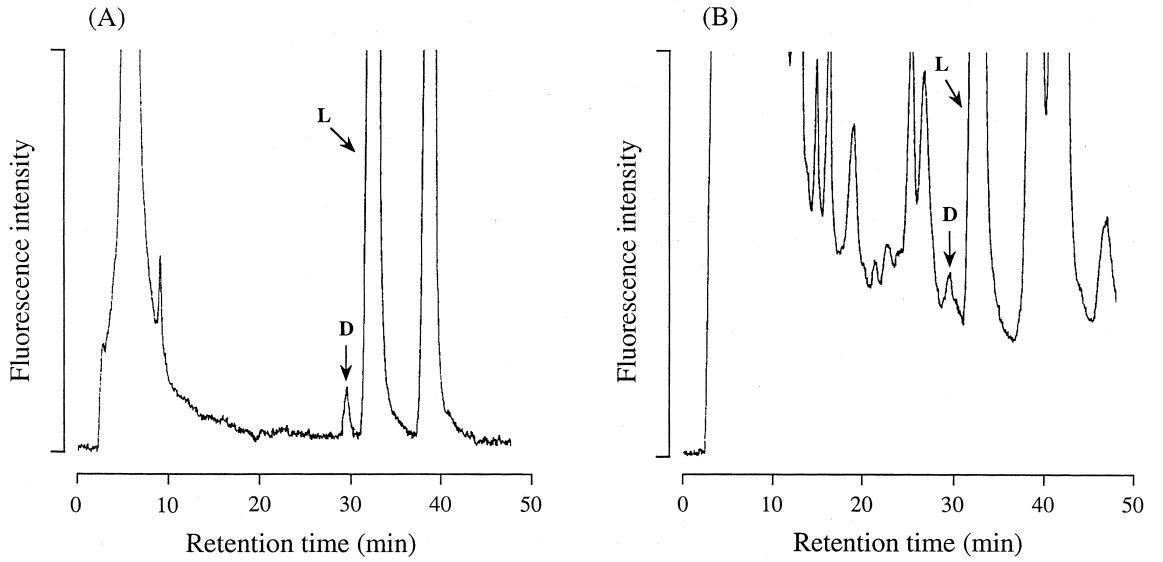

Fig. 4. Separation of NBD-D- and L-Leu in rat hippocampus on the Sumichiral OA-2500S column. (A) Present method; (B) sample solution of rat hippocampus derivatized and directly injected onto the Sumichiral OA-2500S column. Peaks: D, NBD-D-Leu; L, NBD-L-Leu. HPLC conditions as described in the text.

Table 1
Regional distribution of D-Leu in mouse brain[a]

Mouse	Brain region							
	Cerebrum	Cerebellum	Medulla oblongata	Hippocampus	Pituitary gland	Hypothalamus	Others	Pineal gland
a	0.62 (0.53)	0.15 (0.23)	0.42 (0.38)	0.45 (0.50)	1.30 (0.52)	0.65 (0.62)	0.41 (0.41)	1.70 (1.48)
b	N.D.	N.D.	N.D.	0.16 (0.18)	0.83 (0.40)	0.26 (0.30)	N.D.	1.36 (0.80)
c	0.13 (0.12)	N.D.	N.D.	0.23 (0.25)	1.42 (0.29)	0.33 (0.34)	0.17 (0.16)	1.08 (0.36)
Mean	0.25 (0.22)	0.05 (0.08)	0.14 (0.13)	0.28 (0.31)	1.18 (0.40)	0.41 (0.42)	0.19 (0.19)	1.38 (0.88)

[a] Values represent the tissue content of D-Leu (nmol/g wet tissue) except for pineal gland. The values for pineal gland represent the content of D-Leu in whole tissue (pmol/whole tissue). N.D.: Not detected. Values in parentheses are the proportions of D-Leu $[D/(D+L)\cdot 100]$.

D-Leu was not detected for two mice. Because the D-Leu content in the mouse brain was exceptionally small, and this is the first report demonstrating the wide distribution of D-Leu in the mammalian brain, the peaks observed at the retention time of D-Leu were confirmed by enzymatic degradation with DAAO. The enzyme has high specificity toward D-amino acids over the corresponding L-enantiomers and is widely used for the confirmation of D-amino acids. Fig. 5 shows the degradation of D-Leu in the mouse hypothalamus. On the chromatogram, the peak observed at the retention time of D-Leu decreased with the increase of the enzymatic reaction period, and almost completely disappeared by the treatment for 120 min with DAAO, while the L-enantiomer was scarcely affected. D-Leu in other regions also disappeared and was successfully confirmed by the treatment with this highly specific enzyme. In the present investigation, D-Leu was detected in all regions investigated for one mouse (Table 1, a). The commercially available ddY mouse includes the mutant ddY/DAAO− mouse, which lacks DAAO activity in the whole brain. The mutant could not metabolize D-amino acids, and a higher content of D-Ala and D-Ser was observed than that of a normal mouse [18,19]. The ddY strain also includes the normal ddY/DAAO+ mouse, in which high DAAO activity was observed in the cerebellum

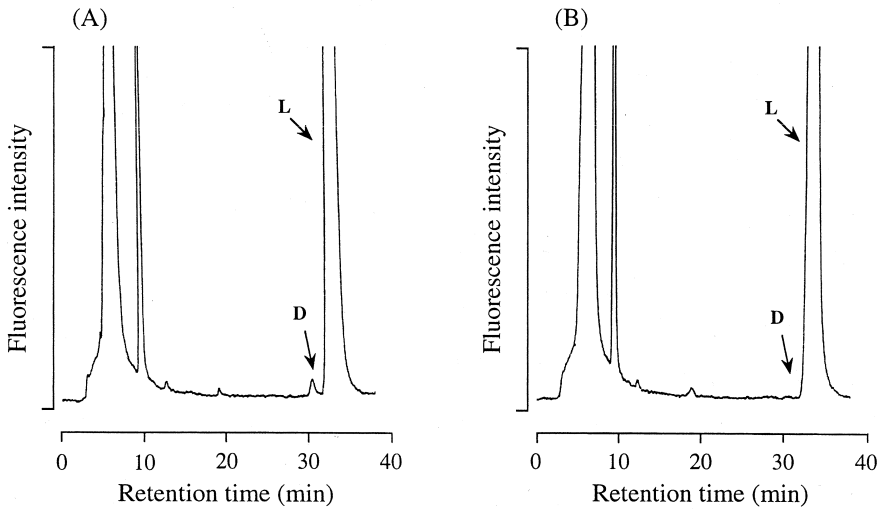

Fig. 5. Separation of NBD-D-Leu in mouse hypothalamus and degradation of D-Leu with DAAO. D- and L-Leu in mouse hypothalamus were determined before (A) and after (B) the treatment with DAAO. Peaks: D, NBD-D-Leu; L, NBD-L-Leu. Chromatographic and enzymatic conditions as described in the text.

and medulla oblongata. The individual variation and the regional difference of D-Leu content obtained in the present investigation might be caused by the variation of DAAO expression, which remains to be solved.

4. Conclusions

In the present investigation, a precise and selective method for the determination of D-Leu was established using the column-switching system of a micro ODS column and a chiral column. The method was successfully applied to the determination of D-Leu in the rat hippocampus and ascertained the wide distribution of D-Leu in the mammalian brain. Because the method could be applicable to the determination of all other D-amino acids with some modifications, the present method should be a powerful tool for various biological samples.

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