Review

Enantioselective Two-Dimensional High-Performance Liquid Chromatographic Determination of Amino Acids; Analysis and Physiological Significance of D-Amino Acids in Mammals

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Abstract

D-Amino acids are now increasingly recognized as physiologically functional molecules and biomarkers in mammals. However, the amounts of D-amino acids are extremely low in most cases, thus highly sensitive and selective analytical methods are practically essential.   In the present review article, enantioselective two-dimensional high-performance liquid chromatographic (2D-HPLC) methods are introduced and their applications to the determination of D-amino acids in mammalian samples are described.   For the sensitive determination, amino acid enantiomers are derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and are determined by a fluorescence detector.   The 2D-HPLC system consists of the reversed-phase non-enantioselective, but chemoselective separation of NBD-amino acids in the first dimension, and the sequential chiral separation in the second dimension.   By using the system, neuroactive D-amino acids (D-Ser and D-Ala) were determined in the brain of various mammalian species and the regulation of their intrinsic amounts are also demonstrated.   The physiological and diagnostic meanings of the trace amounts of D-Ser are also discussed.   The 2D-HPLC systems for the acidic D-amino acids (D-Asp and D-Glu), proline analogues (D-Pro, *trans*- and *cis*-4-hydroxy-D-Pro) and hydrophilic D-amino acids (D-Arg, D-Asn, D-Asp, D-Gln, D-Glu, D-His, D-Ser, D-*allo*-Thr and D-Thr) are also shown.

Keywords: D-Amino acids; Enantiomer analysis; 2D-HPLC

1. Introduction

   Along with the progress in analytical technologies, several D-amino acids of the free form and D-amino acid residues in the proteins were found in higher animals including humans [1-3].   Free D-amino acids are now increasingly recognized as novel physiologically active molecules in the central nervous system and endocrine tissues, and are also demonstrated to be the biomarkers of the pathophysiological conditions [4-6].   D-Amino acid residues in the proteins are shown to have a close relation to aging and also to various diseases [3].   In the present review article, we focus on the free D-amino acids in the higher animals, especially in mammals.   Concerning the D-amino acid residues in the proteins, please refer to the detailed articles already published elsewhere [3,7,8].

   In mammals, incredibly high amounts of free D-amino acids were found in specific cases; e.g., D-Ser in the cerebrum (30% of the L-Ser [9]) and D-Asp in the pineal gland (almost the same as or higher than L-Asp [10]). These D-amino acids are now revealed as a modulator of the *N*-methyl-D-aspartic acid (NMDA) subtype of glutamate receptors (D-Ser [6]), and a regulator of melatonin secretion (D-Asp [5]).   However, the amounts of free D-amino acids in mammalian tissues and physiological fluids are extremely low in most cases, and sensitive and selective analytical methods are practically essential. Enantioselective and two-dimensional high-performance liquid chromatography (2D-HPLC) is one of the most powerful techniques for the determination of trace amounts of D-amino acids in real world samples [2,11].   In the present focusing review, 2D-HPLC analysis of D-amino acids in mammals and the biological significance are discussed.

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2. Enantioselective 2D-HPLC analysis of Ser and Ala in mammals

D-Ser and D-Ala bind to the glycine site of NMDA receptors in the central nervous system, and are indicated as co-agonists of the receptor together with L-Glu [6]. These two D-amino acids modulate the neurotransmission of the Glu receptors, and novel therapies of neuronal diseases focusing on these two D-amino acids are expected. Therefore, the distribution of D-Ser and D-Ala in mammals and their enzymatic regulation should be clarified in detail. Although the amount of D-Ser in the frontal brain area is incredibly high, the amounts in the other brain areas and peripheral tissues are low [9,12-14]. The amounts of D-Ala are also at trace levels in all tissues [13-15]. Therefore, a sensitive and selective determination is necessary, and for that purpose, 2D-HPLC is a reliable and useful technique.
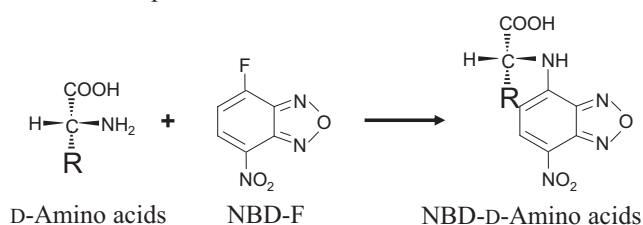

Fig. 1. Derivatization of amino acids with NBD-F.

For the 2D-HPLC determination of D-Ser and D-Ala in mammals, amino acid enantiomers are derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F [16]) prior to the HPLC determination (Fig. 1). The fluorescent NBD-derivatives are first fractionated by a microbore reversed-phase column as a first dimension, and are automatically introduced into an enantioselective column representing the second dimension where the D- and L-enantiomers are separated. The flow diagram of the 2D-HPLC system [13,14] is shown in Fig. 2, and the typical chromatograms obtained using a biological sample (plasma of Wistar rat) are described in Fig. 3. In the first dimension, the fractions of NBD-Ser and NBD-Ala were collected as their D plus L mixtures by a capillary monolithic-ODS column, and the enantiomers were further separated by a narrowbore enantioselective column in the
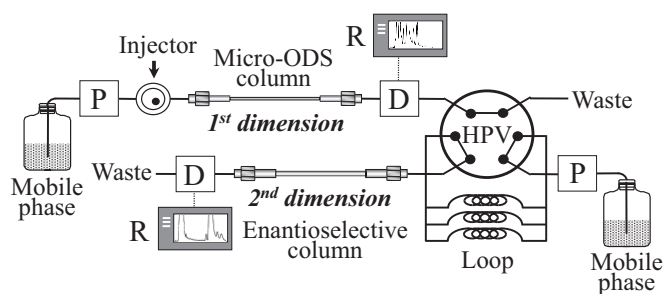

Fig. 2. Flow diagram of the 2D-HPLC system. P: pump, D: detector, R: integrator, HPV: high pressure valve.

second dimension. As an enantioselective column, a Pirkle-type column (Sumichiral OA-2500S, [17,18]) was used. In the rat plasma, trace amounts of D-Ser and D-Ala were clearly determined without severe interference by intrinsic compounds in the tissues. The detection limit of D-Ser using the 2D-HPLC system is 500 amol / injection, and that for D-Ala is also 500 amol / injection.
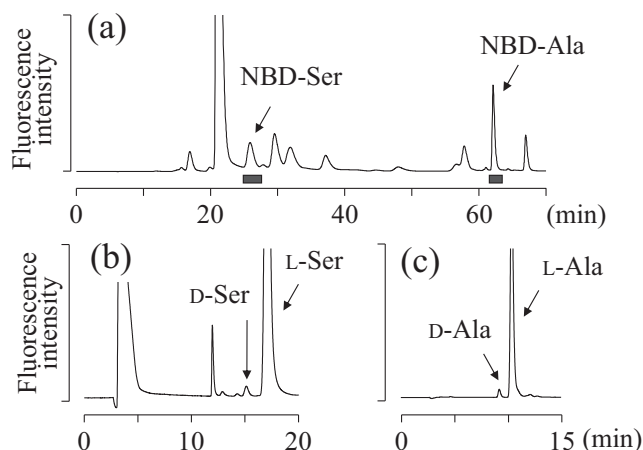

Fig. 3. 2D-HPLC chromatograms of D-Ser and D-Ala in rat plasma. (a) Reversed-phase separation of NBD-Ser and NBD-Ala in the first dimension, and the fractions indicated by closed bars are introduced to the second dimension. (b) Enantiomer separation of NBD-Ser in the second dimension and (c) enantiomer separation of NBD-Ala. Reproduced from ref. [11] with permission.

2.1. Distribution of D-Ser and D-Ala in mammals

By using the 2D-HPLC system described above, the distribution of D-Ser in mammals was clarified [13-15]. As experimental animals, rats and mice were used. The amounts of D-Ser in the central nervous system, peripheral tissues and physiological fluids are shown in Fig. 4(a). In the brain, large amounts of D-Ser were found in the cerebral cortex and hippocampus (the values of %D, (D/(D+L) x 100), was around 20-25% for both the rat and mouse), followed by hypothalamus and olfactory lobe. In the cerebellum and medulla oblongata, the amounts of D-Ser were trace levels (%D was about 1% or lower). Compared to the amounts in the frontal brain areas, the amounts of D-Ser in the peripheral tissues are not significantly high (%D is 0.1-6%). Concerning the physiological fluids, the amount of D-Ser is low in the plasma (%D is 1-2%), however, the amounts are relatively high in the urine (%D is 10-50%).

The distributions of D-Ala in the rat and mouse are summarized to Fig. 4(b). In the brain, the amounts of D-Ala are low for both rodents (%D is lower than 0.5%), especially in the hindbrain areas such as cerebellum and medulla oblongata. On the other hand, the amounts in the pituitary gland and pancreas are relatively high. The
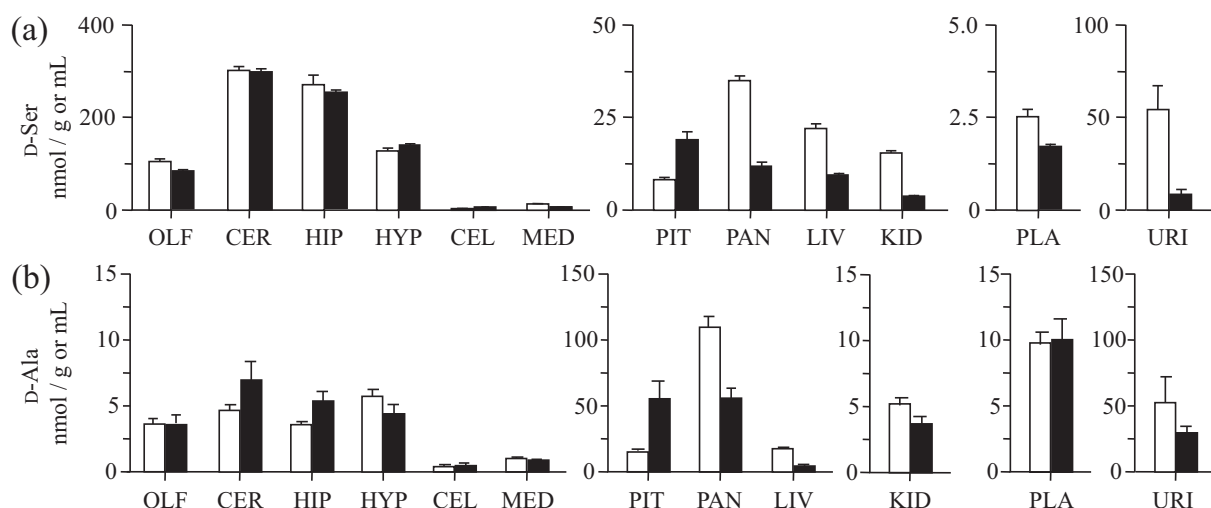
Fig. 4. Distribution of D-Ser (a) and D-Ala (b) in the brain and peripheral tissues. Open bars indicate the values for mice, and closed bars indicate those for rats. OLF, olfactory bulb; CER, cerebral cortex; HIP, hippocampus; HYP, hypothalamus; CEL, cerebellum; MED, medulla oblongata; PIT, anterior pituitary; PAN, pancreas; LIV, liver; KID, kidney; PLA, plasma; URI, urine.

highest amount of D-Ala was observed in the pituitary gland of the rat (%D is 1.3%), and in the pancreas of the mouse (%D is 2.7%). The concentration (%D) of D-Ala in the plasma is about 3% for both animals, however, the %D is over 15% in the urine.

2.2. Enzymatic regulation of D-Ser and D-Ala in mammalian tissues and physiological fluids

As described above, both D-Ser and D-Ala are noted as novel candidate biomarkers of neuronal diseases, and the novel therapies focusing on these two D-amino acids are also expected. Therefore, it is highly required to clarify the regulation mechanisms and their contributions on the intrinsic amounts of D-Ser and D-Ala in mammals. As a degradation enzyme, D-amino acid oxidase (DAO, EC 1.4.3.3) is widely known [1,19,20] and both rat and mouse strains lacking DAO have been established. As a mutant mouse strain lacking the DAO activity, the ddY/DAO⁻ mouse [21] was used, and the LEA/Sen rat was used as a mutant rat lacking DAO [22]. As the control mouse, the ddY/DAO⁺ mouse having a normal DAO activity was used, and the Wistar rat was used as the control rat. By using the 2D-HPLC system described above, the amounts of D-Ser and D-Ala in the tissues and physiological fluids of these rodents were determined in detail. As shown in Fig. 5(a), the amounts of D-Ser are almost the same in the frontal brain areas for all the four strains of rodents; i.e., around 300 nmol of D-Ser / g tissue was observed in the cerebrum and hippocampus. However, in the hind brain areas, the amounts of D-Ser in the mutant rats and mice were drastically higher than those in the control rats and mice. This is because high DAO activity is present in the hind brain areas such as cerebellum and medulla oblongata [23-25] and most of the D-Ser was metabolized by the

enzyme in these tissues. In the peripheral tissues and physiological fluids, the amounts of D-Ser in the mutant animals are significantly higher than those in the control animals in most cases.

The amounts of D-Ala in the ddY/DAO⁺ mouse, ddY/DAO⁻ mouse, Wistar rat and LEA/Sen rat are shown in Fig. 5(b) [13,14,26]. In all the brain areas, peripheral tissues and physiological fluids, the amounts of D-Ala were drastically higher in the mutant rodents than those in the control mice and rats. In the control rats, a high DAO activity is present in the hind brain areas, kidney and liver, and in the control mice, a high DAO activity is present only in the hind brain areas and kidney [27]. However, as shown in Fig. 5(b), the amounts of D-Ala are high in all the tested tissues and physiological fluids regardless of the presence of DAO. This is mainly due to the difference in the D-Ala amounts in the serum/plasma. Because the amounts of D-Ala in the blood are low in the control rodents due to the strong DAO activity in the kidney, the amounts of blood D-Ala are extremely high in the mutant rodents compared to their control animals. D-Ala present in the blood immediately distributed to the tissues [28], therefore, the D-Ala amounts in all the tissues could be upregulated by the decrease in the DAO activity.

As an enzyme synthesizing D-Ser in mammals, Ser racemase (SRR, EC 5.1.1.18) has been reported [29,30], and a knock-out mouse strain lacking SRR (SRR⁻/⁻ mice) has successfully been established [31-34]. Mice with various SRR activities have also been established (SRR⁻/⁻ mice, SRR⁺/⁻ mice and their control SRR⁺/⁺ mice), and the amounts of D-Ser in the entire body of these animals were determined in detail [34]. The amounts of D-Ser in the SRR⁻/⁻ mice were low in all the tested tissues and physiological fluids, especially, a drastic alteration was
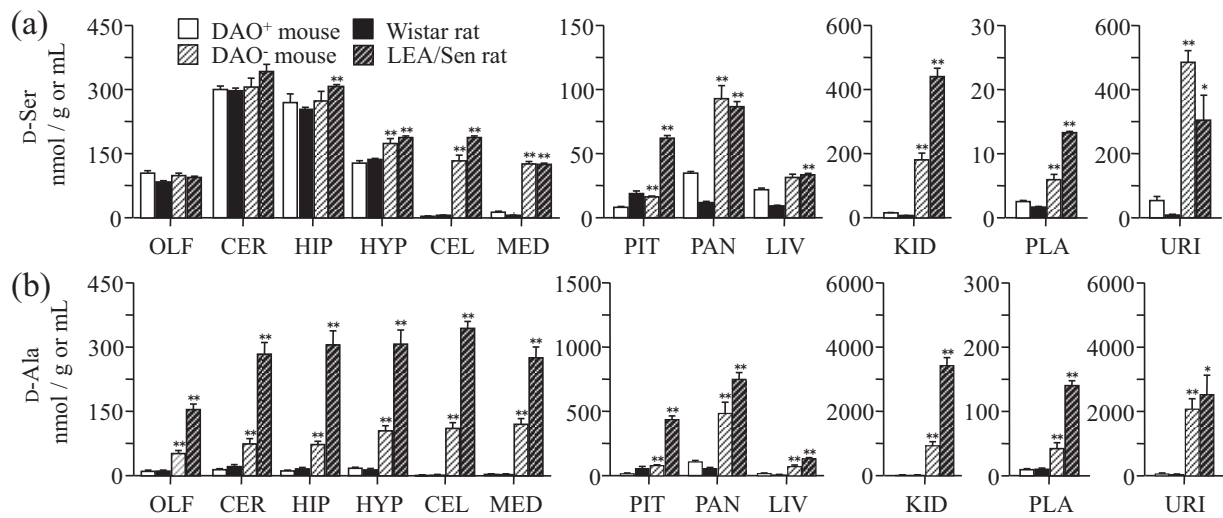
Fig. 5. Alteration of D-Ser (a) and D-Ala (b) amounts in the brain and peripheral tissues of rodents by the lack of DAO activities. OLF, olfactory bulb; CER, cerebral cortex; HIP, hippocampus; HYP, hypothalamus; CEL, cerebellum; MED, medulla oblongata; PIT, anterior pituitary; PAN, pancreas; LIV, liver; KID, kidney; PLA, plasma; URI, urine.

observed in the brain. Fig. 6 shows the results obtained in several brain areas, serum and cerebrospinal fluid. By decreasing the SRR activity, the amounts of D-Ser in most of the brain areas decreased to around 10% of those in the control mice. These results indicated that the amounts of D-Ser in the tissues could be regulated by changing the SRR activity.
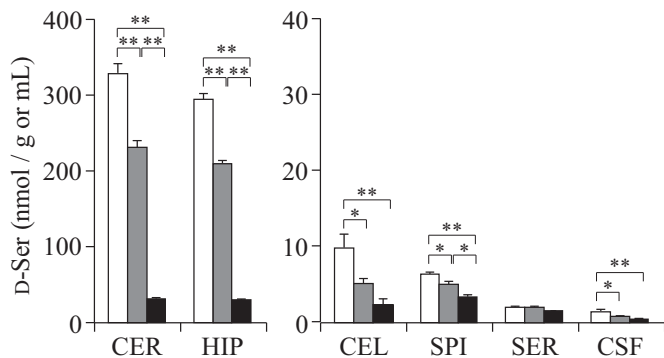

Fig. 6. Alteration of D-Ser amounts in the brain and peripheral tissues of rodents by the lack of SRR activities. Open bars indicate the values for SRR$^{+/+}$ mice, gray bars indicate those for SRR$^{+/-}$ mice and closed bars indicate those for SRR$^{-/-}$ mice. Reproduced from ref. [34] with permission. CER, cerebral cortex; HIP, hippocampus; CEL, cerebellum; SPI, spinal cord; SER, serum; CSF, cerebrospinal fluid.

2.3. Biological significance of trace amounts of D-Ser in mammalian brain

In the cerebrum and hippocampus of mammals, large amounts of D-Ser were observed, and the biosynthesis and physiological function of D-Ser in these tissues have been investigated in detail [6,35]. On the other hand, the amounts of D-Ser in the hindbrain areas, such as the cerebellum and medulla oblongata, are extremely low in the

adult tissue [13,26,36]. Therefore, the biological significance of D-Ser in these hindbrain areas has not been studied for a long time. Especially, in the cerebellum, a significant amount of D-Ser is present only during the immature period [12], and the physiological role of this D-amino acid in the cerebellum has been a matter of interest. In 2011, Kakegawa et al. reported that D-Ser plays a crucial role in regulating the cerebellar long-term depression at the synapses between parallel fibers and the Purkinje cells [37]. By using the 2D-HPLC system described above, the release of D-Ser in the cerebellum of the immature mouse has been clearly demonstrated.

The amounts of D-Ser are changed in several neuronal diseases. D-3-Phosphoglycerate dehydrogenase (Phgdh; EC 1.1.1.95) is an enzyme catalyzing the formation of 3-phosphohydroxypyruvate in the first step of the L-Ser biosynthesis [38]. The lack of activity of this enzyme causes severe neurological symptoms as well as a drastic decrease in the L-Ser amounts. Because D-Ser in the brain is synthesized from L-Ser by SRR, the amount of D-Ser is also likely to be decreased. In the brain of the conditional Phgdh knock-out mouse, the amount of D-Ser has decreased and only 10% of that in the control mouse was observed [39]. D-Ser is also expected as a biomarker of amyotrophic lateral sclerosis (ALS). ALS is a neurodegenerative disorder with a severe loss of motoneurons in the spinal cord [40,41]. It is a progressive and fatal disease, and an effective biomarker is highly needed. Sasabe et al. determined trace amounts of D-Ser in the spinal cord of the ALS model mouse by the 2D-HPLC system [42]. Compared with the preonset stage of ALS, the amount of D-Ser in the spinal cord has increased in the onset and terminal stages of the ALS. These results clearly indicate that trace amounts of D-amino

acids also have the biological and clinical significance in mammals.

3. Enantioselective 2D-HPLC analysis of Asp and Glu in mammals

D-Asp is widely present in the endocrine tissues of mammals, and it has been revealed to have physiological functions in regulating hormonal secretion [5]. D-Glu is also shown to be present in the urine of various animals [43,44]. In addition to be a regulating molecule of hormonal secretion, D-Asp and D-Glu have effects on neuronal transmission [4], and therefore, the analysis of these two D-amino acids in mammalian tissues and physiological fluids is highly required. As a sensitive and selective method, a 2D-HPLC system combining a microbore ODS column and a narrowbore enantioselective column has been established after derivatization of the Asp and Glu enantiomers with NBD-F [45]. For the reversed-phase separation of NBD-Asp and NBD-Glu, a long microbore monolithic ODS column (0.53 mm i.d. x 750 mm) was used, and a Pirkle-type enantioselective column (Sumichiral OA-2500S, 1.5 mm i.d. x 250 mm) was used for their chiral separations. By using the 2D-HPLC system, trace amounts of D-Asp and D-Glu (500 amol) could be determined in biological matrices without any severe interference by intrinsic substances. As the acidic

amino acid analogs, NMDA, NMLA, *N*-methyl-D-glutamic acid (NMDG) and NMLG are also able to be determined by the 2D-HPLC system with slight modification of the separation conditions [46].

3.1. Distribution of D-Asp and D-Glu in mammals

Detailed distributions of D-Asp and D-Glu in the brain and peripheral tissues of the rat have been clarified [45]. The amounts of D-Asp and D-Glu in 7 brain tissues, 11 peripheral tissues, plasma and urine are summarized in Table 1. Concerning Asp, large amounts of the D-enantiomer were observed in the endocrine tissues, such as the pineal gland, pituitary gland, adrenal gland and testis. However, the amounts of D-Asp in most of the central nervous system, such as the cerebrum, hippocampus and cerebellum, were trace levels (the values of D-form were lower than 1% of total the Asp in the corresponding tissues). On the other hand, the amounts of D-Glu are extremely low in all the tissues tested regardless of the function of the tissue (endocrine tissues or neuronal tissues). The amounts of D-Glu are much lower than those of the L-enantiomer, and the %D values are around 0.1%. Only in the urine, a relatively high amount of D-Glu was observed, and the %D was 3.3%.

3.2. Relationship between D-Asp amount in the pineal gland and melatonin content

D-Asp was reported to inhibit melatonin secretion in mammals [47,48]. Melatonin is an indole hormone synthesized in the pineal gland of mammals [49] mainly by serotonin *N*-acetyltransferace (SNAT). In rodents, several strains of mice are known to have a low enzymatic activity of this enzyme, and the amounts of melatonin are extremely low in the pineal gland of these animals [50,51]. Therefore, it is a matter of interest whether the amounts of D-Asp in the pineal gland of these mice are related to the amounts of melatonin. In the pineal gland of 3 strains of rats and 6 strains of mice, both D-Asp and melatonin were determined [52]. For the determination of D-Asp, a 2D-HPLC system was used, while a pre-column oxidation column-switching HPLC system was used for the determination of melatonin [53]. The results are summarized in Fig. 7. In all the tested rats, high levels of D-Asp (almost the same amounts as those of L-Asp) were observed, and high levels of melatonin were also present. On the other hand, in the pineal gland of mice, the amounts of melatonin were totally different between the strains. In the C3H and CBA strains in which a high SNAT activity was observed [50], large amounts of melatonin were observed in the pineal gland. However, in the pineal glands of ICR, BALB/c, C57BL and ddY mice, the amounts of melatonin were trace levels. Interestingly, the amounts of pineal D-Asp in the ICR, BALB/c, C57BL and ddY mice

Table 1. Amounts of D-Asp and D-Glu in the rat tissues and physiological fluids.

Tissues/fluids	Asp	Glu
Brain Tissues		
Cerebral cortex	12.8±1.1 (0.18)	1.9±0.1 (0.04)
Hippocampus	6.0±0.6 (0.16)	3.9±0.5 (0.05)
Hypothalamus	15.0±1.9 (0.32)	3.2±1.0 (0.08)
Cerebellum	28.4±3.1 (0.60)	4.1±0.3 (0.15)
Medulla oblongata	6.5±0.8 (0.09)	2.3±0.4 (0.10)
Pineal gland	2025.9±147.8 (23.9)	7.2±1.1 (0.05)
Pituitary gland	132.5±32.3 (3.34)	2.6±1.0 (0.05)
Peripheral tissues		
Spinal cord	6.7±0.5 (0.09)	3.1±0.4 (0.08)
Adrenal gland	574.3±231.2 (70.5)	0.7±0.1 (0.05)
Testis	129.6±14.1 (17.9)	2.0±0.4 (0.03)
Thymus	88.1±0.5 (1.15)	9.0±1.3 (0.09)
Pancreas	19.3±1.6 (0.41)	1.8±0.4 (0.01)
Spleen	195.6±17.3 (3.61)	4.4±0.6 (0.05)
Lung	141.1±13.6 (3.99)	1.6±0.3 (0.02)
Heart	20.2±4.3 (1.05)	0.8±0.1 (0.01)
Kidney	34.1±6.6 (0.43)	2.8±0.5 (0.01)
Liver	14.5±3.0 (0.98)	2.3±0.8 (0.03)
Muscle	3.5±0.5 (1.13)	1.9±0.3 (0.12)
Physiological fluids		
Plasma	0.9±0.2 (2.92)	trace (-)
Urine	4.1±0.9 (10.2)	3.7±1.0 (3.33)

Values represent mean ± SE (nmol/g or mL) of 3 rats. Values in parentheses represent %D (D / (D + L) x 100). Rat; Wistar, male, 6 weeks of age. Reproduced from ref. [45] with permission.
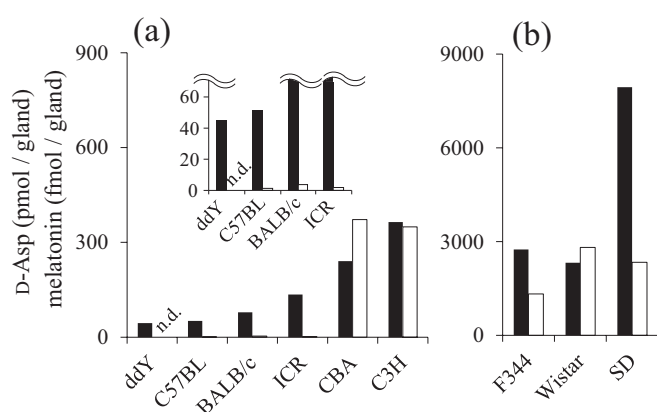
Fig. 7. Amounts of D-Asp and melatonin in the pineal glands of rats and mice. Closed bars indicate the amounts of D-Asp and open bars indicate those of melatonin. Reproduced from ref. [52] with permission.

were also low, while those in the C3H and CBA strains were high. The amounts of D-Asp in other endocrine tissues and the plasma of these rats and mice were not different between the strains.

4. Enantioselective 2D-HPLC analysis of Pro analogues in mammals

D-Pro is one of the D-amino acids widely observed in the plasma and urine in mammalian species [54-56]. The relationship between plasma D-Pro and a renal disorder was reported [54,57], and orally or intraventricularly administered D-Pro affected the hepatic and renal functions [58,59]. Therefore, this D-amino acid is expected as a novel candidate biomarker. A large part of Pro in the mammalian body is present in the collagen, one of the most abundant proteins in the mammalian body, and about half of the Pro in the collagen is converted to hydroxyproline (Hyp). Therefore, a 2D-HPLC method for the enantioselective determination of Pro and Hyp as their fluorescent NBD-derivatives has been established [60]. After the reversed-phase separation as the first dimension, enantiomers of the Pro analogues were separated by a Pirkle-type enantioselective column (Sumichiral OA-2500R) or by a prototype anion-exchange type enantioselective column (Chiralpak QN-2-AX). By using these columns, the enantiomers of Pro, *trans*-Hyp and *cis*-Hyp were nicely separated in about 10 min.

4.1. Distribution of D-Pro in mammals

By using the 2D-HPLC system, the distributions of D-Pro both in the ddY/DAO⁺ mouse and ddY/DAO⁻ mouse have been investigated [61,62]. These values are summarized in Table 2. In the 7 brain areas and 11 peripheral tissues, trace amounts of D-Pro are found (%D is lower than 1%) in the ddY/DAO⁺ mice. In the ddY/DAO⁻ mice, the amounts of D-Pro are generally not very high, however, relatively

high amounts of this D-amino acid were observed in the pancreas (2.0%) and kidney (8.6%). On the other hand, incredibly high amounts of D-Pro were observed in the urine of both strains of the mouse; the value was 25.9% of the total Pro in the ddY/DAO⁺ mouse and 95.1% in the ddY/DAO⁻ mouse. Especially, in the urine of the ddY/DAO⁻ mouse, the amount of D-Pro was almost 20 times higher than that of L-Pro.

Table 2. Distribution of D-Pro in the tissues and physiological fluids of ddY/DAO⁺ and ddY/DAO⁻ mice.

Tissues/fluids	ddY/DAO⁺	ddY/DAO⁻
Brain Tissues		
Cerebral cortex	0.52±0.10 (0.25)	0.54±0.02 (0.14)
Cerebellum	0.45±0.06 (0.38)	1.26±0.06 (1.09)
Medulla oblongata	0.30±0.01 (0.19)	0.95±0.02 (0.82)
Spinal cord	0.50±0.07 (0.36)	0.79±0.02 (0.75)
Pineal gland[a]	3.03±0.53 (0.71)	3.86±0.79 (1.04)
Anterior pituitary[a]	5.13±0.83 (0.23)	5.53±0.26 (0.30)
Posterior pituitary[a]	2.84±0.71 (0.43)	1.98±0.52 (0.50)
Peripheral tissues		
Thyroid gland	1.81±0.18 (0.47)	1.97±0.38 (0.64)
Adrenal gland	1.62±0.23 (0.61)	1.74±0.06 (0.83)
Testis	2.87±0.18 (1.09)	3.73±0.04 (1.24)
Thymus	1.18±0.19 (0.21)	2.21±0.43 (0.49)
Pancreas	2.50±0.42 (0.44)	11.92±2.50 (2.03)
Spleen	1.39±0.14 (0.18)	3.35±0.59 (0.39)
Lung	1.02±0.18 (0.51)	3.15±0.42 (1.77)
Heart	0.86±0.09 (0.63)	2.60±0.21 (1.67)
Kidney	0.45±0.06 (0.09)	19.83±4.81 (8.62)
Liver	1.13±0.21 (0.19)	2.39±0.29 (0.80)
Muscle	1.60±0.20 (0.58)	2.78±0.20 (1.71)
Physiological fluids		
Plasma	0.70±0.15 (0.68)	2.69±0.47 (3.55)
Urine	23.19±5.50 (25.94)	433.11±43.35 (95.09)

Values represent mean ± SE (nmol/g or mL) of 4 mice. [a]The amounts of D-Pro are shown as pmol/whole tissue, because the wet weights of these tissues are about 1 mg or lower. Values in parentheses represent %D (D / (D + L) x 100). Reproduced from ref. [61] with permission.

4.2. Origin of D-Pro in mammalian urine

In order to clarify the origin of large amounts of D-Pro observed in the urine of the ddY/DAO⁻ mouse, the effects of diet and intestinal bacteria have been investigated [61]. The ddY/DAO⁻ mice were starved for 4 days (only water containing 5% sucrose was allowed), and the amounts of urinary D-Pro were determined. As a result, the amount of D-Pro gradually decreased, however, a substantial amount of D-Pro was still observed after starvation for 4 days. The effect of intestinal bacteria was studied using ddY/DAO⁻ mice having a germ-free status. The ddY/DAO⁻ mice inoculated with two strains of bacteria (di-associated), and four strains of bacteria (tetra-associated) were also used. In the urine of the germ-free mice, a large amount of D-Pro was also present, and the amounts of D-Pro did not change after the
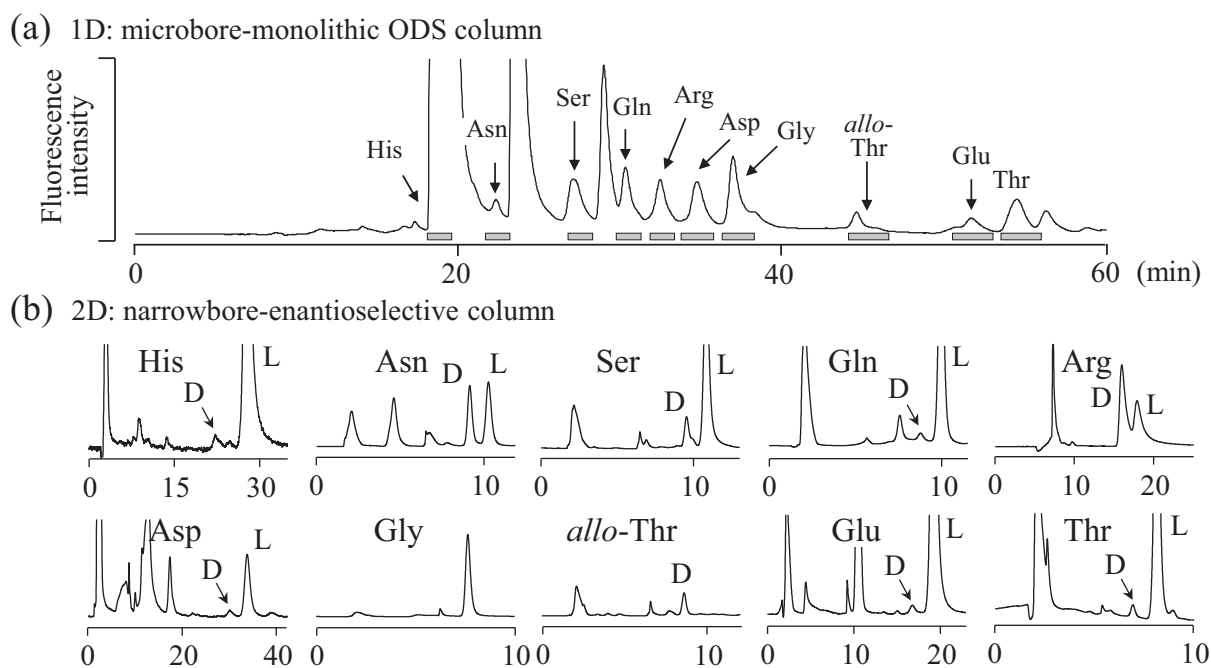
(a) 1D: microbore-monolithic ODS column

(b) 2D: narrowbore-enantioselective column

Fig. 8. 2D-HPLC chromatograms of hydrophilic D-amino acids in rat urine. (a) Reversed-phase separation of NBD-amino acids in the first dimension, and the fractions indicated by gray bars are introduced to the second dimension. (b) Enantiomer separation of NBD-amino acids in the second dimension. Reproduced from ref. [63] with permission.

inoculation of the bacteria. These results strongly indicated that a part of D-Pro was derived from the diet, and also indicated that the rest of the non-negligible amount was possibly produced in the mouse.

5. Enantioselective 2D-HPLC analysis of hydrophilic amino acids

As chiral amino acid metabolomics studies gained attention for the screening of novel physiologically active substances and biomarkers, several 2D-HPLC procedures for multiple amino acid enantiomers have been established [63,64]. For the determination of hydrophilic amino acids, Arg, Asn, Asp, Gln, Glu, His, Ser and Thr were selected as the targets. An achiral amino acid, Gly, and a stereoisomer of Thr, *allo*-Thr, were also determined. After the derivatization with NBD-F, these 10 amino acids were isolated using a long monolithic microbore ODS column, and the enantiomers were separated by a narrowbore Pirkle type enantioselective column (Sumichiral OA-2500S) followed by the determination using a fluorescence detector [63]. Fig. 8 shows the chromatograms of the 10 hydrophilic amino acids in the rat urine obtained by the enantioselective 2D-HPLC system. As shown in the chromatograms, all of the D-isomers of the selected hydrophilic amino acids were present in the rat urine. Especially, the amounts of D-Asn, D-Ser, D-Arg and D-*allo*-Thr were relatively high.

6. Enantioselective 2D-HPLC analysis of branched aliphatic amino acids

Branched aliphatic amino acids (Val, *allo*-Ile, Ile and Leu) are useful biomarkers of metabolic disorders of amino acids [65,66]. For the simultaneous determination of these hydrophobic amino acid enantiomers, a chiral 2D-HPLC system has been established [64]. A reversed-phase HPLC was used for the first dimensional separation of the NBD-derivatives of these amino acids. However, the separation of *allo*-Ile and Ile was difficult because they are structurally similar stereoisomers. The use of tetrahydrofuran was essential as an additive to the mobile phase of the reversed-phase HPLC. For the chiral separation in the second dimension, a cinchona alkaloid-based anion-exchange type enantioselective column (Chiralpak QN-AX, [67]) was used. By using the CSP, the enantiomers of all the branched aliphatic amino acids were separated within 10 min. In the rat urine, drastically high amounts of D-Val (37% of total Val in the urine), D-*allo*-Ile (almost 100% of *allo*-Ile), D-Ile (25% of Ile) and a trace amount of D-Leu (3% of Leu) were observed.

7. Conclusion

As described in the present article, various D-amino acids are indeed present in the higher animals, including humans. Some of the D-amino acids are trace substances in the tissues and physiological fluids, and the 2D-HPLC procedures are useful for their determination. The physiological roles, diagnostic meanings, origins and regulation of these D-amino acids in mammals are now being gradually clarified, and further biochemical and

clinical investigations together with further progress in analytical technologies are continuously required.

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