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Development of a liquid chromatography-tandem mass spectrometry method for quantitative analysis of trace D-amino acids

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D-Amino acids have recently attracted much attention in various research fields including medical, clinical and food industry due to their important biological functions that differ from L-amino acid. Most chiral amino acid separation techniques require complicated derivatization procedures in order to achieve the desirable chromatographic behavior and detectability. Thus, the aim of this research is to develop a highly sensitive analytical method for the enantioseparation of chiral amino acids without any derivatization process using liquid chromatography-tandem mass spectrometry (LC–MS/MS). By optimizing MS/MS parameters, we established a quantification method that allowed the simultaneous analysis of 18 D-amino acids with high sensitivity and reproducibility. Additionally, we applied the method to food sample (vinegar) for the validation, and successfully quantified trace levels of D-amino acids in samples. These results demonstrated the applicability and feasibility of the LC–MS/MS method as a novel, effective tool for D-amino acid measurement in various biological samples.

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[**Key words:** D-Amino acids; Quantitative analysis; Enantioseparation; Underivatization; Liquid chromatography-tandem mass spectrometry]

Amino acids are ubiquitous compounds of significant importance in life science due to their diverse vital functions in living organisms. Most amino acids are main components of proteins and have important roles in maintaining balances of fundamental mechanisms, including gene expression (1), metabolic regulation (2), immune system (3) and hormone secretion (4). In α-amino acids, both the carboxylic acid group and amino group are bonded to the same carbon center and there are many variations of the side chains. Due to this configuration, the structure is able to form enantiomers or mirror images. Generally, in order to distinguish these structures, they are commonly referred to as L- or D-amino acid. L-Amino acids are the predominant building blocks of proteins while D-amino acids cannot be incorporated into proteins via ribosomal synthesis (5) and it was believed for long time that D-amino acids are not present in mammals (6). However, recent technological advances in separation techniques have promoted studies of D-amino acids. D-Serine exists in mammal body, especially in the brain, and activates the *N*-methyl-D-aspartic acid (NMDA) receptor as its co-agonist (7,8) leading to mental disorders such as schizophrenia (9–11). D-Aspartic acid is present in invertebrate and vertebrate neuroendocrine tissues (12) and is involved in positive or negative regulation of hormone secretion (13–15).

Recently, there has been an increasing need to develop rapid, robust, highly sensitive methods for separation and quantification of both configurations of amino acids (16). For the enantiomeric separation, two main strategies have evolved: (i) an indirect method, based on the formation of diastereomers by the reaction of amino acids with a chiral derivatizing agent and separation of the diastereomeric derivatives on an achiral stationary phase; and (ii) a direct method, based on the formation of diastereomers on a chiral stationary phase or with a chiral selector on an achiral stationary phase in a mobile phase (17–19). A number of reports demonstrated the enantioseparation of chiral amino acids by chromatographic technologies such as thin layer chromatography (TLC) (20,21), gas chromatography (GC) (22,23) and high performance liquid chromatography (HPLC) (24–26) using the indirect method. This method requires a specific derivatization procedure considering the avoidance of undesirable side reactions, formation of decomposition products and racemization (27). On the other hand, the direct method needs no chemical derivatization prior to the separation process and employs various types of chiral selectors that provide stereospecific interactions such as π–π, *n*–π, hydrogen bonding, ion–ion and ion–dipole interactions (16). Among various chiral selectors, chiral crown ether has been widely utilized for the enantioseparation of racemic compounds containing a primary amino group (28). Most recently, we have developed an analytical method for simultaneous analysis of eighteen chiral proteinogenic amino acids using a combination of a chiral crown ether column and liquid chromatography-time of flight mass spectrometry (LC–TOFMS) (29). This method enabled the baseline enantioseparation of amino acids while maintaining excellent peak resolution. Moreover, the method requires no derivatization steps and thus can avoid undesirable issues that may occur during derivatization. However, the method was constructed based on accurate mass measurement using a high-end TOFMS system, which hinders its versatility and usability. Compared to TOFMS, tandem mass spectrometry (MS/MS) is widely recognized as a powerful analytical tool in terms of providing quantitative data. Multiple reaction

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monitoring (MRM) mode in MS/MS employs two stages of mass filtering that can remove impurity ions, which enables high sensitive and selective detection of trace target compounds.

Therefore, we aimed to develop an effective method for the simultaneous analysis of chiral amino acids using a high-sensitivity liquid chromatography-tandem mass spectrometry (LC–MS/MS) configured to the MRM mode. First, we established the analysis system that could measure trace levels of amino acid by LC–MS/MS. Then, we verified the feasibility of the method by measuring D-amino acids in real samples and comparing our results to those obtained by LC–TOFMS analysis using the same LC conditions.

MATERIALS AND METHODS

Reagents The following amino acid standards were used in this study. DL-Alanine (DL-Ala), DL-arginine hydrochloride (DL-Arg), DL-asparagine monohydrate (DL-Asn), DL-aspartic acid (DL-Asp), DL-glutamic acid (DL-Glu), DL-glutamine (DL-Gln), DL-histidine (DL-His), DL-isoleucine (DL-Ile, mixture of four stereoisomers containing DL-*allo*-Ile), DL-leucine (DL-Leu), DL-lysine monohydrochloride (DL-Lys), DL-methionine (DL-Met), DL-phenylalanine (DL-Phe), DL-serine (DL-Ser), DL-threonine (DL-Thr), DL-tryptophan (DL-Trp), DL-tyrosine (DL-Tyr), and DL-valine (DL-Val) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). DL-Cysteine hydrochloride monohydrate (DL-Cys), ultrapure water for LC/MS (Water), and 0.1 mol/L hydrochloric acid for volumetric analysis were obtained from Wako Pure Chemical Industries (Osaka, Japan). Methanol-Plus for LC/MS (MeOH), acetonitrile-Plus for LC/MS (ACN), and trifluoroacetic acid for HPLC (TFA) were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Chloroform for HPLC was obtained from Kishida Chemical Co., Ltd. (Osaka, Japan). DL-Alanine-2,3,3,3-d_4 (DL-Ala-d_4) were obtained from Santa Crutz Biotechnology (Dallas, TX, USA).

Preparation of standard solution For the preparation of 20 μmol/mL standard solution, each amino acid standard was dissolved in 50% MeOH (MeOH/Water = 1/1), 50% MeOH–0.02 mol/mL-HCl (for DL-Glu, DL-His and DL-Trp), or 50% MeOH–0.05 mol/mL-HCl (for DL-Asp and DL-Tyr). Eighteen DL-amino acid standard solutions (20 μmol/mL) were mixed to obtain mixture solutions at 0.005, 0.01, 0.05, 0.5, 1, 5, 50 and 100 nmol/mL. A solution of DL-Ala-d_4 (1000 nmol/mL) was prepared as described above and used as an internal standard (IS).

Sample pretreatment Sample extraction was performed in accordance to the previous extraction method (30). Three types of commercially available vinegars (vinegar A, B and C) were prepared. One hundred microlitre of sample was mixed with 350 μL of MeOH, 20 μL of IS solution, and 50 μL of Water in a 1.5-mL tube, followed by mixing with vortex and centrifugation at 10,000 rpm for 10 min at 4°C. Then, 360 μL of supernatant was transferred to a new 1.5-mL tube and mixed with 180 μL of water and 360 μL of chloroform. After vortexing and centrifugation in the same condition as described above, 50 μL of supernatant was diluted with 200 μL of ACN-EtOH mixed solution. Approximately 100 μL of diluted sample was transferred to a vial and subjected to LC/MS analysis. Chiral amino acid standard solutions were also pretreated using the same method as vinegar samples to obtain calibration curves for quantification.

LC–MS analysis LC–MS/MS analysis was performed using the Nexera HPLC System (Shimadzu Corporation, Kyoto, Japan) connected to LCMS-8060 (Shimadzu Corporation) with dual ion source of electrospray ionization and atmospheric pressure chemical ionization in MRM mode. The ion source was operated in positive mode and the interface parameters were optimized under the following conditions: nebulizer gas: 3 L/min; heating gas: 5 L/min; drying gas: 15 L/min; interface temperature: 230°C; desolvation line temperature: 250°C; heat block temperature: 310°C and interface voltage: 4 kV. For the optimization of MRM transition, a 100 nmol/mL amino acid standard solution was injected into the triple quadrupole mass spectrometry. For LC–TOFMS analysis, the Nexera HPLC system coupled to TripleTOF 5600 System (AB SCIEX, Concord, Canada) was used under these conditions: ion source gas 1:50 psi; ion source gas 2:50 psi; curtain gas 1:30 psi, temperature: 600°C; ion spray voltage floating: 5500 V; declustering potential: 60 V, CE: 5 V; and mass range: 60–600 *m/z*. Chromatographic separation was achieved with CROWNPAK CR-I(+) and CR-I(−) (3.0 mm i.d. × 150 mm, 5 μm) (Daicel CPI, Osaka, Japan) as the analytical columns. The injection volume was 1 μL and the oven temperature was kept at 30°C. The mobile phase consisted of a mixture of ACN, EtOH, Water and TFA (80/15/5/0.5) and the flow rate was set to 0.4 mL/min in isocratic condition. Data acquisition and processing were performed using LabSolutions (Shimadzu) for LC–MS/MS analysis and MultiQuant (AB SCIEX) for LC–TOFMS analysis.

RESULTS AND DISCUSSION

Construction of a chiral amino acid analysis system with LC–MS/MS In order to construct an analytical system for the simultaneous identification of chiral amino acids using LC–MS/MS, we first optimized the MRM transitions of targeted amino acids. MS/MS detected $[M + H]^+$ ion as the precursor ion of both D and L-amino acids. The product ions were highly dependent on the collision energy (CE) and the MS/MS electrical parameters including CE, Q1 Pre Bias, and Q3 Pre Bias were optimized to obtain the highest sensitivity (Table 1). MRM transitions of the targeted amino acids were monitored only around the expected retention time (±0.75 min) to decrease the number of concurrent MRM transitions in a single analysis for the high sensitivity and reproducibility. The electrospray probe position was also adjusted because the position was closely related to desolvation and ionization efficiency. The sensitivity increased dramatically by taking more distance from the default probe position. Finally, we fine-tuned the interface parameters such as nebulizer gas, heating gas, drying gas, interface temperature, desolvation line temperature, heat block temperature, and interface voltage.

Next, we confirmed the enantioseparation of chiral amino acids using the optimized method. Chromatographic conditions were determined according to our previous report using LC–TOFMS analysis (29). In the LC–TOFMS method, amino groups in the targeted amino acids are protonated by the highly acidic mobile phase to make ammonium ions. Amino acids in acidic condition generate strong interaction with crown ether in the chiral stationary phase, which causes stereospecific retention to vary between D and L-amino acids. We applied this separation principle in this study and results showed that most chiral amino acids were enantiomerically separated successfully with high resolution ($R_s > 1.5$) (Fig. 1A). However, MS/MS detected the peaks of L-Gln and D-Lys as an identical compound because they had the same MRM transition and similar retention time. To solve this problem, we employed another chiral crown ether column. CROWNPAK CR-I(+) had been selected as a enantioseparation column since it was able to retain D-amino acids longer and elute them prior to L-amino acids. We previously confirmed the elution order of D- and L-amino acids using each L-amino acid standard (not racemic standard). Moreover, the elution order of enantiomers can be reversed by using CR-I(−), which allowed the analysis of L-Gln and D-Lys (Fig. 1B).

We evaluated the developed LC–MS/MS analytical system for linearity of the dilution curve, practicality of the linear range, limits of detection (LOD) and reproducibility (Table 2). The linearity of the dilution curve and the usable linear range were assessed with a standard amino acid mixture at concentrations of 0.005, 0.01, 0.05, 0.5, 1, 5, 50 and 100 nmol/mL. The linearity was evaluated by the

TABLE 1. Optimized MRM transition and parameters for 18 amino acids and the internal standard (IS).

	Precursor ion (*m/z*)	Product ion (*m/z*)	Q1 Pre Bias (V)	CE (eV)	Q3 Pre Bias (V)
Ala	90.10	44.10	−10	−14	−17
Arg	175.10	70.10	−17	−24	−12
Asn	133.10	74.05	−10	−17	−14
Asp	134.20	74.10	−16	−15	−14
Cys	122.05	59.00	−10	−24	−22
Gln	147.10	84.10	−18	−18	−16
Glu	148.10	84.10	−10	−17	−18
His	156.10	110.10	−12	−15	−11
Ile	132.10	86.15	−13	−12	−18
Leu	132.10	86.15	−11	−12	−18
Lys	147.10	84.10	−17	−18	−21
Met	150.10	56.10	−10	−17	−18
Phe	166.10	120.10	−10	−14	−12
Ser	106.10	42.10	−11	−20	−16
Thr	120.10	74.00	−11	−12	−13
Trp	205.10	185.15	−11	−10	−12
Tyr	182.10	136.00	−10	−14	−23
Val	118.10	72.05	−11	−12	−13
Ala-d_4 (IS)	94.15	48.20	−13	−19	−15

CE, collision energy.

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