Focusing Review

Multi-Dimensional HPLC Analysis of Metabolic Related Chiral Amino Acids -Method Development and Biological/Clinical Applications-

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Abstract
The existence of D-amino acids in mammals has been increasingly reported, and clarified that D and L enantiomers have different biological functions, distributions and metabolic pathways. Some D-amino acids were also reported to have a correlation with diseases, and the screening of biomarkers based on quantitative enantioselective metabolomics is highly expected. However, the amounts of most D-amino acids, especially the metabolic-related chiral amino acids, are usually at trace levels in biological samples. Therefore, highly selective and sensitive analytical methods are essential for their accurate determination. In this focusing review, multi-dimensional high-performance liquid chromatographic (HPLC) systems for the precise determination of metabolic-related chiral amino acids are introduced with their application to biological samples. For the highly-sensitive determination, target amino acids were precolumn-derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The multi-dimensional HPLC systems were composed of a reversed-phase column in the 1st dimension and an enantioselective column in the 2nd dimension. By using these systems, various proteinogenic amino acids were clearly observed in the tissues and physiological fluids of rodents. The existence of metabolic-related chiral amino acids in various biological samples were also elucidated.

Keywords: D-Amino acids; Multi-dimensional HPLC; Enantioselective determination; Metabolic-related amino acids

1. Introduction

Besides being the components of peptides and proteins, amino acids play various biological functions, such as neurotransmission and hormone modulation, in living creatures. Although most amino acids have D and L enantiomers, it has long been considered that only L-form was incorporated into those biological functions in higher animals [1]. However, along with the advances in enantioselective chromatographic techniques, the existence of various D-amino acids in mammals, even in humans, has gradually been elucidated [2-7]. Concerning the D-forms of some proteinogenic amino acids, the differences in the relations to diseases [8-12] and the functions [13-17] from those of the L-amino acids have also been clarified. Therefore, these D-amino acids are increasingly recognized as the candidates of novel physiologically-active substances and biomarkers.

In addition to the proteinogenic amino acids, there are various metabolic-related amino acids that play diverse roles in living organisms. Most of these non-proteinogenic amino acid enantiomers are usually present at much lower levels than the D-forms of the proteinogenic amino acids. Hence, the determinations of these metabolic-related chiral amino acids in biological samples are easily disturbed by interfering compounds, and their precise detection and quantification are extremely difficult in many cases. The enantio-discriminated distributions and content changes with diseases of metabolic-related amino acids are still mostly unknown, and highly sensitive and selective analytical methods for getting new insights through the enantioselective metabolomics are expected.

For the enantioselective amino acid analysis in biological samples, various analytical methods have been reported.

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Regarding capillary electrophoresis, the methods with a buffer containing chiral additives, typified by β-cyclodextrin, enable the direct analysis of amino acid enantiomers using small amounts of reagents and samples [18,19]. The GC systems with a chiral capillary column, such as Chirasil-L-valine [20-22], and HPLC methods with chiral derivatization reagents, as represented by 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey’s reagent) [23,24], (+ or −)-1-(9-fluorenyl)ethyl chloroformate (FLEC) [25,26] and o-phthaldialdehyde (plus chiral thiols) [27-29], are traditionally and widely used for the simultaneous determination of various amino acid enantiomers with relatively short analysis times. However, these methods are only one-dimensional separation systems, and sometimes difficult to apply to the determination of trace amounts of amino acid enantiomers in complex biological samples with the lack of sensitivity and selectivity even using a mass spectrometer as the detector. The detection/identification and precise quantification of small amounts of substances, such as peptides and amino compounds, the precise detection and quantification of various amino acid enantiomers in complex biological samples with the lack of sensitivity and selectivity even using a mass spectrometer as the detector. The detection/identification and precise quantification of small amounts of substances, such as peptides and amino compounds, the precise detection and quantification of various amino acid enantiomers in complex biological samples with the lack of sensitivity and selectivity even using a mass spectrometer as the detector.

2. Chiral 2D-HPLC analysis of proteinogenic amino acids

Due to the presence of a great variety of interfering substances, such as peptides and amino compounds, the precise detection and quantification of small amounts of D-amino acids in biological samples are often difficult. To solve the selectivity issues, two-dimensional-HPLC (2D-HPLC) methods combining reversed-phase and enantioselective columns are some of the most powerful options. In this review, we focus on the chiral multi-dimensional HPLC analysis of amino acids including metabolic related ones in various biological samples.

For the highly-sensitive determination, the target amino acids were pre-column derivatized with NBD-F (Fig. 1). The derivatization reaction proceeded at 60°C for 2 min in the presence of an alkaline buffer solution, then an aliquot was injected into the 2D-HPLC system after adding an aqueous TFA solution to terminate the labeling reaction. For the highly-selective determination, 2D-HPLC systems combining reversed-phase separation and enantioselective separation were designed and developed (Fig. 2). The NBD-derivatized target amino acids were first separated from the interference compounds as D and L mixtures by the reversed-phase column and fractionated respectively in the 1st dimension. Each fraction was then introduced to the enantioselective column in the 2nd dimension and the D- and L-forms were separated and detected by a fluorescence detector. The structures of typical chiral selectors introduced in this review are shown in Fig. 3.

![Flow diagram of the 2D-HPLC system](image)

**Fig. 2.** Flow diagram of the 2D-HPLC system. AS, auto sampler; CO, column oven; D, detector; HPV, high pressure valve; P, pump; W, waste.

![Structures of typical chiral selectors](image)

**Fig. 3.** The structures of typical chiral selectors introduced in this review.

By using the 2D-HPLC system described above, the presence of the D-forms of various proteinogenic amino acids in mammals has been clarified. D-Ser and D-Ala have been reported to exist at relatively high concentrations in the central nervous system and both are known as the modulator of the N-methyl-D-aspartic acid (NMDA) receptor-mediated neurotransmission [13,32-34]. For the simultaneous 2D-HPLC determination of D-Ser and D-Ala,
a monolithic ODS column (0.53 mm i.d. x 750 mm) was used as the reversed-phase column in the 1st dimension. NBD-derivatized Ser and Ala were nicely separated with water-acetonitrile gradient elution. Concerning the enantiomer separation in the 2nd dimension, a Pirkle-type column, Sumichiral OA-2500S (1.5 mm i.d. x 250 mm), gave baseline separations with mixed solutions of methanol and acetonitrile containing citric acid as the mobile phase. The system was applied to the brain and peripheral tissues, plasma and urine of mice and rats, and a wide distribution of D-Ser and D-Ala in the mammalian body was unveiled [35,36]. As for D-Ser, a very high amount (around 300 nmol/g) was found in the cerebral cortex and the hippocampus; the hypothalamus and the olfactory bulb also contained a relatively high amount of D-Ser (100-200 nmol/g). On the other hand, the highest levels of D-Ala were found in the pancreas of mice (about 100 nmol/g) and in the pituitary gland of rats (about 50 nmol/g). The presence in the whole frontal brain areas (around 5 nmol/g for each tissue) was also elucidated.

Concerning the acidic amino acids, a wide distribution of D-Asp in the endocrine tissues of mammals has been reported where the D-amino acid is involved in the synthesis/secretion of various hormones [14,17]. As for Glu, however, most of its issues are still not known except for its existence in the urine of various creatures [21,37]. The D-enantiomers of both Asp and Glu are known to have some effects on neuronal transmission [38], and metabolized by the same enzyme, D-aspartate oxidase [39]. Likewise the method for D-Ser and D-Ala, the 2D-HPLC system was composed of a monolithic ODS column (0.53 mm i.d. x 750 mm) and a Pirkle-type column, Sumichiral OA-2500S (1.5 mm i.d. x 250 mm) [40]. In the 1st dimension, NBD-Asp and NBD-Glu were nicely separated from other proteinogenic amino acids in 60 min with the isocratic elution of the aqueous solution containing 8% acetonitrile and 0.05% TFA. In the 2nd dimension, a sufficient enantiomer separation and also separation from interfering substances were provided by using a mixed solution of methanol and acetonitrile (50/50, v/v) containing 2 mM citric acid as the mobile phase. With the 2D-HPLC system, the intrinsic amounts of Asp and Glu enantiomers in the tissues and physiological fluids of rats were reported. The pineal gland was found to contain quite high amounts of D-Asp (more than 2000 nmol/g) and relatively high levels of D-Asp were observed in the endocrine tissues such as the pituitary and adrenal glands (around 100 nmol/g for each tissue). Concerning D-Glu, its detailed distribution in mammals was also revealed. Except in the urine, the intrinsic amount of D-Glu was quite low in the central nervous system and also in the peripheral tissues.

D-Pro is mentioned as one of the D-amino acids widely reported to exist in the plasma and urine of mammals [41-43]. Moreover, its intrinsic amount in the plasma was shown to have a correlation with a renal disorder [12,44]. 2D-HPLC systems with a reversed-phase column and a Pirkle-type column, Sumichiral OA-2500R (4.6 mm i.d. x 500 mm), or an anion-exchange type column, Chiralpak QN-2-AH (1.5 mm i.d. x 150 mm) were reported for the enantioselective determination of Pro [45,46]. The system was applied to the tissues and physiological fluids of mice, and the highest amount of D-Pro was found in the urine of mice (23.19 nmol/mL). A wide distribution of D-Pro was elucidated, although the amounts in most of the tissues and physiological fluids were very low (less than 5 nmol/g).

The chiral 2D-HPLC systems for the branched aliphatic amino acids and hydrophilic amino acids have also been reported [47,48]. As for the branched aliphatic amino acids (Val, Leu, Ile and allo-Ile), the reversed-phase separation of the NBD-amino acids was demonstrated with the mobile-phase containing tetrahydrofuran. For the enantiomer separation, an anion-exchange type column, Chiralpak QN-AX, was used due to its ability to separate all the target enantiomer pairs within 10 min. By using this system, a precise determination of their enantiomers in the rat urine was achieved [47]. In addition to the existence of D-Val and D-Leu in the urine, a relatively high amount of D-allo-Ile in excess of that of D-Ile was observed. Concerning the hydrophilic amino acids, the system targeting 10 amino acids including an achiral amino acid, Gly, was designed [48]. With the established system, the contents of the target amino acid enantiomers in the cerebrum, plasma and urine of rats were elucidated. In the cerebrum, D-Ser, D-Asp, D-Thr and D-allo-Thr were found, and in the plasma, D-Ser and D-Asp were present. On the other hand, the D-enantiomers of all the target chiral amino acids (His, Asn, Ser, Gln, Arg, Asp, allo-Thr, Glu and Thr) were observed in the urine.

3. Chiral 2D-HPLC analysis of NMDA analogues

NMDA is one of the non-proteinogenic metabolic-related amino acids well-known as an agonist of the NMDA subtype of the glutamate receptor [49]. NMDA has also been reported to regulate some hormonal releases such as the growth hormone, in addition to its role as a neuromodulator [50,51]. It had been thought that NMDA was an artificial neuroactive amino acid, however, its existence in S. broughtonii was first reported in 1987 [52]. With that as a start, the presence of NMDA and its analogues including the enantiomer, N-methyl-L-aspartic acid (NMLA), in various living organisms has been revealed using enantioselective chromatographic techniques [50-55]. However, their reported amounts are quite different even in the same species, and the precise determination method for the quantitative analysis of
naturally-occurring NMDA analogues in biological samples is expected.

For the accurate determination of NMDA and its analogues, NMLA, N-methyl-D-glutamic acid (NMDG) and N-methyl-L-glutamic acid (NMLG), a 2D-HPLC system combining a monolithic ODS column and an enantioselective column was designed [56]. Concerning the reversed-phase separation in the 1st dimension, a microbore monolithic ODS column (0.53 mm i.d. x 1000 mm) was used due to its ability to separate NMA and NMG as their NBD-derivatives at a relatively low pressure. As for enantioselective separations, various Pirkle-type columns (Sumichiral OA-2000S, OA-2500S, OA-2500R, OA-3100S, OA-3200S, OA-3300S, OA-4100SR, OA-4500SR, OA-4600SS, OA-4700SR and OA-4900SR, 1.5 mm i.d. x 250 mm) and anion exchange-type columns (Chiralpak QN-AX, QD-AX, QN-2-AX and QD-2-AX, 1.5 mm i.d. x 150 mm) were checked. Among these columns, Sumichiral OA-2500S and OA-2500R columns having 1-naphthyl glycine as the chiral selector, and Chiralpak QN-AX column having the quinine derivative as the chiral selector gave practically sufficient separations. The developed 2D-HPLC system was comprised of a microbore monolithic ODS column (0.53 mm i.d. x 1000 mm) and a Sumichiral OA-2500S column (1.5 mm i.d. x 250 mm). Acetonitrile-TFA-water (6/0.05/95, v/v/v) and 1.5 mM citric acid in a mixed solution of methanol-acetonitrile (50/50, v/v) were used as the mobile phases for the 1D and 2D, respectively.

By using the 2D-HPLC system, precise determination without severe interference in a biological sample, the mantle of *S. broughtonii*, was demonstrated (Fig. 4). A 2D-HPLC system with an ESI-tandem mass spectrometer (2D-HPLC-MS/MS) having a superior selectivity was also developed, and the presence of NMDA analogues in the mantle of *S. broughtonii* was clearly elucidated (Fig. 5). The intrinsic amounts of NMDA analogues in the mantle and foot of bivalves were clarified by these systems and are summarized in Fig. 6. A considerably high amount of NMDA (170.1 nmol/g) and modicum levels of NMG enantiomers (NMDG 17.0 nmol/g, NMLG 11.0 nmol/g) were found in the mantle of *S. broughtonii*. *M. lusoria* contained NMDA (29.3 nmol/g) and NMLG (13.8 nmol/g), whereas *R. philippinarum* contained only one analogue, NMLG (2.6 nmol/g). On the other hand, none of the target NMDA analogues were observed in the rat tissues and plasma, in which NMDA had been reported to exist.

Fig. 4. 2D-HPLC separations of NMDA analogues in the mantle of *S. broughtonii*. The fractions indicated by the closed bars were collected on-line and automatically introduced to the 2nd dimension, with enantiomer separation using enantioselective columns.

Fig. 5. 2D-HPLC-MS/MS determination of NMDA analogues in the mantle of *S. broughtonii*.

Fig. 6. Amounts of NMDA analogues in the mantle and foot of bivalves. Values represent means ± SE of 3 bivalves. n.d., not detected.
4. Chiral 2D-HPLC analysis of metabolic-related aromatic amino acids

Phenylalanine (Phe), tyrosine (Tyr) and 3,4-dihydroxyphenylalanine (DOPA) are aromatic amino acids, and in the mammalian body, Tyr and DOPA are both biosynthesized from Phe [57]. As for Phe and Tyr, they are well known for their relation to some metabolic disorders represented by phenylketonuria [58]. Concerning DOPA, the L-enantiomer has been widely used in the treatment of Parkinson’s disease. DOPA is also known as the precursor of some neurotransmitters, and metabolized to dopamine, noradrenaline and adrenaline [57].

The presence of the Phe and Tyr enantiomers in mammals has been partially elucidated using various chiral analytical methods [59,60], however, little is known about DOPA and also about their content changes under disease conditions because of the difficulty in their precise analysis in biological samples.

For the accurate, precise and simultaneous determination of Phe, Tyr and DOPA enantiomers, a 2D-HPLC system was developed consisting of a monolithic ODS column and an enantioselective column [61]. Regarding the reversed-phase separation with a microbore monolithic ODS column (0.53 mm i.d. x 1000 mm), the linear gradient elution with the acetonitrile concentration gave nice separation of the three target amino acids with sharp peak shapes. Concerning the enantiomer separations, several Pirkle-type columns (Sumichiral OA-2500S, OA-3100S, OA-3200S, OA-4100SR, OA-4700SR and KSAACSP-001S, 1.5 mm i.d. x 250 mm) and an anion exchange-type column (Chiralpak QN-AX, 1.5 mm i.d. x 150 mm) gave a substantial enantiomer separation for all three target amino acids. Among them, KSAACSP-001S column having N-(3,5-dinitrophenylaminocarbonyl)-L-Leu as the chiral selector showed the highest theoretical plates using 0.4% formic acid in a mixed solution of methanol-acetonitrile (60/40, v/v) as the mobile phase.

By using the above mentioned 2D-HPLC system, Phe and Tyr enantiomers were nicely separated from interfering substances and clearly detected in the urine of mice. To clarify the enantioselective metabolism of the metabolic-related aromatic amino acids, their content differences with a D-amino acid oxidase (DAO) deficit were demonstrated using the control C57BL mice and mutant DAO deficient B6DAO⁻ mice (Fig. 7). DAO is an enzyme metabolizing almost all D-amino acids other than the acidic ones, and it has been reported that changes in its enzymatic activity cause some neurodegenerative diseases such as amyotrophic lateral sclerosis and schizophrenia [11,62]. The D and L enantiomers of Phe and Tyr were found in the urine of both the control mice and DAO deficient mice. The %D values of Phe were 9.65% for C57BL mice and 30.28% for B6DAO⁻ mice. Regarding Tyr, the %D values were 4.45% for C57BL mice and 10.06% for B6DAO⁻ mice. While there was no significant difference in the %D values of Tyr, the %D values of Phe increased more than three-fold with the absence of DAO activity and showed significant difference from the control mice. As for the DOPA enantiomers, peaks were not detected in both mice groups. The intraperitoneal administration of the DOPA enantiomers was performed using the control mice, and only D-DOPA was observed in the urine after the administration.

![Fig. 7. 2D-HPLC separation of Phe and Tyr enantiomers in the urine of the control mouse and DAO deficient mouse.](image)

5. Chiral 2D-HPLC analysis of citrulline and ornithine

Citrulline (Cit) and ornithine (Orn) are the well-known non-proteinogenic and metabolic related amino acids included in the urea cycle [63]. Their L-enantiomers are related to various functions, such as vasodilation or anti-fatigue effects, and utilized as food additives and supplements [64,65]. Moreover, without discriminating the enantiomers, their content in the urine and plasma have been used as the biomarkers of urea cycle disorders [58]. However, likewise the metabolic-related amino acids described in the previous chapters, the enantioselective distributions, functions and metabolic pathways of these amino acids have scarcely been elucidated except for the intrinsic amounts of Orn enantiomers in the urine of rodents [59]. Therefore, a highly-sensitive and selective analytical method for the accurate determination of the intrinsic Cit and Orn enantiomers is a matter of concern in the chiral metabolomics.

For the enantioselective and precise analysis of Cit and Orn in biological samples, a 2D-HPLC system was reported [66]. As for the reversed-phase separation with a monolithic ODS column (0.53 mm i.d. x 1000 mm), a linear
gradient elution using an aqueous acetonitrile mixture provided sufficient separation of the NBD-derivatized target amino acids from other proteinogenic ones within 180 min. Regarding the enantiomer separation of NBD-Cit, all the investigated Pirkle-type columns, Sumichiral OA-2500S, KSAACSP-001S, KSAACSP-102S and KSAACSP-105S (1.5 mm i.d. x 250 mm) and anion-exchange type columns, Chiralpak QN-AX and QD-AX (1.5 mm i.d. x 150 mm), gave sufficient resolution factors (the Rs values are about 2.0 or higher). These columns, except for the QN-AX column, also provided an adequate enantiomer separation of NBD-Orn. The columns were further evaluated with the mouse urine, and the KSAACSP-105S column having N-(3,5-dinitrobenzoyl)-3-(2-naphthyl)-L-Ala) as the chiral selector was used in terms of the separation from interfering substances in the biological samples. A mixed solution of methanol and acetonitrile (90/10, v/v) containing 0.1% formic acid and a mixed solution of methanol and acetonitrile (50/50, v/v) containing 0.5% formic acid were used as the mobile phases for the enantiomer separations of Cit and Orn, respectively.

The intrinsic amounts of Cit and Orn enantiomers in the control C57BL mice and mutant DAO deficient B6DAO- mice were also reported by using the 2D-HPLC system described above. These results were useful to elucidate their enantioselective metabolism. In the urine of both the control and DAO deficient mice, all of the 4 target Cit and Orn enantiomers were found (Fig. 8). The 2D-HPLC-MS system equipped with a QDa detector was also developed for a higher selective determination, and the enantiomers of Cit and Orn were nicely demonstrated without interferences by monitoring the negatively charged ions; m/z = 337 for NBD-Cit and m/z = 457 for NBD-Orn (Fig. 9). The enantioselective intrinsic amount of Cit was first clarified by the 2D-HPLC systems. The %D values of Cit were 12.5% for C57BL mice and 43.5% for B6DAO- mice, and the values significantly increased with the DAO deficiency. As for Orn, the %D values for C57BL mice and B6DAO- mice were 40.5% and 61.8%, respectively, and also had a tendency to increase with the absence of the DAO activity.

6. Conclusions
This review summarizes the multi-dimensional HPLC analyses of various chiral amino acids mostly focusing on our recent works. The systems have a high selectivity and sensitivity, and are useful for the accurate determination of trace amounts of amino acid enantiomers in complicated biological matrices. As introduced in this review, various D-amino acids, including non-proteinogenic ones, were clearly elucidated to be present in living organisms. The multi-dimensional HPLC systems are expected to contribute to human health/wellness through the clarification of enantio-discriminated distributions, functions and diagnostic values of amino acids.

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