Multi-Dimensional High-Performance Liquid Chromatographic Determination of Chiral Amino Acids and Related Compounds in Real World Samples

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Abstract
Chiral amino acid analysis, especially the determination of trace levels of D-enantiomers, is currently gathering attention in a variety of research areas including the food/clinical sciences. These D-amino acids had long been believed to be absent in the higher animals. However, by the advances of analytical technologies, some of the D-enantiomers are found in mammals including humans and increasingly recognized as novel physiologically-active substances and/or biomarkers. For the determination of these D-amino acids and related compounds in real world samples, utilization of sensitive and selective methods is essential and multi-dimensional HPLC is one of the straightforward approaches. In the present review, two/three-dimensional HPLC methods and biological/medical applications focusing on our current studies are summarized.

Keywords: Chiral separation; Multi-dimensional HPLC; Amino acids; Hydroxy acids

1. Introduction
All proteinogenic amino acids, except for glycine (Gly), have the chiral carbon at the alpha position, and D/L enantiomers are chemically present. Whereas, in the living organisms on the Earth, a high excess of the L-enantiomers is observed and their antipodes, D-amino acids, had long been believed to have no biological significance especially in the higher animals. However, along with the progress of analytical technologies, especially by the advances of the highly sensitive and chiral separation methods, various D-amino acids are found in mammals including human beings [1-3]. Some of the D-amino acids are indicated to have crucial roles in the regulation of neuronal and hormonal systems [4,5]. The relationships between these D-amino acids and various diseases are also being increasingly clarified [1,5]. However, the amounts of D-amino acids in the higher animals are trace in most cases, and the precise determination of these D-enantiomers is difficult due to the presence of uncountable intrinsic molecules. Therefore, the development/utilization of highly sensitive and selective methods is essential for D-amino acid analysis and multi-dimensional high-performance liquid chromatography (HPLC) is one of the straightforward approaches. In the present article, two-dimensional (2D) and three-dimensional (3D) HPLC methods mainly for the analysis of proteinogenic amino acids and hydroxy acids are summarized, and applications in various real world matrices including clinical/biological/extraterrestrial samples are described. Concerning the multi-dimensional analysis of non-proteinogenic amino acids, please refer to the following review [6].

2. 2D-HPLC analysis of proteinogenic amino acid enantiomers as their NBD derivatives
For the determination of amino acid enantiomers, high sensitivity is required. Because the amino acids normally do not have chromophores and fluorophores, precolumn derivatization for one of the functional groups is useful. 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) is a fluorescence derivatization reagent for primary and secondary amino groups [7], and the resultant NBD-amino acids have intense fluorescence. Concerning the separation issues, the alpha amino group is blocked by the NBD moiety, and the NBD-amino acids are separated as the corresponding fluorescent chiral anions (Fig. 1). For the chiral separation of NBD-amino acids, Pirkle-type enantioselective columns are reported to be effective [8].
However, the determination of D-amino acids are frequently interfered due to the co-elution of other amino acids and/or unknown intrinsic compounds. To overcome these co-elution problems, utilization of other separation modes is a useful strategy, and in the 2D-HPLC concept, the reversed-phase separation is performed prior to the chiral separation. In the first step to design the chiral 2D-HPLC method, conventional size reversed-phase and enantioselective columns are adopted [9]. For the first dimension, an ODS column (J’sphere ODS-M80, 4.6 mm ID x 250 mm, YMC, Wilmington, NC, USA) is used at 35°C with the mobile phase containing trifluoroacetic acid (TFA), tetrahydrofuran (THF), acetonitrile (MeCN) and water. Using the gradient elution of MeCN, 18 proteinogenic amino acids, except for tryptophan (Trp) and cysteine (Cys), are separated within 100 min. Because NBD-F is a non-chiral fluorescence derivatization reagent, the NBD-D- and NBD-L-amino acids are also enantiomers. Therefore, these NBD-amino acids are separated as their scalemic D plus L mixtures by the reversed-phase separation, and all 18 peaks are collected. As an example, a chromatogram obtained when analyzing the rat pineal gland is shown in Fig. 2. After the collected fractions are evaporated to dryness under reduced pressure, the residues are dissolved using 1% acetic acid in methanol (MeOH), and subjected to the enantiomer separation using Pirkle-type columns.

As the enantiomer separation representing the second dimension, Sumichiral OA-2500S and R columns (4.6 mm ID x 250 mm, Sumika Chemical Analysis Service, Osaka, Japan) are used at 25°C. These columns are the Pirkle-type ones having N-3,5-dinitrobenzoyl-L or D-naphthylglycine as their chiral selectors [10-13], and the enantiomers of NBD-amino acids are nicely separated. Figure 3A shows the chromatogram obtained using the standard D/L-alanine (Ala), proline (Pro) and aspartic acid (Asp). All of these 6 amino acid enantiomers are nicely separated within 60 min. Figure 3B shows the chromatogram analyzing the fractions obtained from the rat pineal gland. The fractions of Ala, Pro and Asp are mixed and injected together into the Sumichiral 2500R column. Although the D forms are not observed for Ala and Pro, the D-Asp peak is clearly observed. Quantification of the amino acid enantiomers is carried out by the measurement of total amounts of the amino acids (as their D plus L forms) and the proportions of the D forms. By using the 2D-HPLC concept, non-negligible levels of D-Ser, D-Asp, D-Ala and D-leucine (Leu) are found in a variety of rat tissues.

3. Enantioselective on-line micro 2D-HPLC analysis of specific amino acid

By using the off-line and conventional size 2D-HPLC, the presence of various D-amino acids has been clarified [9]. However, for the quantification of the D-amino acids, the above mentioned off-line method requires the determination of the target amino acid in the first dimension (as the D plus L form), and also requires the determination of the D/L ratio in the second dimension. Therefore, for the accurate

![Diagram](https://via.placeholder.com/150)

**Fig. 1.** Precolumn derivatization of amino acid enantiomers with NBD-F.

![Diagram](https://via.placeholder.com/150)

**Fig. 2.** Reversed-phase separation of proteinogenic amino acids in the rat pineal gland as their NBD derivatives. Amino acids are indicated by their single letter abbreviations.

![Diagram](https://via.placeholder.com/150)

**Fig. 3.** Enantiomer separations of Ala, Pro and Asp as their NBD-derivatives. (A) Chromatogram obtained for authentic racemic mixtures of Ala, Pro and Asp. (B) Ala, Pro and Asp fractions obtained from the rat pineal gland indicated in Fig. 2 are mixed and analyzed.

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Chromatography 2020, 41, 1-17
determination, the target amino acid enantiomers should be completely separated from other compounds both in the first and second dimensions. However, separation of the target amino acids is frequently disturbed by the co-elution of various intrinsic substances, thus the direct quantification of amino acid enantiomers in the second dimension is highly expected. In order to realize the direct quantification of amino acid enantiomers in the second dimension, an on-line micro 2D-HPLC system using the whole fraction transfer concept has been designed/developed [14]. For the whole fraction transfer, the volume of the target fraction should be reduced in the second dimension to avoid the mobile phase incompatibility issues. Therefore, a micro ODS column was used in the first dimension, and a wider size (conventional or narrowbore) enantioselective column is used in the second dimension.

For the determination of Leu enantiomers, a micro 2D-HPLC system has been developed [14]. As the first dimension, a micro ODS column (Mightysil RP-18 GP, 1.0 mm ID x 100 mm, Kanto Chemical, Tokyo, Japan) is used at 35°C with the mobile phase of MeCN/THF/TFA/water = 32.5/1/0.02/66.5 (50 µL/min). For the second dimension, a Sumichiral OA-2500S column (4.6 mm ID x 250 mm) is used at 35°C with the mobile phase of 2 mM citric acid in MeOH at the flow rate of 0.8 mL/min. The flow diagram is shown in Fig. 4. Following the pre-column derivatization with NBD-F, Leu is separated by a micro-ODS column (position A). When the peak of NBD-Leu is observed, the column selection unit (CS) is changed and the sample collection loop is connected to the micro-ODS column (position B). After the elution of the whole NBD-Leu fraction is completed, the CS position is again changed from B to A, and the target fraction is transferred into the second dimension (enantioselective column). Figure 5A shows the chromatogram obtained for the rat hippocampus, and the trace level of D-Leu is nicely observed without interference. On the other hand, Fig. 5B shows the chromatogram analyzing the same sample without using the 2D-HPLC (directly injected into the enantioselective column); various intrinsic compounds are eluted around the target amino acid enantiomers, and the precise determination is practically difficult. By using the on-line 2D-HPLC system, trace levels of D-Leu are observed in a variety of brain tissues.

Using the same concept, the 2D-HPLC methods for D-Pro [15] and D-Ala [16] have been developed. For the determination of D-Pro, a Mightysil RP-18 GP column (1.0 mm ID x 100 mm) is used at 35°C as the first dimension with the mobile phase of MeCN/THF/TFA/water = 15/1/0.02/84 (50 µL/min). For the second dimension, a Sumichiral OA-2500R column (4.6 mm ID x 250 mm) is used at 35°C with the mobile phase of 5 mM citric acid in MeOH (1.2 mL/min). By using the 2D-HPLC system, the presence of small amounts of D-Pro (0.1 - 1% of total Pro) is demonstrated in various brain areas of the mice. Because the amounts of D-Pro in the mouse brain are extremely low, the presence of D-Pro is confirmed by the enzymatic degradation by D-amino acid oxidase (DAO). DAO catalyzes the oxidative deamination of neutral D-amino acids, and D-Pro is one of the best substrates. Therefore, by the treatment of DAO, the amounts of D-Pro is considered to be reduced. As shown in Fig. 6A, a small amount of D-Pro in mouse pituitary gland is clearly observed without DAO treatment. On the other hand, only the peak of D-Pro disappears after the treatment of DAO (Fig. 6B), indicating that D-Pro is actually present in the tissue.

Concerning D-Ala, a Mightysil RP-18 GP column (1.0 mm ID x 100 mm) is also used at 40°C as the first dimension with the mobile phase of MeCN/THF/TFA/water = 10/1/0.02/89 (50 µL/min). For the second dimension, a Sumichiral OA-2500S column (4.6 mm ID x 250 mm) is used at 40°C with the mobile phase of 5 mM citric acid in
MeOH (0.8 mL/min). Figure 7 shows the chromatogram obtained using a rat cerebrum sample. In the first dimension (A), the peak of NBD-Ala is collected as the D plus L mixture (as indicated by a closed bar), and injected into the second dimension. In the second dimension (B), the enantiomers of NBD-Ala are separated and a trace amount of D-Ala is clearly observed. Using the 2D-HPLC method, the distribution of D-Ala in the whole rat body is clarified. As shown in Fig. 8, among the 10 brain/neuronal tissues and 12 peripheral tissues, the highest amount of D-Ala is observed in the anterior pituitary gland, and the second highest amount is observed in the pancreas. To confirm the presence of D-Ala in the tissues, replacement of the enantiomeric Sumichiral OA-2500S column with the OA-2500R column is performed. By using the enantiomeric column having the opposite chiral center, the elution order of the D- and L-amino acids is reversed. Figure 9 shows the chromatograms analyzing D- and L-Ala in the rat pancreas using both Sumichiral OA-2500S and 2500R columns. As shown in the Figs. 9A and 9B, the elution order of the two peaks (D-Ala and L-Ala) is clearly reversed, and the presence of trace levels of D-Ala in the rat tissues are nicely confirmed.

In the pituitary gland and pancreas, and also in the plasma, the amounts of D-Ala have clear circadian changes [17]. In the two tissues and in the plasma of normal rats, the circadian profiles of D-Ala are similar; increasing in the daytime and decreasing in the nighttime (Fig. 10A). In order to clarify the reasons underlying these circadian changes, the activity rhythms of the rats are changed. Normally, rats are animals with nocturnal habits. However, by keeping these animals under restricted feeding conditions, the rats with diurnal habits can be made. In these rats with diurnal habits, the circadian changes of D-Ala are totally reversed, increasing in the nighttime and decreasing in the daytime (Fig. 10B). These circadian changes are also observed in mice [18] and humans [19], suggesting that D-Ala has some universal functions in mammals. In order to clarify the biological significance of D-Ala in the pituitary gland and in the pancreas, the cellular localization of D-Ala in these tissues has been examined. For the immunohistochemical staining, a mouse monoclonal antibody against D-Ala is raised, and cellular localization is identified by the double staining technique.
Fig. 10. Circadian changes of D-Ala amounts in the anterior pituitary gland (AP, circles), pancreas (PN, diamonds) and plasma (PL, triangles). The values represent means ± SE of three animals, and the asterisks indicate significant changes ($P < 0.01$) from the values at 13:00. (A) Normal rats with nocturnal habits; (B) rats with the diurnal habits made by restricted feeding.

Fig. 11. Immunofluorescence staining of D-Ala in the rat pancreas and double staining of D-Ala with insulin, glucagon, somatostatin and pancreatic polypeptide in a rat Langerhans islet.

Fig. 12. Immunofluorescence staining of D-Ala in the rat pituitary gland and double staining of D-Ala with adrenocorticotropic hormone, growth hormone, prolactin, gonadotropin hormone and thyroid-stimulating hormone in the anterior lobe.
In the rat pancreas, intense staining is observed only in the Langerhans islets. The Langerhans islet mainly contains 4 principal cells secreting insulin, glucagon, somatostatin and pancreatic polypeptide. By the immunofluorescence double staining, D-Ala is clearly demonstrated to be present only in the β-cells secreting insulin (Fig. 11). Cellular localization of D-Ala in the rat pituitary gland is also identified by the immunofluorescence double staining [21]. In the pituitary gland, intense staining is observed in the anterior lobe (the result is consistent with that obtained by the 2D-HPLC method [16]). The anterior pituitary gland contains 5 types of cells secreting growth hormone, adrenocorticotropic hormone (ACTH), gonadotropic hormone, prolactin, and thyroid-stimulating hormone. Figure 12 shows the double staining results indicating that D-Ala is present in the ACTH secreting cells. Insulin and ACTH are the hormones regulating the blood glucose level. Considered together with the results that D-Ala has clear circadian changes controlled by the activity rhythm, D-Ala is suggested to have relevant functions in blood glucose regulation.

4. Micro 2D-HPLC analysis of multiple amino acid enantiomers

The 2D-HPLC analysis is practically useful for the determination of chiral amino acids in real world samples, and the relationships between the structurally/metabolically related amino acids frequently provide biological/clinical insights. A variety of micro 2D-HPLC methods for multiple chiral amino acids have thus been designed/developed. In order to analyze multiple chiral amino acids, a multi-loop 2D-HPLC system has been designed (Fig. 13). In the first dimension, NBD-amino acids are separated by a reversed-phase column, and the target fractions are collected in the respective loops of the multi-loop valve. The fractions are then successively transferred to the second dimension, and the D/L forms are separated by the enantioselective column. For the D-amino acids related to the neuronal regulation, D-Ser and D-Ala should be simultaneously analyzed [22,23]. As the first dimension, a microbore-monolithic ODS column (0.53 mm ID x 750 mm, a capillary monolithic ODS column, designed by the collaboration with Shiseido, Tokyo, Japan) is used at 40°C. In order to separate/fractionate both NBD-Ser and Ala, the gradient elution is performed using the mobile phase of 5% MeCN 0.05% TFA in water and 20% MeCN 0.05% TFA in water. After separating these target amino acids, the fractions are on-line transferred to the second dimension, where a Sumichiral OA-2500S column (1.5 mm ID x 250 mm, 25°C) is connected. For the mobile phases, 3 mM citric acid in a mixed solution of MeOH/MeCN (25/75) is used for separating the NBD-Ser enantiomers, and 4 mM citric acid in a mixed solution of MeOH/MeCN (50/50) is used for separating the NBD-Ala enantiomers. By using these conditions, NBD-Ser and Ser are similar, Gln enantiomers should be separated from Ser enantiomers in the second dimension.

Fig. 13. Flow diagram of the 2D-HPLC system for the enantioselective determination of multiple amino acids. P, pump; C1, reversed-phase column; C2, enantioselective column; CO, column oven; D, detector; R, integrator; HPV, high-pressure valve; W, waste.

Fig. 14. On-line 2D-HPLC separation of Ser and Ala enantiomers as their NBD derivatives. (A) First dimension; (B) second dimension. Amino acids are indicated by their single letter abbreviations. Because the retention times of NBD-Gln and Ser are similar, Gln enantiomers should be separated from Ser enantiomers in the second dimension.
dimension, and a Pirkle-type enantioselective column, KSAACP-001S (1.0 mm ID x 250 mm, particle size 5 µm, having N,3,5-dinitrophenylaminocarbonyl-L-Leu as a chiral selector, designed by the collaboration with Shiseido) is used as the second dimension [23]. The mobile phases for both dimensions are thoroughly tested and mobile phase compatibility issues are negligible as shown in Fig. 14.

The simultaneous on-line chiral 2D-HPLC analysis of acidic amino acids, namely, aspartic acid (Asp) and glutamic acid (Glu), has also been reported [24,25]. For the reversed-phase separation, a microbore-monolithic ODS column (0.53 mm ID x 750 mm) is used at 40°C with the mobile phase of 8% MeCN 0.05% TFA in water. For the chiral separation, a narrowbore Sumichiral OA-2500S column (1.5 mm ID x 250 mm) is used at 25°C with the mobile phase of 2 mM citric acid in a mixed solution of MeOH/MeCN (50/50). Under these conditions, NBD-Asp and Glu are separated within 60 min in the first dimension, and their enantiomers are separated within 80 min in the second dimension. By using the present 2D-HPLC system, the distributions of D-Asp and D-Glu in the tissues/physiological fluids of rats [24] and mice [25] are demonstrated.

For the determination of branched aliphatic amino acid (BCAA) enantiomers, a Capcell pak C18 MG II column (1.0 mm ID x 150 mm, Shiseido) is used at 40°C with 25% THF 0.05% TFA in water as the mobile phase [26]. For the second dimension, a Chiralpak QN-AX column or a Chiralpak QD-AX column (4.0 mm ID x 150 mm, prototype, materials are from Chiral Technologies Europe) is used. These columns have a quinine (QN) or quinidine (QD) tertiarybutylcarbamate moiety as chiral selectors [27-29], and the NBD-amino acid enantiomers are effectively separated. By using this method, the NBD derivatives of valine (Val), isoleucine (Ile), allo-Ile and Leu are separated within 60 min in the first dimension, and their enantiomers are rapidly separated in the second dimension within 10 min.

The 2D-HPLC method is successfully applied to the analysis of the BCAA enantiomers in the rat tissues and physiological fluids. Concerning Ile, both the alpha and beta positions are chiral carbons, and the conversion of the alpha carbon of L-Ile results in the formation of D-allo-Ile. Therefore, in the biological matrices, the amounts of D-allo-Ile are higher than that of D-Ile (both of the alpha and beta carbons of L-Ile should be converted to form D-Ile). Similar trends are observed for threonine (Thr), and the amounts of D-allo-Thr are higher than those of D-Thr in the rat tissues and physiological fluids [30].

As for the Pro analogs, D/L-Pro and 4-hydroxyproline (4-Hyp) are present in mammals. Concerning 4-Hyp, D- and L-enantiomers of both trans and cis forms exist. Therefore, for the simultaneous analysis of Pro and Hyp, 6 isomers should be analyzed [31]. As the first dimension, a microbore-monolithic ODS column (0.53 mm ID x 500 mm) is used at 40°C. For the mobile phase, gradient elution using 4% MeCN 0.02% TFA and 8% MeCN 0.02% TFA in water is used. As the second dimension, a Chiralpak QN-2-AX column or a Chiralpak QD-2-AX column (1.5 mm ID x 150 mm, prototype, materials are from Chiral Technologies Europe) is used. These columns have a QN/QD diisopropylphenylcarbamate moiety as the chiral selectors. Using these conditions, 3 target NBD-amino acids are separated in the first dimension within 50 min, and their enantiomers are separated in about 10 min.

The simultaneous and enantioselective analysis of hydrophilic amino acids [32] is practically useful because several physiologically active D-forms, such as Asp, Ser, Ala and Glu, are included. For the reversed-phase
separation of hydrophilic amino acids as their NBD derivatives, a microbore-monolithic ODS column (0.53 mm ID x 1000 mm) is used as the stationary phase. As the target amino acids, arginine (Arg), asparagine (Asn), Asp, glutamine (Gln), Glu, histidine (His), Ser, Thr, allo-Thr and the nonchiral amino acid, Gly, are selected. The simultaneous and complete separation of these 10 amino acids are practically difficult, and the separation conditions are thoroughly investigated including the MeCN, TFA concentrations and column temperature. As a result, 6% MeCN 0.06% TFA in water is used as the mobile phase, and the separation is performed at 45ºC. Under these conditions, the target 10 NBD-amino acids are separated within 60 min; all of the 10 fractions are collected in the multi-loop device and successively transferred to the second dimension. For the chiral separations of these NBD-amino acids, a narrowbore Sumichiral OA-2500S column (1.5 mm ID x 250 mm) is used at 25ºC. Concerning the mobile phases, mixed solutions of MeOH and MeCN containing citric acid are used, and the target enantiomers are separated within about 30 min. Figure 15 shows the chromatograms obtained using a rat urine sample. As shown in the figure, all of the D-forms of the 9 target amino acids are observed in the rat urine, and their origin and biological significance are expected to be clarified.

5. Enzymatic regulation of intrinsic D-amino acids in mammals

Since the D-amino acids are now gathering attention as physiologically active substances and/or biomarkers, the regulation of these D-forms in the tissues and physiological fluids are the subjects of interest. In mammals, D-amino acids are metabolized by DAO and D-aspartic acid oxidase (DDO). The amounts of several D-amino acids are measured in rodents lacking these enzymes. DAO is an enzyme degrading neutral D-amino acids [33], and the changes in the amounts of intrinsic D-amino acids including Ala [22,34], Leu [15], Phe [35], Pro [15,36] and Ser [22,34] are demonstrated. The amounts of D-Ala significantly increase in all the brain regions, peripheral tissues, plasma and urine of both mice and rats lacking DAO activity. However, in the same animals, the amounts of D-Ser significantly increase only in the limited tissues/regions/fluids such as cerebellum, medulla oblongata, kidney, serum/plasma and urine. Figure 16 shows the amounts of D-Ala in the brain and periphery of the DAO−/− mice, and Fig. 17 shows those of D-Ser in the same mice. D-Ala is mainly produced by intestinal bacteria, and the alteration of the plasma/serum D-Ala level strongly affects those in the whole body [37]. On the other hand, D-Ser is mainly produced by an enzyme, i.e., Ser racemase.

![Fig. 16. Amounts of D-Ala in the tissues and physiological fluids of DAO+/+ (open bars), DAO+/− (gray bars) and DAO−/− (closed bars) mice. Values represent mean±SE (nmol/g wet tissue or mL) of 3-5 mice. CER, Cerebral cortex; HIP, hippocampus; OLF, olfactory bulb; HYP, hypothalamus; CEL, cerebellum; MED, medulla oblongata; PIT, pituitary gland; LIV, liver; PAN, pancreas; KID, kidney; SER, serum; URI, urine. **P < 0.01, significant increase from the values of DAO+/+ mice.](image1)

![Fig. 17. Amounts of D-Ser in the tissues and physiological fluids of DAO+/+ (open bars), DAO+/− (gray bars) and DAO−/− (closed bars) mice. Values represent mean±SE (nmol/g wet tissue or mL) of 3-5 mice. **P < 0.01, significant increase from the values of DAO+/+ mice. Abbreviations are the same as those in Fig. 16.](image2)
Therefore, in the frontal brain regions where D-Ser is produced, high levels of D-Ser are observed. In contrast, in the hindbrain regions where DAO is expressed, the amounts of D-Ser significantly change by the difference in the DAO activity. Concerning Leu, higher amounts of D-Leu are observed in the whole brain regions and also in the serum of the DAO−/− mice [15]. On the other hand, the amounts of D-Pro are mostly the same between the two strains (DAO−/− mice and control DAO+/+ mice) in all the brain and peripheral tissues [15,36], although extremely high levels of D-Pro are observed in the urine of the DAO−/− mice (20 times higher than those in the DAO+/+ mice). In the urine of DAO−/− mice, D-forms of several other proteinogenic amino acids, such as Phe and Tyr [35], and also non-proteinogenic amino acids, such as citrulline and ornithine, are likely to increase [38].

Acidic D-amino acids (D-Asp and D-Glu) are metabolized by DDO [39]. In the mice lacking DDO, the amounts of D-Asp significantly increase in all of the brain regions and peripheral tissues [40]. In the physiological fluids (plasma and urine), the amounts of D-Asp also significantly increase in the DDO−/− mice compared to those in the control DDO+/+ mice (Fig. 18). Considering the substrate specificity of DDO, D-Glu is also a nice substrate for DDO like D-Asp. However, in the tissues and physiological fluids of DDO−/− mice, the amounts of D-Glu are almost the same as those in the DDO+/+ mice. These results suggest that the origin and regulation of these two acidic D-amino acids are different, and further studies are expected.

As the enzyme synthesizing D-amino acids in mammals, Ser racemase has been reported [41,42]. Ser racemase catalyzes the conversion from L-Ser to D-Ser, and from D-Ser to L-Ser. Because L-Ser is abundant in mammalian tissues, the enzyme works to synthesize D-Ser in the tissues. In the brain of SRR+/+ mice having high SRR activity, large amounts of D-Ser are present in the frontal brain including the cerebral cortex and hippocampus [43]. In contrast, the amounts of D-Ser are low in the whole brain regions in the mice lacking SRR activity, indicating that D-Ser is truly biosynthesized by an enzyme, SRR, in our body.

6. Application of the 2D-HPLC analysis of amino acid enantiomers to clinical, food and extraterrestrial samples

The 2D-HPLC systems are practically useful for the screening of new biomarkers and/or physiologically active D-amino acids. For the biomarker screening, the relationships between the D-amino acids and several diseases including amyotrophic lateral sclerosis (ALS) and renal failure have been reported [44-46]. In the spinal cord of the ALS model mice, the amounts of D-Ser significantly increase compared to those in the control mice, and the alteration is associated with the progress of the disease [44]. In the serum and urine of acute kidney injury (AKI) model mice, drastic changes in the D-Ser amounts are observed [45]. In the serum, the amounts of D-Ser gradually increase after renal ischemia-reperfusion injury (IRI), whereas, the amounts of L-Ser rapidly decrease. In the urine, large amounts of D-Ser are observed before IRI (twice higher than those of L-Ser). However, the amounts reduce immediately after IRI and the amounts of L-Ser increase, resulting in the drastic change in the D/L ratio. In the case of human chronic kidney disease (CKD) patients, the levels of several D-amino acids including Ser, Pro and Ala significantly increase along with the progression of the disease [46]. These results indicate the potential of D-amino acids as the new biomarkers of renal failure including AKI and CKD and further clinical evaluations are highly expected.

D-Amino acids are also gathering attention as the physiologically active substances in mammals. In the cerebellum, D-Ser is clearly demonstrated to regulate cerebellar long-term depression (LTD) through the glutamate receptor [47]. The LTD is the key to obtain the cerebellar memory and improve the athletic ability, and D-Ser would be an effective supplement for cerebellar function. D-Amino acids are associated with the mucosal defence [48]. Gut microbiota produce high levels of D-Ala, D-Asp, D-Glu and D-Pro and some of them are good substrates of DAO. Oxidative deamination of these D-amino acids by intestinal DAO results in the production...
of H₂O₂ that can protect the mucosal surface from pathogens.

Concerning other applications, the 2D-HPLC methods can be used for the enantioselective determination of amino acid residues in peptides [49]. Some of the peptides produced by microorganisms contain D-amino acids and also non-proteinogenic amino acids. Lysoclin E, a new antibiotic peptide, contains the D-form residues of Arg, Gln and Trp. Also, a non-proteinogenic amino acid, D-N-methylphenylalanine, is observed. In food samples, D-amino acids are frequently observed in the fermented products. In the Japanese traditional black vinegar (amber rice vinegar, Kurozu), high levels of D-Ala, D-Asp, D-Glu, D-Leu and D-Ser are observed [50]. The developmental changes of these D-amino acids are specific to the respective amino acids, and the relationships between their amounts and fermentation/aging processes are expected to be clarified. The 2D-HPLC system is also applicable to the determination of extraterrestrial amino acids [51]. As the target, non-proteinogenic chiral amino acids (2-aminobutyric acid, 3-aminobutyric acid, 3-aminoisobutyric acid, norvaline and isovaline) are also selected, because the contamination from the terrestrial environment is always the problem for proteinogenic amino acids. As a result, significant amounts of non-proteinogenic chiral amino acids are observed in the carbonaceous chondrite. The D/L ratios of these non-proteinogenic amino acids are almost racemic, and their origins and relation to the terrestrial life are expected to be clarified.

7. 2D HPLC-MS/MS analysis of chiral amino acids

Combining the reversed-phase separation and the enantioselective separation by using 2D-HPLC is effective for the selective determination of intrinsic chiral amino acids. However, the practical selectivity of the 2D-HPLC separation is sometimes insufficient depending on the complexity of the target matrices. Therefore, the development of higher selective methods has been expected to accelerate further investigations about the physiological meaning of trace levels of the intrinsic D-amino acids. To have higher analytical selectivity, the connection of additional dimensions is one of the suitable approaches. Based on this concept, an enantioselective 2D-HPLC is on-line connected with a triple quadrupole mass spectrometer (MS/MS). The developed system is composed of the reversed-phase separation (first dimension), the enantioselective separation (second dimension) and the filtering of target ion pairs by a multiple reaction monitoring mode of MS/MS (third and fourth dimensions). As the target, Ala, Asp, Glu, Leu, Pro and Ser are selected because their D-forms are frequently observed in biological samples.

The developed system is applied to the simultaneous determination of chiral amino acids in human urine and plasma; the most commonly used samples as clinically obtained matrices [52]. The results for a healthy human urine sample is shown in Fig. 19. After NBD-derivatization, urinary amino acids are isolated from interfering compounds by the hydrophobicity using a C18 column (KSAARP, 1.0 mm ID x 500 mm, Fig. 19A). The isolated fractions containing the target analytes are automatically collected in the multi-loop device, then transferred to the next dimension by changing the valve position. The enantioselective separations are nicely performed by an original Pirkle-type column (KSAACSP-001S, 1.5 mm ID x 250 mm, Fig. 19B). The isolated fractions containing the target analytes are automatically collected in the multi-loop device, then transferred to the next dimension by changing the valve position. The enantioselective separations are nicely performed by an original Pirkle-type column (KSAACSP-001S, 1.5 mm ID x 250 mm, Fig. 19C). The isolated fractions containing the target analytes are automatically collected in the multi-loop device, then transferred to the next dimension by changing the valve position. The enantioselective separations are nicely performed by an original Pirkle-type column (KSAACSP-001S, 1.5 mm ID x 250 mm, Fig. 19C).
samples are quite sufficient without using internal standards. This might be because the 2D HPLC-MS/MS method is mostly matrix-effect-free by virtue of the 2D-LC separation. In human urine, the obtained concentrations of the target L-enantiomers and D-enantiomers are 4.01-407.16 and 0.06-249.52 µmol/L, respectively. The ratios of the D-enantiomers to the respective D- plus L-amino acids are 19.21% for Ala, 8.05% for Asp, 21.74% for Glu, 1.64% for Leu, 0.60% for Pro and 38.00% for Ser. The results indicate that the 2D-LC separation is a powerful tool for the highly selective determination of intrinsic chiral amino acids. It has been clarified that the amounts of several inherent D-amino acids change along with the progress of diseases [45,46], hence, further applications to discover novel biologically significant D-amino acids using the 2D HPLC-MS/MS are eagerly expected.

Japanese traditional black vinegar (amber rice vinegar) and nam pla (fish source) are selected and the chiral amino acids in these foodstuffs are analyzed by the 2D HPLC-MS/MS system [53]. The chromatograms obtained using the black vinegar are shown in Fig. 20. Accordingly, the target amino acids are clearly detected and determined without any interfering peaks (Fig. 20C) in all the tested samples. The amounts of the amino acid enantiomers in the respective samples are summarized in Table 1. High %D values of Ala and Asp are observed in the black vinegar and the cheese samples. In addition, the %D value of Pro is high in the nam pla sample. Since D-amino acids have been regarded as physiologically active substances in mammals, the screening of chiral amino acids in various fermented products using multi-dimensional methods is useful for finding natural functional foods and beverages.

Table 1. The amounts of amino acid enantiomers in fermented products.

| Amino acids | Black vinegar | Cheese | Nam pla |
|-------------|---------------|--------|---------|
|            | D | L | %D | D | L | %D | D | L | %D |
| Ala        | 6.21 | 15.05 | 29.2 | 0.73 | 2.21 | 24.8 | 1.95 | 90.69 | 2.1 |
| Asp        | 0.37 | 1.54 | 19.6 | 0.31 | 1.38 | 18.5 | 0.21 | 33.23 | 0.6 |
| Glu        | 0.32 | 2.69 | 10.6 | 0.33 | 12.36 | 2.6 | 0.73 | 17.26 | 0.2 |
| Leu        | 0.05 | 6.38 | 0.7 | 0.04 | 17.26 | 0.2 | 0.39 | 49.37 | 0.8 |
| Pro        | 0.02 | 2.58 | 1.0 | tr | 2.51 | - | 2.30 | 7.49 | 23.5 |
| Ser        | 0.22 | 3.84 | 5.5 | 0.02 | 2.31 | 1.1 | 0.07 | 13.56 | 0.5 |

Values represent µmol/g or mL. tr: less than LLOQ. %D: D/(D+L) x 100.

The 2D HPLC-MS/MS system is also used to evaluate the formation of D-amino acid residues in proteins. It has been discovered that specific L-amino acid residues in proteins turn into the D-forms during the aging process, and the stereo-inversion leads to the structural and functional changes of the proteins [54]. Since the D-amino acid residues are potential biomarkers of the quality/deterioration of proteins and also age-related diseases, the precise enantiomeric analysis of amino acid residues in the proteins has been expected. The acid hydrolysis has been widely adopted for the screening of D-amino acid residues in the proteins, however, the chemical racemization of amino acids during the hydrolysis is not negligible. To overcome this problem, adopting the $^3$HCl/$^2$H$_2$O hydrolysis is a promising approach. By the $^3$HCl/$^2$H$_2$O hydrolysis, the α proton of amino acids racemized during hydrolysis is replaced with deuterium as shown in Fig. 21, therefore, the naturally-occurring D-amino acids in the proteins are distinguished by the difference in the molecular weight (α proton is a hydrogen). Combining the $^3$HCl/$^2$H$_2$O hydrolysis and the 2D HPLC-MS/MS system, selective determination of D-amino acid residues in the proteins is carried out [55,56].
Concerning the denaturation of proteins, the pH conditions have been regarded as contributing factors. To evaluate the effect of the pH conditions on the isomerization of amino acid residues, Ala, Asp, Glu, Pro and Ser in the model protein are investigated using the 2D HPLC-MS/MS system following the 2HCl/2H2O hydrolysis. As a model protein, ovalbumin (OVA) is selected based on a previous report [57]. The protein samples are individually stored at 37ºC for a week under acidic (pH 4.0), neutral (pH 7.4) and basic (pH 9.5) conditions. After the 2HCl/2H2O hydrolysis, the obtained amino acids are derivatized with NBD-F and injected into the 2D HPLC-MS/MS system. As a result, remarkably high amounts of D-Ser are observed not only in the stored OVA but also even in the native protein (lyophilized OVA is dissolved in water, and hydrolyzed) as shown in Fig. 22A. Furthermore, the ratio of D-Ser to total Ser increases during the storage under all the tested pH conditions, and the %D Ser in OVA stored at pH 9.5 is approximately 10 times higher than that in the native OVA. In the case of Asp residues, the %D value in the native OVA and also those stored at pH 4.0 and 7.4 are negligible as shown in Fig. 22B. On the other hand, the formation of D-Asp residues is suggested when stored at pH 9.5. These results indicate that amino acid residues in proteins are likely to be inverted to the D-forms, and the relationships between the formation of the D-form residues and various diseases are expected to be clarified.

8. 3D-HPLC analysis of chiral amino acids and peptides

A 3D-HPLC system combining three different columns is one of the promising methods with highest selectivity. In the 2D-HPLC system, a reversed-phase column is used to separate the target amino acids from other amino acids and coexisting compounds, and an enantioselective column is employed for the chiral separation. In some complex matrices, however, co-eluted compounds are fractionated together with the target amino acids in the first dimension, and disturbed the precise determination of the amino acid enantiomers in the second dimension. Therefore, an additional column is placed between the reversed-phase and enantioselective columns for further separation of the target amino acids from interfering compounds [58,59].

A 3D-HPLC system has been developed for the determination of kidney disease related chiral amino acids, namely, Asn, Ser, Ala and Pro [59]. For the multi-dimensional HPLC, the orthogonality of the separation mode is the key to obtain the high selectivity [60]. Therefore, an anion-exchange column is used for the second dimension. The flow diagram of the system is shown in Fig. 23. After derivatization of the amino acids with NBD-F, an aliquot of the reaction mixture is injected into the reversed-phase column in the first dimension. The target amino acid enantiomers are separated and determined. By using the on-line 3D-HPLC system, all of the target amino acid enantiomers are nicely separated without having mobile phase incompatibility issues. The obtained resolution values in the third dimension are 2.18 for NBD-Asn, 2.96 for Ser, 1.92 for Ala and 2.09 for Pro.
The 2D- and 3D-HPLC systems are compared by analyzing the human plasma. Figure 24 shows the chromatograms obtained by the 2D-HPLC system without adopting the anion-exchange column. In the second dimension, the large peaks of the L-forms are clearly observed. However, the determination of trace amounts of the D-forms is difficult due to a number of interfering peaks. The results obtained by the 3D-HPLC are shown in Fig. 25. In the second dimension (anion-exchange column), various peaks are present and separated from the target amino acids. Therefore, the trace amounts of the D-forms are observed without interfering compounds in the third dimension.

By using the 3D-HPLC system, the human plasma samples of 25 CKD patients are analyzed. For all 25 samples, all of the target amino acid enantiomers are observed without interfering compounds in the third dimension.

The 2D- and 3D-HPLC systems are compared by analyzing the human plasma. Figure 24 shows the chromatograms obtained by the 2D-HPLC system without adopting the anion-exchange column. In the second dimension, the large peaks of the L-forms are clearly observed. However, the determination of trace amounts of the D-forms is difficult due to a number of interfering peaks. The results obtained by the 3D-HPLC are shown in Fig. 25. In the second dimension (anion-exchange column), various peaks are present and separated from the target amino acids. Therefore, the trace amounts of the D-forms are observed without interfering compounds in the third dimension.

By using the 3D-HPLC system, the human plasma samples of 25 CKD patients are analyzed. For all 25 samples, all of the target amino acid enantiomers are observed without interfering compounds. Figures 25A-C show the chromatograms obtained from three patients with high, middle and low estimated glomerular filtration ratio (eGFR) values. The %D values of all the target amino acids (especially for Asn and Ser) increase along with the renal disfunction, showing a nice inverse correlation with the eGFR values (Fig. 26). These results indicate that D-amino acids are expected as the novel biomarker of CKD in the future, and the detailed studies using the 3D-HPLC system is expected.

9. 2D/3D-HPLC analysis of hydroxy acids
Not only chiral amino acids, chiral hydroxy acids have also gained increased attention due to their relationships to various diseases. Especially, lactate (LA) and 3-hydroxybutyrate (3HB), indicated in Fig. 27, are the focus because of their roles in energy circulation and signaling in mammals [61,62]. The changes in their concentrations can be observed under certain disease conditions including...
To the homochirality features in the biological environment, like amino acids, LA and 3HB have been considered that only the major enantiomers (L-LA and D-3HB) exist in mammals and the minor forms (D-LA and L-3HB) are absent and have no biological significance. However, with the progress of analytical technologies, the existence of the minor enantiomers in living beings including humans has been reported and the differences in their concentrations have been found in various diseases [67-69].

Until now, various methods including CE [70,71], GC [72,73] and LC [74-80] have been developed for the enantioselective analysis of LA and 3HB, and multi-dimensional HPLC is useful like chiral amino acid analysis. An on-line 2D-HPLC method has been developed for the simultaneous analyses of LA and 3HB enantiomers in the human plasma and urine [81]. After the pre-column derivatization using 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), the LA and 3HB enantiomers are separated by the 2D-HPLC system having a Capcell Pak C18 ACR column (1.5 mm ID x 250 mm, Shiseido) used at 40°C in the first dimension and a Chiralpak AD-H column (2.0 mm ID x 250 mm, Daicel, Osaka, Japan) used at 25°C in the second dimension.

For the mobile phase in the first dimension, aqueous 0.01% TFA 25% MeOH is used at the flow rate of 100 μL/min. For the mobile phase used in the second dimension, the mixed solution of MeCN/MeOH (5/95, v/v) is used for NBD-LA and the mixed solution of MeCN/EtOH (5/95, v/v) is used for NBD-3HB at the flow rate of 200 μL/min. The detection is carried out by the fluorescence emission at 530 nm with the excitation at 470 nm. Using the narrowbore columns and whole-fraction transfer, the accurate quantification and simultaneous determination are achieved, and high α and Rs values are also observed (α = 1.82 and Rs = 7.06 for LA; α = 1.53 and Rs = 4.27 for 3HB).

This method is validated (by the calibration line, intra-day precision, inter-day precision and accuracy) and applied to human plasma (Fig. 28) and urine (Fig. 29). The levels of D-LA and L-3HB are found to be much lower than those of L-LA and D-3HB in the plasma. In the urine, D-LA is the trace form while the 3HB is almost racemic.
Although the 2D-HPLC system has been considered as one of the suitable methods for the trace analysis of LA and 3HB enantiomers, it still faces some problems of insufficient selectivity, especially for the human urine [81]. In order to increase the selectivity, a 3D-HPLC composed of a reversed-phase separation, a mixed-mode separation and an enantioselective separation has been developed and applied to mammalian urine samples [82]. In the first dimension, a KSAAMX column (1.0 mm ID x 250 mm, 15% MeCN and the flow rate is 50 μL/min. In the second dimension, a KSAARP column (1.0 mm ID x 250 mm) is used at 40°C. The mobile phase is an aqueous 0.05% TFA and the flow rate is 100 μL/min. In the third dimension, a Chiralpak AD-H column (2.0 mm ID x 250 mm, having N-3,5-dinitrophenylaminocarbonyl-Gly as a selector, particle size 5 µm, designed by the collaboration with Shiseido) is used at 25°C. The mobile phase is EtOH and the flow rate is 200 μL/min. NBD-LA and NBD-3HB are detected by fluorescence emission at 530 nm with the excitation at 470 nm. Concerning the newly designed second dimension, the mixed-mode column has various interactions including π-π, hydrogen-bonding and hydrophobicity. Therefore, the separation profiles are different from those in the first dimension, which helps to remove most of the compounds co-eluted with LA and 3HB and the interfering peaks in the third dimension are drastically reduced in the human urine (Fig. 30). These studies indicate that the multi-dimensional HPLC methods would be useful for the enantioselective determination of the trace hydroxy acids and the further investigations focusing on LA and 3HB enantiomers in various diseases including metabolic disorders are eagerly expected.

10. Conclusion

The enantioselective determination of amino acids and related compounds has the possibility to provide novel insights especially in the medical area for the screening of new physiologically active substances and biomarkers. The key is the quantitative and precise analysis of trace levels of minor enantiomers, and the multi-dimensional HPLC is one of the most straightforward and promising approaches. The methods introduced in the present review would be useful and the obtained results are the milestones for the studies of chiral amino acids and hydroxy acids, and further contributions are expected.

Acknowledgements

The authors express their deepest gratitude to all of the colleagues, graduates and students who worked hard to promote the present research in the Department of Bio-Analytical Chemistry / Drug Discovery and Evolution, Graduate School of Pharmaceutical Sciences, Kyushu University. The authors would also like to thank all of the collaborators in the reference list. The technical support from the KAGAMI members are greatly appreciated. A part of the study was financially supported by several KAKENHI projects (22390007, 25293007, 16H05080, 19H03359).

References

[1] Hamase, K.; Morikawa, A.; Zaitsu, K. J. Chromatogr. B 2002, 781, 73-91.
[2] Miyoshi, Y.; Koga, R.; Oyama, T.; Han, H.; Ueno, K.; Masuyama, K.; Itoh, Y.; Hamase, K. J. Pharm. Biomed. Anal. 2012, 69, 42-49.
[3] Miyoshi, Y.; Oyama, T.; Itoh, Y.; Hamase, K. Chromatography 2014, 35, 49-57.
[4] Katane, M.; Homma, H. J. Chromatogr. B 2011, 879, 3108-3121.
[5] Nishikawa, T. J. Chromatogr. B 2011, 879, 3169-3183.
[6] Koga, R.; Yoshida, H.; Nohta, H.; Hamase, K. Chromatography 2019, 40, 1-8.
[7] Imai, K.; Watanabe, Y. Anal. Chim. Acta 1981, 130, 377-383.
[8] Fukushima, T.; Kato, M.; Santa, T.; Imai, K. Biomed. Chromatogr. 1995, 9, 10-17.
[9] Hamase, K.; Homma, H.; Takigawa, Y.; Fukushima, T.; Santa, T.; Imai, K. Biochim. Biophys. Acta 1997, 1334, 214-222.
[10] Welch, C. J. J. Chromatogr. A 1994, 666, 3-26.
[11] Pirkle, W. H.; House, D. W.; Finn, J. M. J. Chromatogr. 1980, 192, 143-158.
[12] Öi, N.; Kitahara, H.; Matsumoto, Y.; Nakajima, H.; Horikawa Y. J. Chromatogr. 1989, 462, 382-386.
[13] Öi, N. Chromatography 2005, 26, 1-5.
