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Review

HPLC analysis of naturally occurring free D-amino acids in mammals

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ABSTRACT

D-Amino acids are currently recognized as naturally occurring physiologically active substances and biomarkers in mammals. The progress of analytical technologies, mostly high resolution chromatographic or electrodriven separation methods, has significantly contributed to the advances in D-amino acid research in real biological matrices. In this review, we would like to describe the D-amino acid research, from the discovery of appreciable amounts of free D-amino acids in mammals to the current metabolomics study focusing on amino acid enantiomers. The liquid phase enantioselective analytical methods utilized for the determination of D-amino acids in mammals including human beings will be discussed.

Contents

1. Introduction

D-Amino acids are the enantiomers of L-amino acids. Although all proteinogenic L-amino acids are abundantly present in living organisms, the amounts of their D-enantiomers are usually at trace levels, especially in the higher animals. However, along with the progress in sensitive and enantioselective analytical methods mostly depending on the chromatographic separation technologies, various D-amino acids were found even in mammals including humans [1]. These D-amino acids are frequently localized to specific tissues in relatively high concentrations, and they are expected as novel drug candidates and biomarkers [2,3]. In this review, we focus on the HPLC analysis of naturally occurring free D-amino acids in mammals.

2. Discovery of large amounts of D-amino acids in mammals

Before the early 1980s, most scientists believed that free D-amino acids are not present in the higher animals. D-Amino acids were often called "unnatural" amino acids, and they were thought to be the by-products of some chemical reaction or derived from the contamination of bacteria or other lower species. However, in 1986, Dunlop et al. discovered the presence of a large amount of D-aspartic acid (D-Asp) in the cerebrum of a newborn rat, and in the pituitary gland of an adult rat [4]. It was the first report to show the presence of appreciable quantities of free D-amino acids in mammalian tissues; the amounts of D-Asp were 164 nmol/g (8.4% of total Asp) in the cerebral hemisphere of the newborn rat, and 127 nmol/g (3.8% of total Asp) in the pituitary gland of the adult rat. In those days, it was unbelievable that such high amounts of D-amino acids were present in these higher animals. Therefore, the authors reported their results with the greatest care.

Concerning the methodology, they developed and utilized three different HPLC techniques. The first one was the use of a chiral

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Table 1
The concentration of D-aspartic acid and the %D in various tissues of young and adult rats.

	Young (36-h old)						
	Blood	Cerebral hemisphere	Cerebellum	Spinal cord	Pituitary	Liver	Kidney
D-Aspartic acid (nmol/g)	8.9	100	28	38	19	28	49
%D (D/(D+L) x 100)	16.0	5.1	1.9	2.0	3.8	0.9	1.4
	Adult (55-days old)						
D-Aspartic acid (nmol/g)	2.2	13	22	3.3	127	11	12
%D (D/(D + L) × 100)	4.5	0.43	0.79	0.13	3.8	1.0	0.5

Values are obtained from Ref. [4].

derivatization reagent, (+)-1-(1-naphthyl)ethyl isocyanate. Under alkaline conditions, the isocyanate reacts with amino acids to form naphthylethylcarbamoyl amino acids. With the use of enantiomerically pure isocyanate, the D- and L-amino acids are converted into the diastereomers, and then resolved on a reversed-phase column. The second one was the use of an optically reversed enantiomer reagent, (−)-1-(1-naphthyl)ethyl isocyanate, for preparing the derivatives. By using the (−) enantiomer as a chiral derivatizing reagent, the elution order of the diastereomers derived from the D- and L-amino acids was reversed, and the identity of naphthylethylcarbamoyl D-Asp was effectively verified. The third one was the use of the optically inactive derivatizing reagent, 1-naphthyl isocyanate. After the derivatization, naphthylcarbamoyl Asp was isolated by reversed-phase HPLC as a mixture of the D- and L-forms, and the fraction was then further resolved on another reversed-phase column saturated with L-aspartyl-L-phenylalanine methyl ester. This is a two-dimensional HPLC system combining a reversed-phase separation and an enantioselective separation using the chiral mobile phase technique. By adopting the above three different HPLC methods, the presence of D-Asp in mammalian tissue was definitely confirmed.

In this paper, Dunlop et al. reported that a high concentration of D-Asp was observed in the young rat with a specific tissue distribution. In the 36-h old rat, the highest concentration was observed in the cerebral hemisphere (100 nmol/g), followed by the kidney, spinal cord, and cerebellum (Table 1). In the 55-day old adult rat, the distribution of D-Asp was different from that of the young rat, and the highest amount was present in the pituitary gland (127 nmol/g), followed by the cerebellum and cerebral hemisphere. The concentration of D-Asp in the cerebral hemisphere decreased with the increasing age, and approached the adult value within one week. In the same paper, they also reported that the amount of D-Asp in the human blood obtained from a placental cord was much higher than that in the blood of an adult male, suggesting that the free D-Asp and its developmental changes are also relevant to humans.

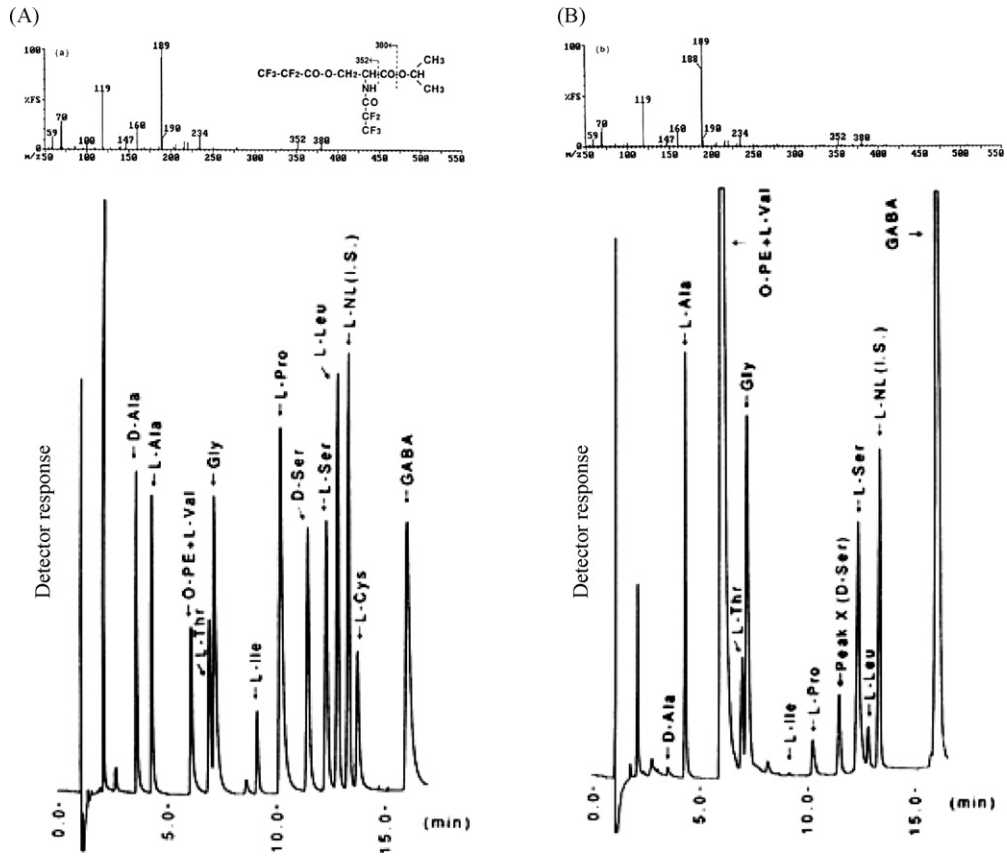


Fig. 1. Chiral GC chromatograms of standard amino acids (A) and amino acids in rat brain (B). The electron impact mass spectra of *N,O*-PFP-isopropyl derivative of authentic D-Ser and the peak X are also shown.
Reproduced from Ref. [5] with permission.

The next significant impact on the amino acid research is the finding of D-serine (D-Ser) in the mammalian brain. In 1992, an extremely high amount of D-Ser was found in the rat brain [5,6]. Concerning the methodology, a GC system with a Chirasil-L-Val chiral capillary column [7], and a reversed-phase HPLC system following the chiral derivatization [8] were used. For the GC analysis, the enantiomers of Ser were derivatized with isopropanol and pentafluoropropionic anhydride to form *N,O*-pentafluoropropionyl isopropyl derivatives. It was first thought that an unknown compound (in the paper, they indicated the unknown compound as peak X) was present in the rat brain extract which exhibited the same retention time as that of the authentic D-Ser. However, by adopting a GC–MS analysis, the electron impact mass spectrum of peak X was perfectly identical to that of the authentic D-Ser, which strongly support the identification that peak X is really naturally occurring D-Ser (Fig. 1).

For the HPLC analysis of the serine enantiomers, a chiral derivatization method using *o*-phthalaldehyde and *N*-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) was used. The diastereomers derived from D- and L-Ser were separated by an ODS column, and detected by a fluorescence detector at 443 nm with excitation wavelength at 344 nm. By this HPLC method, they also tested the rat brain extract and an extremely high level of D-Ser was again observed. The amount of D-Ser in the rat brain determined by the chiral GC analysis was 270 nmol/g, and the ratio of D-Ser to total Ser was 0.23. The amount of D-Ser determined by the reversed-phase HPLC analysis was 220 nmol/g, and the ratio of D-Ser to total Ser was 0.25. These values are almost consistent and thus, the presence of unexpectedly high levels of naturally occurring D-Ser (approximately 30% of L-Ser) was clearly demonstrated in the rat brain. At that time, exogenous D-Ser was known to be a potent co-agonist of the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor, and their findings had a significant influence on the neurochemical field. Today, it is widely accepted that D-Ser is an endogenous neuroactive D-amino acid in mammals.

3. Determination of D-amino acids in mammals using 1D-HPLC

After the discovery of large amounts of D-Ser and D-Asp in mammals, many researchers started investigations of naturally occurring D-amino acids in mammals. Most of the investigations were performed using one-dimensional HPLC methods by applying the chiral derivatization reagents or chiral stationary phases. In the following Section 3.1, we discuss the chiral derivatization reagents, and in Section 3.2. focus on the chiral stationary phases.

3.1. Determination of D-amino acids in mammals using chiral derivatization reagents

Chiral derivatization reagents in combination with reversed-phase HPLC methods have been widely used for the determination of D-amino acids in mammalian tissues and physiological fluids. Because the efficiency of a reversed-phase column is usually much higher than that of an enantioselective column, the separation of diastereomers by a reversed-phase column following the chiral derivatization of amino acids is favorably used. The use of an effective RP-HPLC system is also suitable to obtain high sensitivity due to the great theoretical plate number of the analytical column. Although chiral derivatization method has some limitations (determination of a trace amount of D-amino acid is difficult due to the enantiomeric impurity of the reagent, physico-chemical properties of the diastereomers are not equal), it is practically useful for the determination of relatively large amounts of D-amino acids. As described above, the discovery of D-Asp and D-Ser in mammals was also performed using the chiral derivatization methods [4,6]. Especially, the OPA reagent in combination with chiral thiol compounds is practically and widely used for the determination of D-amino acids in mammalian samples. Originally, the OPA chiral derivatization method was established by Aswad in 1984 using *N*-acetyl-L-cysteine (NAC) as a chiral thiol [9], and was investigated in detail by Buck [10] and Nimura [11]. OPA reacts with primary amino acids (but not secondary amino acids such as Pro) together with chiral thiol compounds within 2 min at room temperature to form diastereomeric isoindole derivatives (Fig. 2). These derivatives are highly fluorescent and are able to be determined by a sensitive fluorescence detector.

Concerning the application of the OPA-NAC method to mammalian samples, D'Aniello et al. determined D-Asp in the testis and pituitary gland of the rat, and a drastic increase in the D-Asp levels in these tissues after birth was demonstrated [12]. Kera et al. also used the OPA-NAC method, and found high levels of acidic D-amino acids (D-Asp and D-Glu) in the liver, kidney and brain of mature rats [13]. As the thiol compounds, various chiral thiols were reported [14–20]. An NAC analog, Boc-L-Cys, contributed to find out D-Ser in the mammalian brain [6], and was also used to clarify the distribution of D-Ser and D-Asp in the mammalian central nervous system and periphery [16–18]. Using Boc-L-Cys as a chiral thiol, the simultaneous determination of D-Ser, D-Asp and D-Ala was also performed, and the distribution of these D-amino acids in the mouse brain was demonstrated [19]. Brückner et al. reported *N*-isobutyryl-L-cysteine (IBLC) and its optically reversed analog, *N*-isobutyryl-D-cysteine (IBDC), as the thiol compounds [20]. By using a set of chiral thiols, the enantioselective analysis of 41 amino acid components could be accomplished, and various naturally occurring D-amino acids were found in mammalian serum and urine. In the serum of a human, dog and stallion, relatively large amounts of D-Asp (around 5% of total Asp) and D-Ser (around 2% of total Ser) were found, and in the urine, large amounts of D-Ser (60% of total Ser) and D-Ala (20% of total Ala) were found. Replacement of IBLC with IBDC results in reversal of the elution orders of the D- and L-amino acids, which is a powerful tool to confirm the presence of D-amino acids in mammals. By the reversal of the elution order, D-amino acids are distinguishable from various chemical interferences observed in real biological matrices.

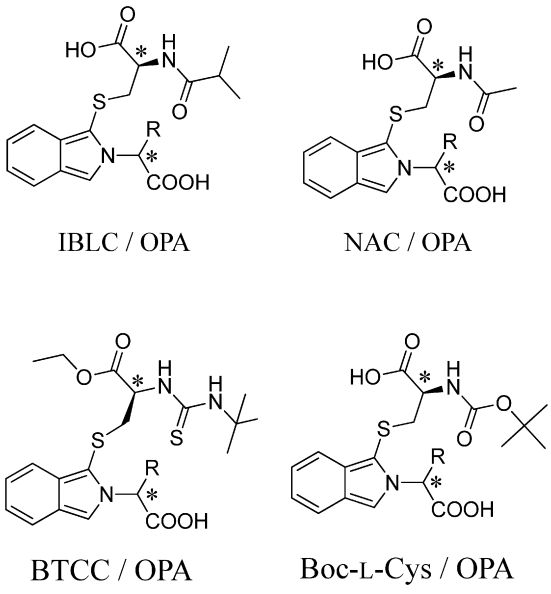


Fig. 2. Derivatization of amino acids with OPA plus chiral thiols.

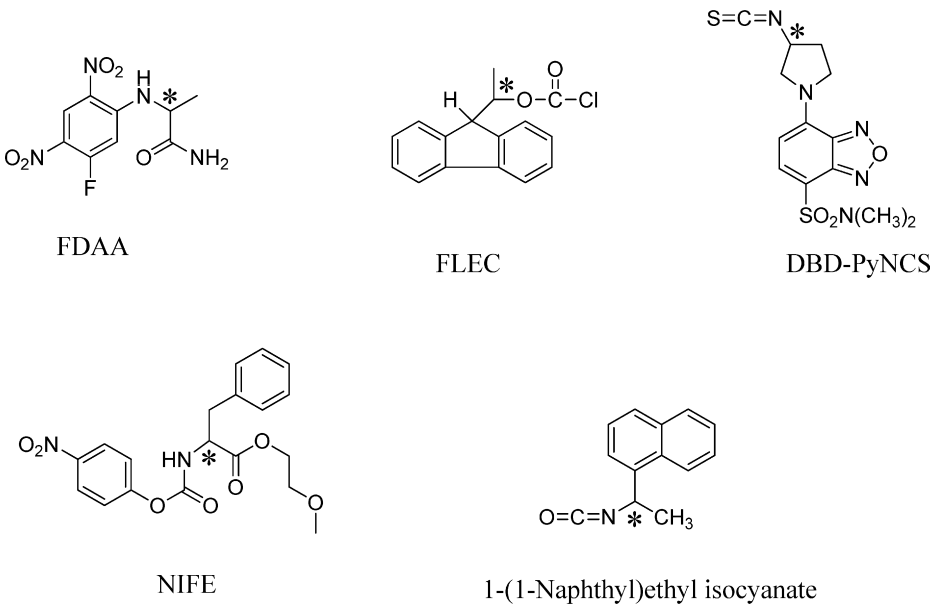


Fig. 3. Chiral derivatization reagents for amino acids.

Many other chiral derivatization reagents including the traditionally used 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent [21,22]) and (+ or −)-1-(9-fluorenyl)ethyl chloroformate (FLEC [23,24]) were also reported for the enantioselective determination of amino acids (Fig. 3). Today, the combination of diastereomer separation using reversed-phase HPLC and the highly selective determination using MS or MS/MS detectors has been established. Min and Toyo'oka et al. reported R(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (R(−)-DBD-PyNCS) as a chiral derivatization reagent, and the enantioselective determination of 18 amino acids was performed [25]. By using this method, D-Ala, D-Val, D-Pro, D-Ile and D-Leu were found in human nail samples and the relationship between the amounts of these D-amino acids and diabetes were demonstrated. (S)-*N*-(4-Nitrophenoxycarbonyl)-L-phenylalanine-2-methoxyethyl ester ((S)-NIFE) has also been reported as a sensitive chiral derivatization reagent for the RP-HPLC-MS/MS method [26]. By using this method, the enantiomers of 19 proteinogenic amino acids except Gly could be nicely separated, and relatively high levels of D-Ser, D-Asn, D-Ala and D-Phe were observed in human cerebrospinal fluid. Small amounts of D-Asp, D-Ser, D-Ala and D-Pro were found in human serum, while relatively large amounts of D-Asp, D-Ser, D-Asn, D-Ala, D-Arg and D-Met were found in human urine.

3.2. Determination of D-amino acids in mammals using chiral stationary phases

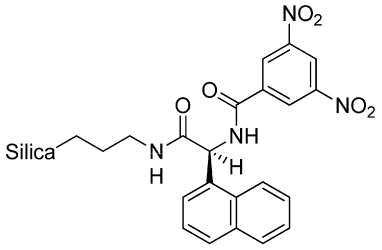
Chiral stationary phases are also used for the determination of D-amino acids in mammals. Normally, the separation efficiency of the chiral stationary phase is not sufficient for the determination of D-amino acids in real biological samples. However, the one-dimensional chiral HPLC method is applicable for several specific cases. In 1995, Imai et al. reported that an unbelievably high amount of D-Asp was present in the pineal gland of the adult rat [27]. The amount of D-Asp was around 1000 nmol/g tissue, and was higher than that of L-Asp in the tissue. In the study, the amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F [28]), and the enantiomers were separated using a Pirkle-type chiral stationary phase, Sumichiral OA-2500S [29]. By using this enantioselective column, the selective determination of NBD-Asp enantiomers could be performed. The presence of D-Asp was confirmed using the chiral stationary phase with inversed configuration and reversed elution order, Sumichiral OA-2500 R. The other Pirkle-type chiral stationary phase, Sumichiral OA-4600SS, was also reported to be suitable for the selective enantiomer determination of NBD-Ser [30]. By using the enantioselective column, NBD-D-Ser in the cerebrum of a bovine and rat could be determined. Recently, a highly sensitive and selective one-dimensional chiral HPLC-MS/MS method was reported by Reischl and Lindner et al. [31]. In the report, amino acids were derivatized with succinimidyl ferrocenyl propionate (SFP), and the enantiomers of the SFP-amino acids were separated by a cinchona alkaloid-based chiral stationary phase, Chiralpak QD-AX [32]. The structures of the chiral stationary phases are shown in Fig. 4. By using this method, the enantiomers of 19 amino acids were separated and selectively determined by MS/MS detection, and relatively high amounts of D-Ala, D-Lys, D-Ser, D-Thr and D-Ile were found in human urine.

4. Determination of D-amino acids in mammals using 2D-HPLC

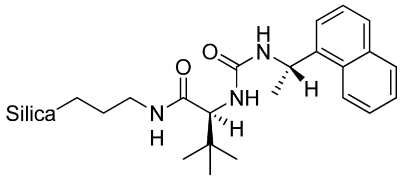
One-dimensional HPLC techniques have been widely utilized for the determination of D-amino acids in mammalian tissues and physiological fluids because of their simplicity and practically acceptable sensitivity in real biological samples. However, the determination of trace amounts of D-amino acids (about 1% of L-amino acids or lower) are difficult, because thousands or more interfering substances are present in real biological matrices. Therefore, the selectivity of the analytical method is a more important factor, and various two-dimensional HPLC methods as well as 1D-HPLC-MS/MS methods have been established. In the following Section 4.1, we will describe relatively simple 2D-HPLC procedures for the determination of a single D-amino acid, and the 2D-HPLC systems for multiple D-amino acids will also be explained in Section 4.2.

4.1. 2D-HPLC determination of a specific D-amino acid

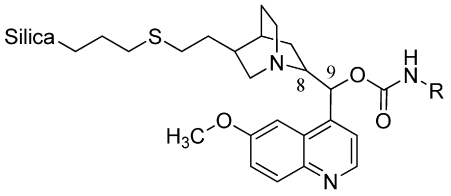
For the selective determination of trace amounts of D-amino acids in mammalian samples, various 2D-HPLC procedures have been established. Armstrong et al. reported a coupled-column HPLC



Sumichiral OA-2500S



Sumichiral OA-4600SS



QN; 8S 9R QD; 8R 9S
QN-1 and QD-1; R = *tert*-butyl
QN-2; R = diisopropylphenyl

Chiralpak QN-1-AX, QD-1-AX and QN-2-AX

Fig. 4. Chiral stationary phases used for the determination of amino acid enantiomers.

(2D-HPLC) system for the determination of Pro enantiomers using an ODS column and a β-CD bonded enantioselective column [33]. In this system, D- and L-Pro were isolated using an ODS column following the pre-column derivatization with 9-fluorenylmethyl chloroformate (Fmoc-Cl [34]), and a part of the Fmoc-Pro fraction was heart-cut and transferred to the next chiral stationary phase in which the D- and L-forms were separated. By using this 2D-HPLC system, a small amount of D-Pro was determined in human urine, plasma, cerebrospinal fluid and amniotic fluid. This method was also applied to the determination of D-Pro in the urine and plasma of rodents including rats and mice [35]. Armstrong et al. also developed a 2D-HPLC system [36] combining an ODS column and a chiral crown ether column, Crownpak CR(+) column [37]. In this system, Phe, Tyr, Trp and Leu were isolated by using an ODS column in their native forms, and the fraction was introduced to the enantioselective column to separate the enantiomers. Following the enantiomer separation step in the second dimension, the amino acids were post-column derivatized with OPA plus mercaptoethanol [38] in order to perform the sensitive fluorescence detection. A Crownpak CR(−) column was used to confirm the presence of D-amino acids in the real world samples. By using this procedure, relatively large amounts of D-Phe, D-Tyr and D-Trp were found in the urine of rodents, and a small amount of D-Phe was also found in their plasma. In the human physiological fluids, small amounts of D-Phe, D-Trp and D-Tyr were found in the urine and plasma, however, only D-Phe was found in the cerebrospinal fluid and amniotic fluid.

2D-HPLC procedures combining a reversed-phase column and a Pirkle-type enantioselective column following the sensitive fluorescence pre-column derivatization with NBD-F have also been reported. For the determination of Asp, Long and Homma et al. reported a column-switching method using an octyl-column and a Sumichiral OA-3100S column, and a small amount of D-Asp in the blood of the adult rat was successfully determined [39]. Fukushima et al. established a method combining an ODS column and the tandem series of two Sumichiral OA-2500S columns (4.6 mm ID × 500 mm (250 mm × 2)) for the determination of the NBD-Ser enantiomers [40]. By using the 2D-HPLC procedure, the sensitive and selective determination of D-Ser in the brain microdialysis sample obtained from the prefrontal cortex could be accomplished. Concerning the determination of a small amount of D-Ser, a more sensitive micro-2D-HPLC system was also established [41]. For the procedure, a microbore-monolithic ODS column (0.53 mm ID × 750 mm) was used for the isolation of NBD-Ser, and the total volume of the NBD-Ser fraction was transferred to the narrowbore-enantioselective column (Sumichiral OA-2500S, 1.5 mm ID x 250 mm) via a loop device (Fig. 5). By using the micro-2D-HPLC system, the detailed distribution of D-Ser in mice with various D-amino acid oxidase (DAO) activities (ddY/DAO$^{+/+}$ mouse, ddY/DAO$^{+/-}$ mouse and ddY/DAO$^{-/-}$ mouse [42]) was demonstrated. DAO is an enzyme which catalyzes the oxidation of D-amino acids to the corresponding α-keto acids [43], and as expected, the amount of D-Ser drastically increased in the cerebellum, medulla oblongata and kidney in the DAO$^{-/-}$ mice. This method could be applied to the determination of D-Ser in the brain of D-3-phosphoglycerate dehydrogenase (Phgdh, the enzyme that catalyzes the L-Ser synthesis via the phosphorylated pathway) knock-out mice, showing that the amount of D-Ser, not only L-Ser, decreased in these mice [44]. By using this method, a trace amount of D-Ser in the spinal cord [45] and that of extracellular D-Ser in the cerebellar slices [46] have also been successfully determined. Concerning naturally occurring D-amino acids other than D-Asp and D-Ser, a small amount of D-Ala in mammalian tissues was clearly determined by the similar 2D-HPLC system [41,47]. D-Ala is localized in the anterior pituitary gland [48] and in the pancreas [49], and the amounts show a clear circadian rhythm being higher in the daytime. By using the food-restricted rats, the circadian changes of D-Ala were shown to be closely related to the activity rhythms of the animals [50]. Small amounts of D-Leu and D-Pro were also selectively determined by the 2D-HPLC system [51–53]. D-Leu was localized in the pituitary gland and pineal gland in the mouse brain, and D-Pro was localized in the pituitary gland, pineal gland, pancreas and testis in the brain and periphery of the mouse. In the urine, a high concentration of D-Pro was found. Especially, 95% of the Pro excreted in the urine was the D-form in the ddY/DAO$^{-/-}$ mouse, and 25% of Pro was the D-form in the control ddY/DAO$^{+/+}$ mouse.

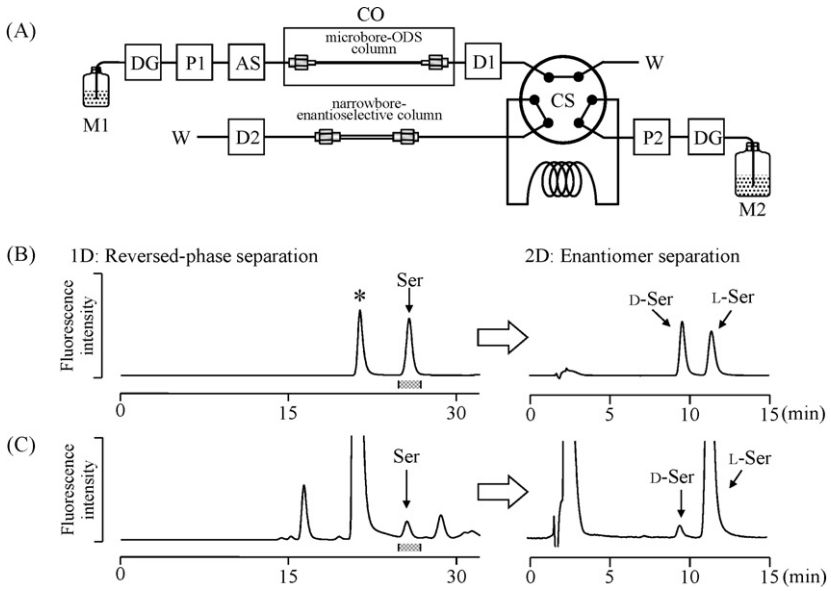


Fig. 5. 2D-HPLC separation of D- and L-Ser. (A); Flow diagram of the 2D-HPLC system, (B); separation of the standard DL-Ser, (C); separation of D- and L-Ser in the mouse serum.
Reproduced from Ref. [41] with permission.

4.2. Simultaneous 2D-HPLC determination of multiple D-amino acids

For the simultaneous two-dimensional determination of multiple D-amino acids in mammalian tissues and physiological fluids, a 2D-HPLC system equipped with a multi-loop valve device was developed. Prior to the HPLC determination, the amino acids were derivatized with a fluorescence derivatization reagent, NBD-F, for the sensitive analysis of the D-amino acids. In the first dimension, the NBD-amino acids were separated by a microbore-ODS column as their D plus L mixtures, and their elution was monitored by a fluorescence detector. The target amino acid fractions were then independently collected in the multi-loop device by appropriately switching the high-pressure 6-port valve and a multi-loop valve. These valves were automatically controlled and the fractions were continuously transferred to the enantioselective column representing the second dimension. As the enantioselective column, a Pirkle-type chiral stationary phase (Sumichiral OA-2500S) or cinchona alkaloid based chiral anion-exchange type stationary phases (Chiralpak QN-AX and prototype QN-2-AX) were used, and the values of D-amino acids were confirmed by the replacement of enantioselective columns to their optically inversed ones.

By using the multi-loop 2D-HPLC system, simultaneous determination of the acidic amino acid enantiomers (Asp and Glu) was performed [54]. In the 7 brain areas and 11 peripheral tissues, the highest amount of D-Asp (higher than 2000 nmol/g tissue) was observed in the pineal gland of rats [55], and relatively high amounts of D-Asp were observed in the pituitary gland, adrenal gland, testis spleen and lung (higher than 100 nmol/g tissue). Although an appreciable amount of D-Asp was present in mammalian brain and periphery, the amount of D-Glu was extremely small. The highest amount of D-Glu was observed in the thymus (9 nmol/g tissue), and the amounts were much lower than those of D-Asp in all the tested samples, indicating that these two acidic D-amino acids are recognized and handled differently in mammalian body. The widely known neuroactive D-amino acids (D-Ser and D-Ala [56,57]) could also be determined by the simultaneous 2D-HPLC procedure [58]. By using this procedure, enzymatic regulation of these two D-amino acids in the 7 brain areas, 4 peripheral tissues, plasma and urine was demonstrated. As the enzyme controlling the amount of D-amino acids in mammals, DAO was selected, and the change in these D-amino acid amounts was described using rats with various DAO activities. As a result, the amount of D-Ala drastically increased in all the tested tissues and physiological fluids in the rats having no DAO activity (LEA/Sen rats), however, the amount of D-Ser did not change in the frontal brain areas. LEA/Sen rats would be a nice model animal with increasing levels of D-Ser and D-Ala in their tissues.

More complex 2D-HPLC procedures for analyzing the enantiomers of Pro and 2 Pro analogs [59], 4 branched aliphatic amino acids [60] and 10 hydrophilic amino acids [61] have also been established. Concerning the Pro analogs, D- and L-Pro, the enantiomers of *trans*- and *cis*-4-hydroxyproline were selected as the target analytes [59]. For the enantiomer separation, a cinchona alkaloid-based chiral stationary phase, Chiralpak QN-2-AX (a prototype chiral stationary phase having diisopropylphenylcarbamoyl quinine moiety as a chiral selector) was used. By using the method, D-Pro, L-Pro and *trans*-L-hydroxyproline were found in the mouse serum and skin. Concerning the branched aliphatic amino acids, Val, *allo*-Ile, Ile and Leu were selected, and a multi-loop 2D-HPLC system has also been established [60]. For the enantiomer separation, a cinchona alkaloid-based chiral stationary phase, Chiralpak QN-AX (having *tert*-buthylcarbamoyl quinine moiety as a chiral selector) was used. As a result, relatively large amounts of D-Val, D-*allo*-Ile and D-Ile were observed in the rat urine. Especially, only the D-form was present in the NBD-*allo*-Ile fraction. The simultaneous determination of hydrophilic D-amino acids including Ser analogs (Ser, Thr and *allo*-Thr) and Asp analogs (Asp, Glu, Asn and Gln) is an important task for the metabolomics study focusing on D-amino acids, and therefore, a multi-loop 2D-HPLC system for 10 hydrophilic amino acids including all of these analogs has been established [61]. As the target amino acids, His, Asn, Ser, Gln, Arg, Asp, Gly, *allo*-Thr, Glu and Thr were selected. A long microbore-monolithic ODS column (0.53 mm ID × 1000 mm) was used for the first dimension, and a narrowbore-Pirkle-type enantioselective column, Sumichiral OA-2500 S (1.5 mm ID × 250 mm) was used for the second dimension. Replacement of OA-2500 S with an inversed configuration chiral stationary phase, OA-2500R, enables the powerful confirmation of finding D-amino acids in mammalian samples. By using the method, D-Ser, D-Asp, D-*allo*-Thr and D-Thr were found in the cerebrum, and

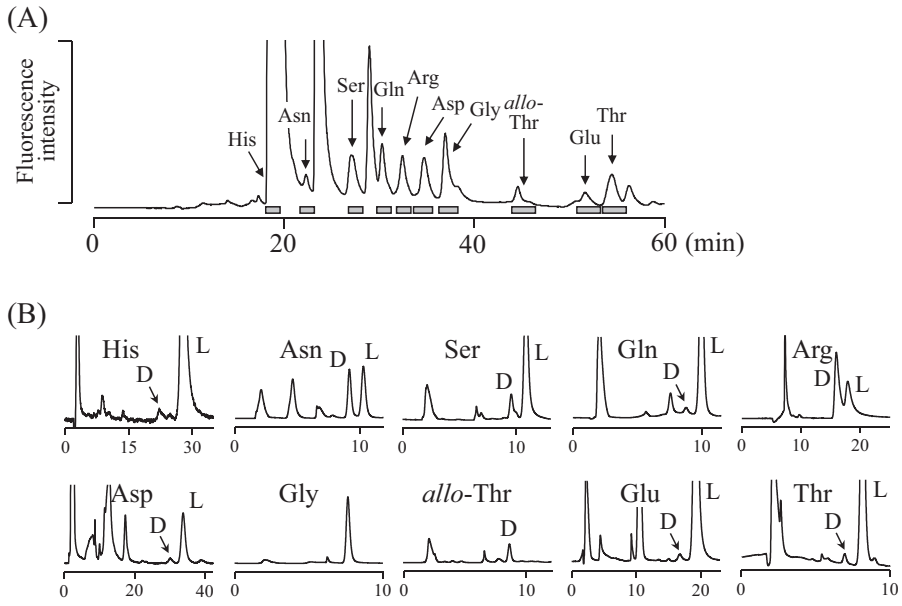


Fig. 6. Simultaneous 2D-HPLC separation of hydrophilic amino acid enantiomers in the rat urine. (A); 1D, reversed-phase separation using microbore-monolithic ODS column, (B); 2D, chiral separation using narrowbore-enantioselective column.
Reproduced from Ref. [61] with permission.

D-Ser and D-Asp were observed in the plasma of the adult rat. In the urine, D-enantiomers of all the tested hydrophilic amino acids were observed in remarkably high amounts for D-Asn and D-Arg (Fig. 6).

5. Conclusion

Free D-amino acids are currently widely accepted as naturally occurring substances, and a variety of free D-amino acids have been found in mammals including human beings. However, the research studies on small amounts of D-amino acids in real biological matrices largely depend on the progress of the selective and sensitive analytical methodologies, and the development of a highly effective analytical method is still expected. Along with the advances in analytical chemistry, the physiological meanings and diagnostic values of various D-amino acids would surely be clarified in the near future, and will contribute to the human life sciences.

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