Review

Sensitive Two-Dimensional Determination of Small Amounts of D-Amino Acids in Mammals and the Study on Their Functions

Kenji HAMASE

Graduate School of Pharmaceutical Sciences, Kyushu University; 3–1–1 Maidashi, Higashi-ku, Fukuoka 812–8582, Japan.

Received October 30, 2006

D-Amino acids are the candidates of novel physiologically active substances and the marker molecules of diseases in mammals. In the present study, the two-dimensional determination of small amounts of D-amino acids in mammals has been performed after sensitive pre-column fluorescence derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The two-dimensional HPLC system includes the isolation of the target amino acids as D+L mixtures using the micro-ODS column, and the determination of the enantiomers using the chiral column. This method enables the sensitive and selective determination of small amounts of D-amino acids in mammals without the interference of L-amino acids and peptides, and the presence and distribution of D-Leu, D-Ala, D-Pro, D-Thr and D-*allo*-Thr has been demonstrated in rats and mice. The regulation and the origins of these D-amino acids, and their relationships to the biological rhythms are also discussed.

Key words D-amino acids; two-dimensional HPLC; fluorescence; enantiomer separation

1. Introduction

D-Amino acids are the enantiomers of L-amino acids, and it is long believed that only the L-enantiomers are present in higher animals. However, since the 1980s, several D-amino acids have been found in mammals including humans, and they are now being noticed as the candidates of novel physiologically active substances and/or the marker molecules of diseases.[1,2] Especially, D-serine (D-Ser) and D-aspartic acid (D-Asp) have widely been investigated. D-Ser is thought to be a neuromodulator of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate (Glu) receptors in the brain,[3—5] and D-Asp is reported to regulate hormonal secretion in various endocrine glands.[6—9] While the amounts of other D-amino acids in mammals are low, and they have not been well investigated due to the lack of appropriate analytical methods.

For the determination of D- and L-amino acids, various chromatographic methods are reported and frequently used for the quantitative analysis of D-Ser and D-Asp in mammals.[10—18] However, in mammalian samples, around 20 extremely large peaks of the L-amino acids and hundreds of small unknown peaks due to peptides often co-elute with the D-amino acids, and most of the methods have limited sensitivity and selectivity for identifying and determining small amounts of D-amino acids in tissues and physiological fluids. For the determination of trace amounts of D-amino acids in mammals, we have established two-dimensional methods using micro-ODS and chiral columns in combination with sensitive fluorescence pre-column derivatization, and demonstrated that various D-amino acids are present in mammals.[19—27] In this article, I would like to introduce our current research on the two-dimensional determination of D-amino acids in mammals and the study on their functions.

2. Two-Dimensional Determination of D-Amino Acids in Mammals

2.1. Fluorescence Derivatization of Amino Acids For the sensitive detection, the D- and L-amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F).[28,29] By this reaction, the enantiomer mixtures of the NBD-D- and L-amino acids were produced (Fig. 1), and these fluorescent derivatives were determined using 2D-HPLC. Briefly, the tissues and physiological fluids were depro-

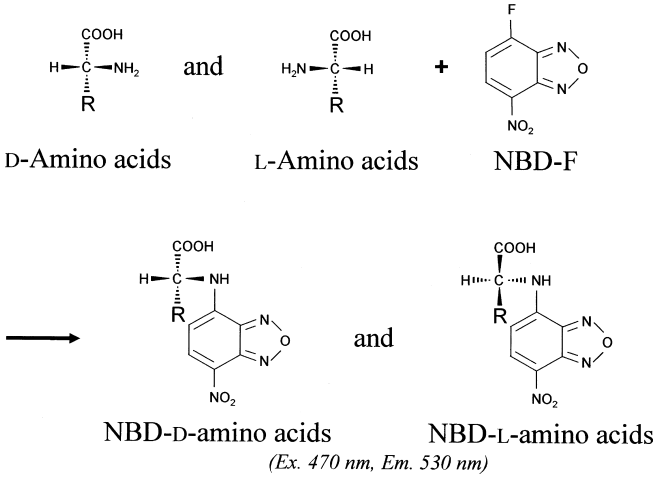

Fig. 1. Fluorescence Derivatization of the Amino Acids with NBD-F

e-mail: hamase@phar.kyushu-u.ac.jp

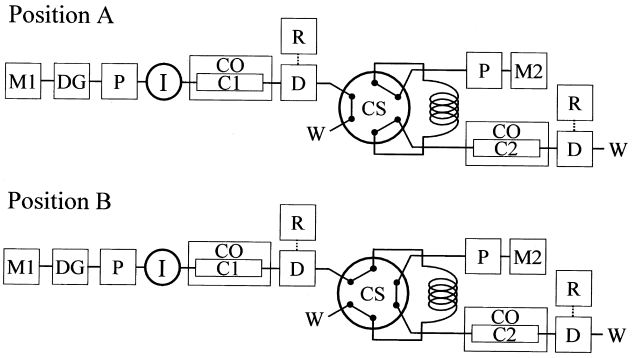

Fig. 2. Flow Diagram of the Column-Switching HPLC System Combining Micro-ODS and Chiral Columns

C1, micro-ODS column; C2, chiral column; M, mobile phase; DG, degasser; P, pump; I, injector; CO, column oven; D, detector; R, integrator; CS, column selection unit; W, waste.

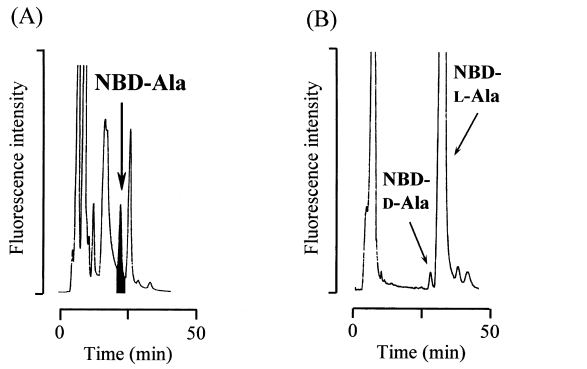

Fig. 3. Separation of NBD-Ala in the Rat Cerebrum by a Micro-ODS Column (A), and the Separation of NBD-D- and L-Ala in the Isolated Fraction by a Chiral Column, Sumichiral OA-2500S, Using a Column-Switching System (B)

The chromatographic conditions are described in the text.

teinized in 20-fold volumes of MeOH on ice (in case of small tissues, larger volumes of MeOH were used) and centrifuged at 4500 ***g*** for 5 min. A portion of the supernatant was evaporated to dryness under reduced pressure at 40 °C. To the residue, 20 μl of 200 mM Na-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. After adding aqueous 2 or 5% trifluoroacetic acid (TFA) to terminate the reaction, an aliquot of this reaction mixture (1—5 μl) was subjected to the HPLC system.

2.2. HPLC System For the selective determination, the 2D-HPLC system combining a micro-ODS column and a chiral column was used. This HPLC system consisted of two pumps, an injector, a column oven, two fluorescence detectors, two integrators, an on-line degasser and a column selection unit. These components were arranged as outlined in Fig. 2 with a micro-ODS column and a chiral column. A stainless steel loop was set in the column selection unit to connect the reversed-phase separation and chiral separation systems. On a micro-ODS column, the amino acids pre-column derivatized with NBD-F were separated from the other materials in the tissues and physiological fluids as D+L mixtures. The fraction of the target amino acid was introduced to the loop by changing the valve position from A to B (Fig. 2), and the isolated fraction was then introduced into a chiral column by changing the valve again (B to A). On the chiral column, the D and L enantiomers were separated and these NBD-D- and L-amino acids were then determined by their fluorescence at 530 nm with excitation at 470 nm. By using a micro-ODS column in the isolation step, the volume of the target amino acid fraction was reduced, therefore, the entire volume of the fraction could be introduced into the chiral column, and highly sensitive determination of D-amino acids could be performed without the loss of sensitivity due to the column-switching procedure. Figure 3 shows the 2D-determination of D-alanine (D-Ala) in the rat cerebrum. As shown in Fig. 3A, NBD-Ala was isolated from the other interfering substances by the micro-ODS column, and after the enantiomer separation by the chiral column, the highly selective determination of a small amount of NBD-D-Ala (about 1% of the L-enantiomer) could be carried out (Fig. 3B).

2.3. Determination of D-Leucine (D-Leu)[21,22] The two-dimensional determination of NBD-D- and L-Leu was carried out by using a Mightysil RP-18 GP (1.0 mm i.d.×100 mm) as the micro-ODS column, and a Sumichiral OA-2500S (4.6 mm i.d.×250 mm) as the chiral column. The separation conditions of both columns were investigated by changing the solvent compositions, organic solvents added to the mobile phase, ionic strength and flow rates. The selected mobile phase for the micro-ODS column was tetrahydrofuran (THF)–TFA–MeCN–H_2O (1/0.02/32.5/66.5, v/v, flow rate 50 μl/min), and that for the chiral column was 2 mM citric acid in MeOH with the flow rate of 0.8 ml/min. Under these conditions, NBD-Leu was eluted within 20 min on the micro-ODS column, and the enantiomer separation of NBD-Leu was completed in about 30 min on the chiral column. This

Kenji Hamase was born in Osaka, Japan, in 1968. He graduated from The University of Tokyo in 1991, and received a Master of Pharmacy in 1993. He then obtained his Ph.D. degree in Pharmaceutical Sciences under the supervision of Professor Kazuhiro Imai in 1996. Throughout the doctoral course, he obtained research fellowships from the Japan Society for the Promotion of Science for Young Scientists (1993–1996). Subsequently, he began his academic carrier as an Assistant Professor in Professor Kiyoshi Zaitsu's group at Kyushu University, and he has been an Associate Professor of the same group since 2001. He received The Japan Society for Analytical Chemistry Award for Young Scientists in 2003, and The Pharmaceutical Society of Japan Award for Young Scientists in 2006. His current research interests focus on the development of analytical methods for D-amino acids and the study on their physiological functions and diagnostic values.

Kenji Hamase

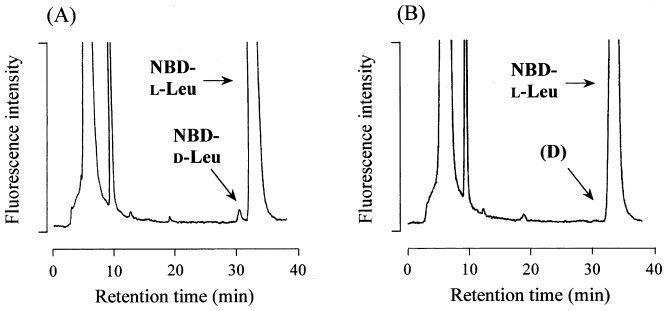

Fig. 4. Separation of NBD-D- and L-Leu in Mouse Hypothalamus before (A) and after the Treatment with DAO (B)

The chromatographic conditions were described in the text.

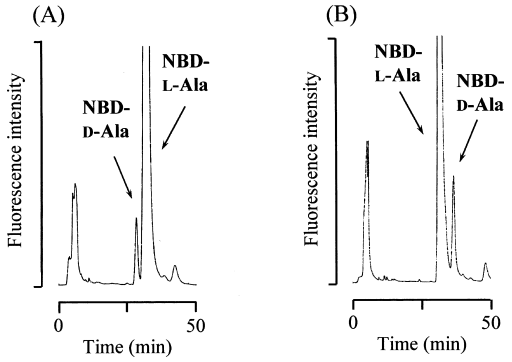

Fig. 5. Separation of NBD-D- and L-Ala in the Rat Anterior Pituitary Gland Using a Sumichiral OA-2500S (A) and a Sumichiral OA-2500 (B, This Column Has the R Conformation at the Chiral Center) as the Chiral Columns

2D-HPLC system was validated using the rat hippocampus. A calibration curve of D-Leu was constructed by the addition of known amounts of D-Leu to the supernatant obtained from a rat hippocampus, and a linear calibration line was observed from 1—1000 fmol (injection amount of spiked D-Leu) with the correlation coefficient of 0.9999. The within-day and day-to-day precisions were examined using a rat hippocampus sample (without adding D-Leu). The within-day precision was determined by derivatizing 5 portions within a day, and the obtained RSD was 5.11%. The day-to-day precision was determined by derivatizing the same sample on each day (5 d), and the obtained RSD was 5.25%. For the confirmation to show the presence of small amounts of D-amino acids in mammals, we frequently use the enzymatic degradation of D-amino acids by D-amino acid oxidase (DAO). This enzyme catalyses the oxidation of neutral D-amino acids, and has a high specificity toward D-amino acids over the corresponding L-enantiomers.[30] Figure 4 shows an example confirming the presence of D-Leu in the mouse hypothalamus. On the chromatogram, the peak observed at the retention time of NBD-D-Leu (Fig. 4A) decreased by increasing the enzymatic reaction period, and completely disappeared after a 2 h treatment with DAO, while the L-enantiomer was scarcely affected (Fig. 4B). These results indicate that the presence of D-Leu in the hypothalamus was successfully confirmed by the treatment with this highly specific enzyme.

2.4. Determination of D-Alanine[23] D-Ala was also determined using a Mightysil RP-18 GP (1.0 mm i.d.×100 mm) as the micro-ODS column, and a Sumichiral OA-2500S (4.6 mm i.d.×250 mm) as the chiral column. The separation conditions for the micro-ODS column were THF–TFA–MeCN–H_2O (1/0.02/10/89, v/v) at the flow rate of 50 μl/min, and the mobile phase for the chiral column was 5 mM citric acid in MeOH at the flow rate of 0.8 ml/min. The retention time for NBD-Ala on the micro-ODS column is about 20 min, and those for NBD-D- and L-Ala on the chiral column were about 30 min. The HPLC system was validated using the rat cerebellum. The cerebellum is useful for the validation of this method, because the cerebellum is well-known to contain low levels of D-amino acids.[19,31] The calibration curve of D-Ala constructed by adding 5—5000 fmol of D-Ala (injected amount) to a rat cerebellum sample is linear with a correlation coefficient of 1.0000, and the slope of this calibration line is approximately the same as that for the standard D-Ala. The within-day and day-to-day precisions of the D-Ala determination obtained by the rat cerebellum sample spiked with 15 fmol of D-Ala are 3.9 and 4.8% (RSD), respectively. Figure 5A shows the chromatogram obtained for the rat anterior pituitary gland. As shown in this figure, the peak of NBD-D-Ala was clearly observed although the amount of D-Ala in this tissue is only 2.5% of that for L-Ala. Figure 5B shows another confirmation of the D-amino acid amounts using a chiral column of the opposite chiral center (in this case, Sumichiral OA-2500). Replacement of Sumichiral OA-2500S with Sumichiral OA-2500 (this column has the R conformation at the chiral center) leads to a reversal in the elution order of NBD-D- and L-Ala, strongly indicating the presence of D-Ala in the anterior pituitary gland. This concept is a very useful chromatographic proof to show the presence of D-amino acids in the tissues and physiological fluids.

2.5. Determination of D-Proline (D-Pro)[22,27] The micro-ODS column for the separation of NBD-Pro was a Mightysil RP-18 GP (1.0 mm i.d.×150 mm), and a mixed solution of THF–TFA–MeCN–H_2O (1/0.02/10/89, v/v) was used as the mobile phase at the flow rate of 50 μl/min. The analytical column for chiral separation was a Sumichiral OA-2500 (4.6 mm i.d.×250 mm). The mobile phase was 2 mM citric acid in MeOH, and the flow rate was 1.2 ml/min. For the determination of D-Pro in the tissue samples, a chiral column of Sumichiral OA-2500 (4.6 mm i.d.×500 mm) was used. The mobile phase was a mixed solution of MeCN–MeOH (2/8, v/v) containing 2 mM citric acid, and the flow rate was 0.9 ml/min. This method was validated using the kidney and urine of a ddY/DAO^+ mouse by checking the calibration lines, within-day precisions and day-to-day precisions. The calibration lines of D-Pro spiked into a mouse kidney sample and a mouse urine sample were linear with good correlation coefficients. The calibration line for the kidney was linear from 5—1000 fmol (r=0.9995); and that for urine was linear from 50—25000 fmol (r=1.0000). The within-day precisions of the D-Pro determination were 1.16 and 3.73% (RSD, n=5) for the kidney (20 fmol of D-Pro as an injection amount was spiked) and urine (intrinsic amount was measured), respectively, and the day-to-day precisions (5 d) were 4.39% (D-Pro, kidney) and 3.75% (D-Pro, urine). The typical chromatograms obtained for the urine of a ddY/DAO^- mice are shown in Fig. 6. The ddY/DAO^- mouse is a mutant mouse strain lacking DAO activity,[32,33] and the urinary excretion of large amounts of D-Pro in these mutant mice is

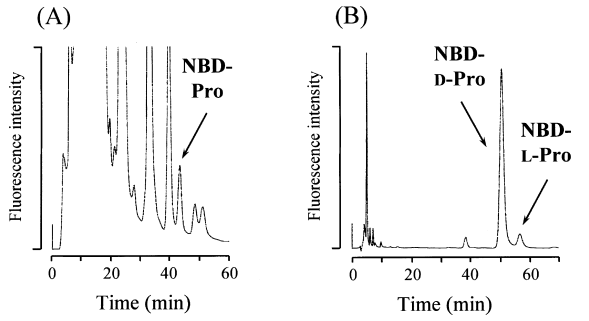

Fig. 6. Separation of NBD-Pro in the Urine of a ddY/DAO$^-$ Mouse Using a Micro-ODS Column (A), and the Separation of NBD-D- and L-Pro in the Isolated Fraction by a Chiral Column, Sumichiral OA-2500, Using a Column-Switching System (B)

The chromatographic conditions are described in the text.

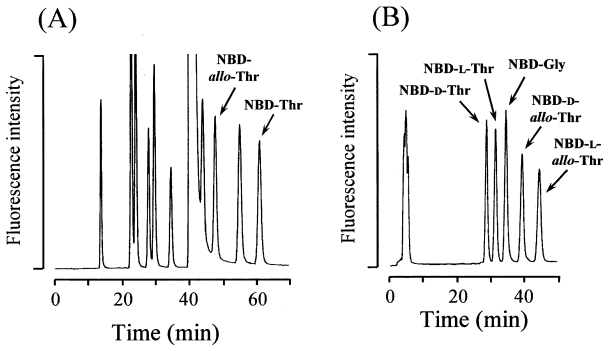

Fig. 7. (A) Separation of NBD-*allo*-Thr and NBD-Thr in the Mixed Solution of Proteinogenic Amino Acids Using a Semimicro-ODS Column

The hydrophobic amino acids that 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 Chiral Column, Sumichiral OA-2500S

The chromatographic conditions are described in the text.

demonstrated for the first time.

2.6. Determination of D-Threonine (D-Thr) and D-*allo*-Threonine[24] Thr has two chiral centers, and therefore, four isomers (L-Thr, D-Thr, L-*allo*-Thr and D-*allo*-Thr) are present. By epimerization of the α-carbon, which is usually observed for other amino acids, L-Thr is converted into D-*allo*-Thr. The conversion of the chirality at the β-carbon of L-Thr produces L-*allo*-Thr, and D-Thr is produced by the reversal of the optical forms on both of the two chiral carbons. Therefore, to investigate the D-enantiomer of Thr, not only D-Thr but also D-*allo*-Thr should be considered. The two-dimensional determination of D-Thr and D-*allo*-Thr was performed using a Capcell pak C18-AQ (2.0 mm i.d.×250 mm) as a semimicro-ODS column, and a Sumichiral OA-2500S (4.6 mm i.d.×250 mm) as a chiral column. The mobile phase for the reversed-phase separation was MeCN–TFA–H_2O (12/0.08/88, v/v) at the flow rate of 0.2 ml/min, and that for the chiral separation was 1 mM citric acid in the mixed solution of MeOH–MeCN (60/40, v/v), at the flow-rate of 1 ml/min. Under these conditions, NBD-Thr and NBD-*allo*-Thr are eluted in about 60 min on the semimicro-ODS column with minimized interference by excess reagents and other amino acids (Fig. 7A). After the isolation of the separated fractions (in the case of Thr and *allo*-Thr, the two fractions were manually collected), the enantiomers were separated by the chiral column. Under the conditions described above, the enantiomers of NBD-Thr, the enantiomers of NBD-*allo*-Thr and NBD-Gly (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) were clearly separated in about 45 min (Fig. 7B). This method was validated using the rat cerebellum. The calibration lines of D-Thr and D-*allo*-Thr were linear from 2.5—5000 fmol with good correlation coefficients (r=1.0000 for both D-Thr and D-*allo*-Thr). The within-day and day-to-day precisions were investigated using a rat cerebellum sample spiked with 100 fmol (as injection amounts on the chiral column) of D-Thr and D-*allo*-Thr, and the obtained RSD values of 5 analyses were 1.99—5.29%. Figure 8 shows the chromatograms obtained for the rat urine. As shown in these chromatograms, small amounts of D-Thr and D-*allo*-Thr were clearly observed in the urine.

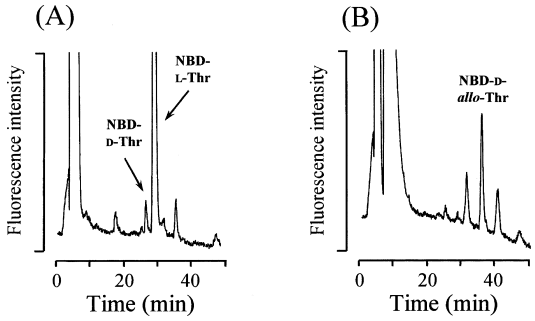

Fig. 8. Separation of NBD-D- and L-Thr (A) and NBD-D-*allo*-Thr (B) in Rat Urine Using a Chiral Column, Sumichiral OA-2500S

The chromatographic conditions are described in the text.

3. Anatomical Distribution of D-Amino Acids in Mammals

Using the methods described above, the anatomical distributions of D-Ala,[23] D-Pro,[27] D-Leu,[22] D-Thr and D-*allo*-Thr[24] were investigated. Concerning the distributions of D-Ser and D-Asp, they have been clearly summarized in other reviews.[1,34] The amounts of naturally occurring D-Ala were determined in 22 tissues and plasma of male Wistar rats (9 weeks of age). The present method enables the sensitive and selective determination of D-Ala in mammalian tissues, and naturally occurring D-Ala could be determined in all tissues examined as shown in Table 1. Among the 22 tissues tested, the highest amount of D-Ala was observed in the anterior pituitary gland (86.4±9.9 nmol/g wet tissue (%D=2.5) using Sumichiral OA-2500S, 87.5±9.4 nmol/g wet tissue using Sumichiral OA-2500); which is 6 times higher than that observed in the posterior pituitary gland (14.7±2.5 nmol/g wet tissue, %D=0.6). The second highest amount of D-Ala was observed in the pancreas (29.2±5.0 nmol/g wet tissue, %D=1.3). The amounts of D-Pro were determined in 7 brain tissues, 11 peripheral tissues, the plasma and urine of mice (ddY/DAO$^+$ strain was used because this strain shows a high DAO activity like other higher animals). Although the amounts of D-Pro in the tissues are low, relatively high amounts were observed in the pituitary and pineal glands in the brain, and in the pancreas and testis in the periphery. Anatomical distribution of D-Leu in the brain of mice was

Table 1. Amounts of D-Amino Acids in Mammalian Tissues and Physiological Fluids

	D-Ala	D-Pro	D-Leu	D-Thr	D-*allo*-Thr
Animal	Rat	Mouse	Mouse	Rat	Rat
Strain	Wistar	ddY/DAO$^+$	ddY/DAO$^+$	Wistar	Wistar
Age	9 weeks	18 weeks	9—10 weeks	8 weeks	8 weeks
N	5	4	3—4	3—5	3—5
Reference	23)	27)	22)	24)	24)
Brain tissues					
Cerebrum	3.3	0.52	0.41	0.56	3.39
Corpus striatum	4.8	—	—	0.85	5.01
Olfactory lobe	4.2	—	—	—	—
Cerebellum	0.6	0.45	0.24	n.d.	n.d.
Medulla oblongata	1.5	0.30	0.27	—	—
Spinal cord	1.8	0.50	—	—	—
Hypothalamus	3.3	—	0.55	n.d.	2.00
Anterior pituitary	86.4	5.13	0.76[a)]	—	—
Posterior pituitary	14.7	2.84	—	—	—
Pineal gland	15.9	3.03	1.94	—	—
Peripheral tissues					
Thyroid gland	7.4	1.81	—	—	—
Adrenal gland	12.1	1.62	—	n.d.	n.d.
Pancreas	29.2	2.50	—	n.d.	n.d.
Testis	7.3	2.87	—	n.d.	n.d.
Thymus	15.2	1.18	—	—	—
Spleen	9.7	1.39	—	n.d.	n.d.
Bone marrow	17.0	—	—	—	—
Muscle	10.6	1.60	—	—	—
Heart	6.7	0.86	—	n.d.	n.d.
Lung	19.9	1.02	—	n.d.	n.d.
Kidney	3.6	0.45	—	n.d.	n.d.
Liver	4.4	1.13	—	n.d.	n.d.
Physiological fluids					
Plasma (P) or Serum (S)	10.9 (P)	0.70 (P)	0.39 (S)	n.d. (S)	n.d. (S)
Urine	—	23.19	—	1.16	5.40

The amounts of D-amino acids represent the mean values (nmol/g wet tissue or ml); *a*) the amount was measured in whole pituitary gland; n.d., not detected (lower than the detection limit); —, not tested.

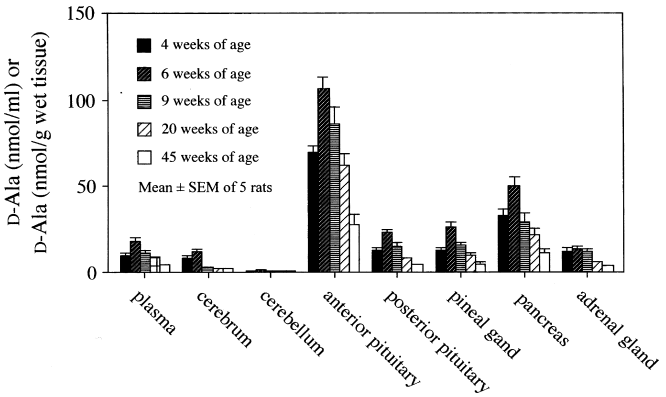

Fig. 9. Postnatal Changes in D-Ala Amount in the Rat Central Nervous System and Periphery

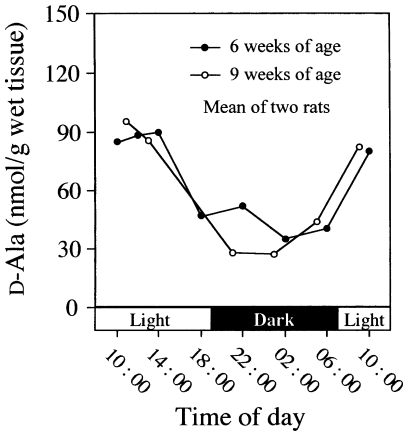

Fig. 10. Day–Night Changes in the D-Ala Amount in the Rat Anterior Pituitary Gland

also investigated and found that the amounts of D-Leu in the pituitary and pineal glands were slightly higher than those in the other brain areas. Concerning D-Thr and D-*allo*-Thr, we could not detect these two D-amino acids in all the tested peripheral tissues; they are localized to the frontal brain areas (cerebrum and striatum). Of special note, the amounts of D-*allo*-Thr are higher than those of D-Thr, suggesting that the reversal of the chiral center at the α-carbon of L-Thr predominantly occurs in mammals.

4. D-Alanine and Biological Rhythms

D-Ala shows clear postnatal changes and day–night changes.[19,23)] Using rats of 4—45 weeks of age, we investigated the postnatal changes in the D-Ala amounts in the 7 tissues and plasma. The tested tissues included the cerebrum, cerebellum, anterior pituitary gland, posterior pituitary gland, pineal gland, pancreas and adrenal gland. The results are summarized in Fig. 9. In all the tested tissues, the amounts of D-Ala reached the maximum at 6 weeks of age and gradually decreased thereafter. At any age, the highest amount of D-Ala was observed in the anterior pituitary gland, and the second highest amount was observed in the pancreas. These results suggest that D-Ala has some yet unknown physiological functions in these two endocrine glands. The day–night changes in the D-Ala amount in the anterior pituitary gland were also investigated during the day. As shown in Fig. 10, the amounts of D-Ala in the anterior pituitary gland show a clear circadian rhythm. The levels of D-Ala are approximately 3 times higher during the daytime than those during the nighttime, and these circadian changes were observed for both the rats of 6 and 9 weeks of age. The physiological meanings of these D-Ala rhythms and the regulation mechanisms are the subjects of further investigation.

5. Effect of D-Amino Acid Oxidase on the Amounts of D-Amino Acids

D-Amino acid oxidase (DAO, EC. 1.4.3.3), which catalyzes the oxidative deamination of D-amino acids to form the corresponding α-keto acids, is one of the major regulatory pathways of D-amino acids.[30)] This enzyme is widely observed in mammals, and most of the wild type animals have DAO in the kidney, liver and brain.[35)] Therefore, the naturally occurring D-amino acids in mammals are frequently diminished by the DAO, and it is important to clarify the D-amino acid amounts in the absence of any DAO activity as well as to investigate the effects of DAO on their amounts. In this context, mutant animals lacking DAO activity are useful, and we determined the amounts of D-Asp, D-Ser, D-Ala, D-Leu and D-Pro in the brains of the mutant ddY/DAO$^-$ mice, and compared the amounts to those in the brains of the control ddY/DAO$^+$ mice having normal DAO activity.[25)] Figure 11

shows the distribution of 5 D-amino acids in the brains of the ddY/DAO$^-$ and ddY/DAO$^+$ mice. As shown in Fig. 11A, the amounts and distributions of D-Asp in the brains of both strains are almost the same, and relatively large amounts of D-Asp were observed in the pineal gland. D-Asp is an acidic D-amino acid, and it is oxidized by D-aspartic acid oxidase (DAspO), not by DAO, and the DAspO activity is reported to be almost equal in the ddY/DAO$^+$ and ddY/DAO$^-$ mice.[36] Therefore, it is likely that no significant difference in the D-Asp amounts was observed between the two strains. Figure 11B shows the amounts of D-Ser in the brains of the two strains. In both strains, large amounts of D-Ser were observed in the frontal brain areas such as the cerebrum and hippocampus, while the amounts were low in the pituitary and pineal glands. In the cerebellum and medulla oblongata, a clear difference was observed between the ddY/DAO$^+$ and ddY/DAO$^-$ mice; large amounts of D-Ser were observed only in the mutant ddY/DAO$^-$ mice. High DAO activities are present in the cerebellum and medulla oblongata of the ddY/DAO$^+$ mice[37]; the amounts of D-Ser in these tissues are, therefore, diminished by DAO. On the other hand, the amounts of D-Ser in the tissues of the ddY/DAO$^-$ mice remained unchanged. Therefore, our results that large amounts of D-Ser were observed in 5 brain areas (cerebrum, hippocampus, hypothalamus, cerebellum and medulla oblongata) of the ddY/DAO$^-$ mice suggest that the accumulation of D-Ser occurs in widespread areas of the mouse brain. Figure 11C shows the amounts of D-Ala. The distribution of D-Ala in the ddY/DAO$^+$ mice was clearly different from that of D-Ser, and relatively large amounts of D-Ala were observed in the pituitary and pineal glands. Since D-Ala is also a good substrate of DAO as well as D-Ser,[38] it was surprising that similar amounts of D-Ala were present in the frontal brain areas and hindbrain. While, in the brains of the ddY/DAO$^-$ mice, the amounts of D-Ala are higher than those in the brains of the ddY/DAO$^+$ mice in all the tested brain areas. A clear difference was also observed in the tissues showing no DAO activity, such as the cerebrum and hippocampus, therefore, the difference would be caused by the DAO activity in the whole body (mainly in the kidneys), and by the difference in the D-Ala levels in the serum (8.8 ± 1.4 nmol/ml in the ddY/DAO$^+$ mice, and 134.6 ± 16.5 nmol/ml in the ddY/DAO$^-$ mice).[31] Up to now, several reports have been published concerning the amounts of D-Asp, D-Ser and D-Ala in the cerebrum and cerebellum of the ddY/DAO$^+$ and ddY/DAO$^-$ mice,[39,40] and our results are consistent with these reports. Figures 11D and 11E show the results for D-Leu and D-Pro. Because the amounts of D-Leu and D-Pro in mammalian tissues are extremely low, our reports are the first cases to show the detailed distributions of these D-amino acids in the mammalian brain.[22,25] Both D-Leu and D-Pro are good substrates of DAO,[38,41] however, the results for D-Leu are quite different from those for D-Pro. The amounts of D-Leu drastically increased (about 10 times) in the ddY/DAO$^-$ mice compared to those in the ddY/DAO$^+$ mice. While, the amounts of D-Pro in the brains of the ddY/DAO$^-$ mice are not significantly different from those in the ddY/DAO$^+$ mice. These results indicate that in mammals, these two D-amino acids are regulated in different ways.

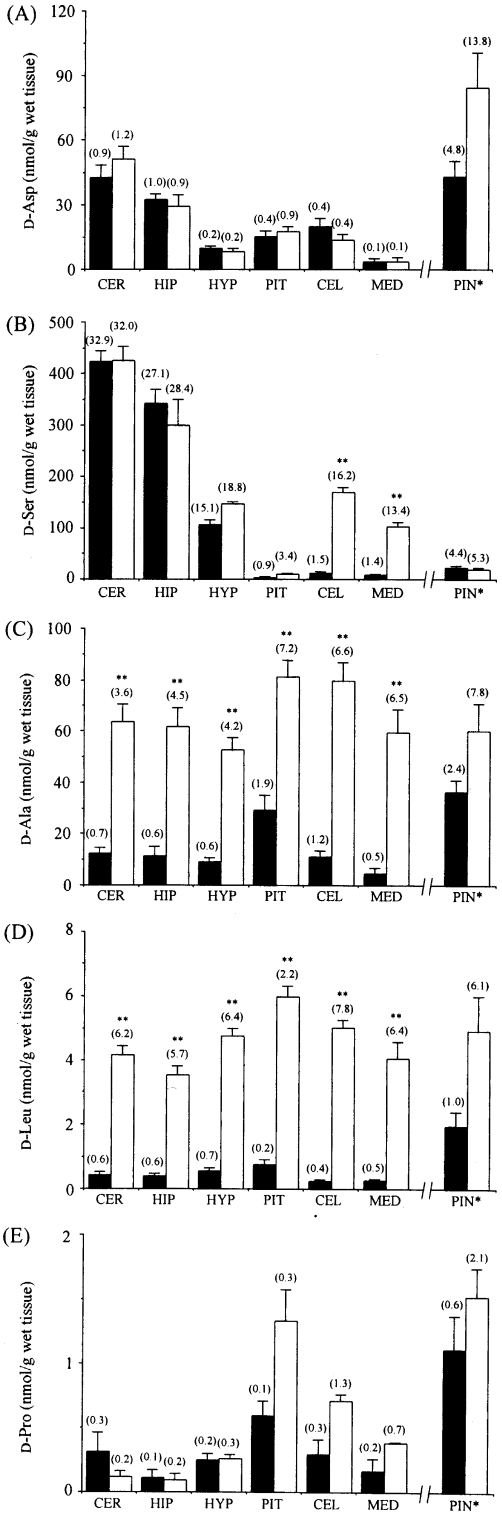

Fig. 11. Amounts of D-Asp (A), D-Ser (B), D-Ala (C), D-Leu (D) and D-Pro (E) in the Brains of ddY/DAO$^+$ Mice (Closed Bars) and ddY/DAO$^-$ Mice (Open Bars)

Values represent means$\pm$S.E.M. of 4 or 5 mice (nmol/g wet tissue). * The values for the pineal gland are expressed as pmol/whole pineal gland because the mouse pineal glands are too small to accurately measure the wet weights. Values in parentheses are the proportions of the D-form (D/(D+L)$\times$100). **$p<0.01$, significant difference from the values of ddY/DAO$^+$ mice. CER: cerebrum, HIP: hippocampus, HYP: hypothalamus, PIT: pituitary gland, CEL: cerebellum, MED: medulla oblongata, PIN: pineal gland.

6. Alteration of D-Amino Acid Levels after the Administration of D-Amino Acids[26]

Certain kinds of foods, beverages and intestinal bacteria contain D-amino acids in relatively large amounts,[42] and these exogenous D-amino acids are proposed to be one of the major origins of D-amino acids in mammals. Therefore, the amounts of D-Asp, D-Ser, D-Ala, D-Leu and D-Pro in the rat brain were determined at 0, 3, 5, 15, and 60 min after the intravenous administration of the five D-amino acids (Fig. 12). The amount of D-Asp markedly increased in the pineal gland; it reached approximately 5000 nmol/g wet tissue 3 min after the administration, and then gradually decreased (Fig. 12A). The amount of D-Ser increased in the pituitary and pineal glands 3 min after the administration, and the levels did not significantly change until 60 min (Fig. 12B). Remarkable increases in the D-Ala levels were also observed only in the pituitary and pineal glands 3 min after the administration, but decreased to almost the control levels at 60 min (Fig. 12C). The D-Leu amounts increased in all the brain regions (Fig. 12D), while notable increases in the D-Pro amounts were observed only in the pituitary and pineal glands (Fig. 12E). These results clearly indicate that each D-amino acid is regulated in a different manner, which should be investigated in the future.

7. Study on the Origin of D-Proline[27]

As described above, we found that ddY/DAO$^-$ mice excrete large amounts of D-Pro into their urine (Fig. 6, about 20 times that of L-Pro). While, in the ddY/DAO$^+$ mice having a high DAO activity, the urinary excretion of D-Pro is only 5% of that in the urine of the ddY/DAO$^-$ mice. These findings strongly suggest that in the absence of DAO activity, a large amount of D-Pro is present in mammals, and also suggest that the D-Pro is metabolized by the enzyme in the wild type animals. Using the ddY/DAO$^-$ mice, the origin of this urinary D-Pro was investigated. As the origin of the free D-amino acids in mammals, intestinal bacteria, diet and biosynthesis have been reported,[1,2] and the intestinal bacterial origin of D-Pro was investigated using the ddY/DAO$^-$ mice having a germ-free status, as well as inoculating them with typical intestinal bacteria. Figure 13 shows the chromatograms obtained using a germ-free mouse (Fig. 13A), a di-associated mouse (Fig. 13B, inoculated with two gram negative bacteria, *Escherichia coli*, and *Bacteroides vulgatus*) and a tetra-associated mouse (Fig. 13C, inoculated with *Bifidobacterium longum* and *Eubacterium aerofaciens* in addition to the 2 bacteria described above). As shown in Fig. 13, the germ-free ddY/DAO$^-$ mice still excrete large amounts of D-Pro into their urine, and the inoculation with 4 typical intestinal bacteria did not result in an increase in the urinary D-Pro amount, strongly suggesting that the D-Pro present in the urine of the ddY/DAO$^-$ mice is not of intestinal bacterial origin. The dietary origin of urinary D-Pro was investigated by

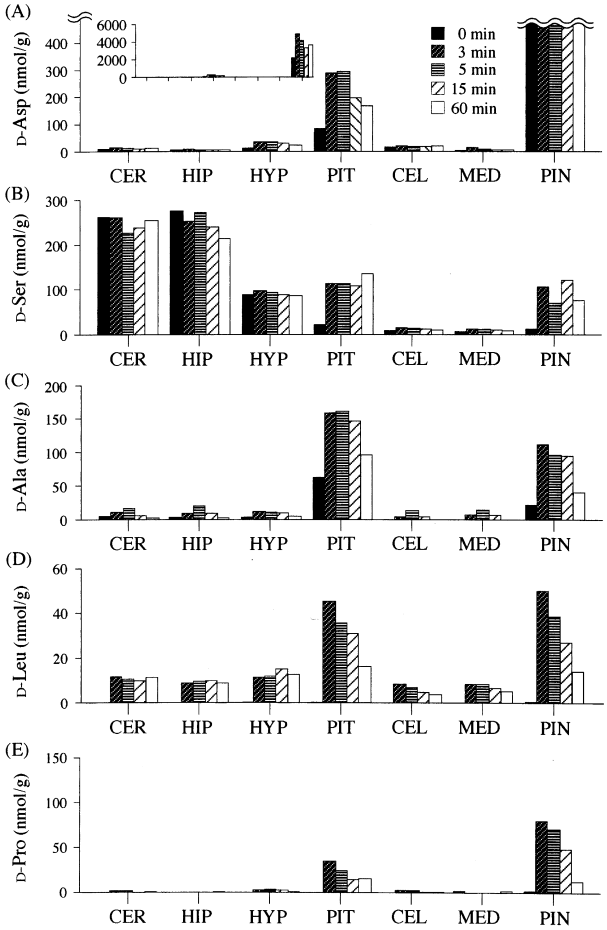

Fig. 12. The Time Courses of the D-Asp (A), D-Ser (B), D-Ala (C), D-Leu (D) and D-Pro (E) Levels in the Various Rat Brain Regions after the Intravenous Administration of the Five D-Amino Acids

Values represent means (nmol/g wet tissue) of 3 (D-Asp, D-Ser, D-Ala) or 2 (D-Leu, D-Pro) rats. The abbreviations of the tissues are the same as shown in Fig. 11.

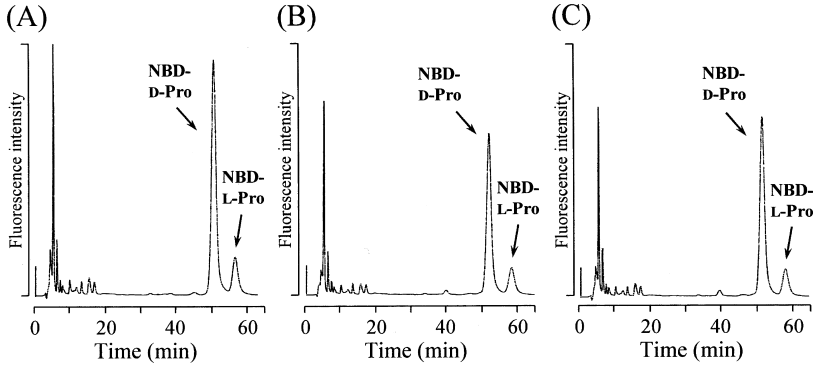

Fig. 13. Separation of NBD-D- and L-Pro in the Urine of a Germ-Free ddY/DAO$^-$ Mouse (A), a Di-associated ddY/DAO$^-$ Mouse (B) and Tetra-Associated ddY/DAO$^-$ Mouse (C)

The chromatographic conditions are described in the text.

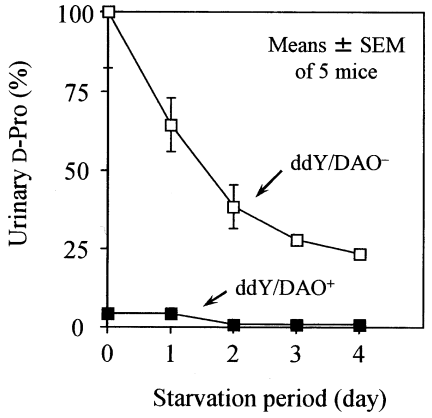

Fig. 14. Effect of Starvation on the Amounts of D-Pro in the Urine of ddY/DAO$^-$ Mice (Open Squares) and ddY/DAO$^+$ Mice (Closed Squares)

the starvation for 4 d. As shown in Fig. 14, the urinary D-Pro of the ddY/DAO$^-$ mice rapidly decreased for 2 d and then slightly decreased or remained rather constant thereafter. On the fourth day of starvation, 22.7% of D-Pro was still excreted (the amount of urinary D-Pro before the starvation was taken as 100%). The amount of urinary D-Pro in the ddY/DAO$^+$ mice also decreased and became a trace amount after starvation for 2 d. The present results strongly suggest that the urinary D-Pro is partially of dietary origin, although, at least some of the D-Pro is not derived from their diet. Considering together that the D-Pro is not derived from intestinal bacteria, a notable amount of D-Pro is likely to be produced in these mice, and detailed investigations on the origin of D-Pro are highly expected.

8. Conclusion

In the present article, we demonstrated that various D-amino acids of small amounts are present in mammals using highly sensitive and selective two-dimensional HPLC. These D-amino acids show particular distributions and alterations in their amounts. The findings described in the present study strongly suggest that each D-amino acid has some physiological roles in mammals, which should be clarified in the future. Other D-amino acids that have not yet been investigated might present in mammals with some physiological functions and/or diagnostic values, and further advances in the analytical methods in combination with biochemical and clinical approaches are highly expected.

Acknowledgements I deeply express my gratitude to Professor Kiyoshi Zaitsu (Kyushu University) for his kind supervision throughout these studies. I also thank emeritus Professor Kazuhiro Imai (Tokyo University), Professors Hiroshi Homma (Kitasato University) and Ryuichi Konno (Dokkyo Medical University) for their advice and encouragement. I would like to express my sincere appreciation to Dr. Morikawa and our lab members for their hard-work and valuable daily discussions, and all of the co-workers for their kind help. The financial supports by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Takeda Science Foundation, and Industrial Technology Research Grant Program in '05 from New Energy and Industrial Technology Development Organization (NEDO) of Japan are greatly appreciated.

References

1) Hamase K., Morikawa A., Zaitsu K., *J. Chromatogr. B*, **781**, 73—91 (2002).
2) Fujii N., *Orig. Life Evol. Biosph.*, **32**, 103—127 (2002).
3) Snyder S. H., Kim P. M., *Neurochem. Res.*, **25**, 553—560 (2000).
4) Nishikawa T., *Biol. Pharm. Bull.*, **28**, 1561—1565 (2005).
5) Schell M. J., Molliver M. E., Snyder S. H., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 3948—3952 (1995).
6) Furuchi T., Homma H., *Biol. Pharm. Bull.*, **28**, 1566—1570 (2005).
7) D'Aniello G., Tolino A., D'Aniello A., Errico F., Fisher G. H., Di Fiore M. M., *Endocrinology*, **141**, 3862—3870 (2000).
8) Nagata Y., Homma H., Lee J.-A., Imai K., *FEBS Lett.*, **444**, 160—164 (1999).
9) Takigawa Y., Homma H., Lee J.-A., Fukushima T., Santa T., Iwatsubo T., Imai K., *Biochem. Biophys. Res. Commun.*, **248**, 641—647 (1998).
10) Aswad D. W., *Anal. Biochem.*, **137**, 405—409 (1984).
11) Hashimoto A., Nishikawa T., Oka T., Takahashi K., Hayashi T., *J. Chromatogr.*, **582**, 41—48 (1992).
12) Brückner H., Haasmann S., Langer M., Westhauser T., Wittner R., Godel H., *J. Chromatogr. A*, **666**, 259—273 (1994).
13) Nimura N., Fujiwara T., Watanabe A., Sekine M., Furuchi T., Yohda M., Yamagishi A., Oshima T., Homma H., *Anal. Biochem.*, **315**, 262—269 (2003).
14) Marfey P., *Carlsberg Res. Commun.*, **49**, 591—596 (1984).
15) Nagata Y., Yamamoto K., Shimojo T., *J. Chromatogr.*, **575**, 147—152 (1992).
16) Einarsson S., Josefsson B., Möller P., Sanchez D., *Anal. Chem.*, **59**, 1191—1195 (1987).
17) Hashimoto A., Nishikawa T., Hayashi T., Fujii N., Harada K., Oka T., Takahashi K., *FEBS Lett.*, **296**, 33—36 (1992).
18) Brückner H., Schieber A., *Biomed. Chromatogr.*, **15**, 166—172 (2001).
19) Hamase K., Homma H., Takigawa Y., Fukushima T., Santa T., Imai K., *Biochim. Biophys. Acta*, **1334**, 214—222 (1997).
20) Hamase K., Homma H., Takigawa Y., Imai K., *Amino Acids*, **17**, 277—283 (1999).
21) Inoue T., Hamase K., Morikawa A., Zaitsu K., *J. Chromatogr. B*, **744**, 213—219 (2000).
22) Hamase K., Inoue T., Morikawa A., Konno R., Zaitsu K., *Anal. Biochem.*, **298**, 253—258 (2001).
23) Morikawa A., Hamase K., Zaitsu K., *Anal. Biochem.*, **312**, 66—72 (2003).
24) Zhao H., Hamase K., Morikawa A., Qiu Z., Zaitsu K., *J. Chromatogr. B*, **810**, 245—250 (2004).
25) Hamase K., Konno R., Morikawa A., Zaitsu K., *Biol. Pharm. Bull.*, **28**, 1578—1584 (2005).
26) Morikawa A., Hamase K., Inoue T., Konno R., Zaitsu K., *Amino Acids*, **32**, 13—20 (2007).
27) Hamase K., Takagi S., Morikawa A., Konno R., Niwa A., Zaitsu K., *Anal. Bioanal. Chem.*, **386**, 705—711 (2006).
28) Imai K., Watanabe Y., *Anal. Chim. Acta*, **130**, 377—383 (1981).
29) Fukushima T., Kato M., Santa T., Imai K., *Biomed. Chromatogr.*, **9**, 10—17 (1995).
30) Pilone M. S., *Cell. Mol. Life Sci.*, **57**, 1732—1747 (2000).
31) Morikawa A., Hamase K., Inoue T., Konno R., Niwa A., Zaitsu K., *J. Chromatogr. B*, **757**, 119—125 (2001).
32) Konno R., Yasumura Y., *Genetics*, **103**, 277—285 (1983).
33) Konno R., Yasumura Y., *Lab. Anim. Sci.*, **38**, 292—295 (1988).
34) Hashimoto A., Oka T., *Prog. Neurobiol.*, **52**, 325—353 (1997).
35) Konno R., Yasumura Y., *Int. J. Biochem.*, **24**, 519—524 (1992).
36) Nagasaki H., Yamada R., Konno R., Yasumura Y., Iwashima A., *Experientia*, **46**, 468—470 (1990).
37) Hamase K., Nagayasu R., Morikawa A., Konno R., Zaitsu K., *J. Chromatogr. A*, **1106**, 159—164 (2006).
38) D'Aniello A., Vetere A., Petrucelli L., *Comp. Biochem. Physiol. B*, **105**, 731—734 (1993).
39) Hashimoto A., Nishikawa T., Konno R., Niwa A., Yasumura Y., Oka T., Takahashi K., *Neurosci. Lett.*, **152**, 33—36 (1993).
40) Nagata Y., Konno R., Niwa A., *Metabolism*, **43**, 1153—1157 (1994).
41) Hasegawa H., Matsukawa T., Shinohara Y., Konno R., Hashimoto T., *Am. J. Physiol.*, **287**, E160—E165 (2004).
42) Friedman M., *J. Agric. Food Chem.*, **47**, 3457—3479 (1999).