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Automatic switching valve system to minimize variation of liquid chromatography-tandem mass spectrometry-based chiral amino acid profiling

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Although D-amino acids are less prevalent in nature, they have been detected in mammals (including humans) and it is widely accepted that they might play important physiological roles. While an analytical method for chiral amino acid profiling is strongly required, it has not been well-established because of the difficulties associated with analysis. A high-sensitivity and high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method was recently reported by our group for chiral amino acids; however, it lacked sufficient repeatability for several D-amino acids. Thus, the aim of this research was to reduce the experimental variation of chiral amino acid analysis. By installing an automatic switching valve system in LC-MS/MS, it was possible to reduce the relative standard deviations of D-amino acid ratios (D/(D+L)) in rat urine obtained from three technical replicates. The results indicated that the automatic switching valve system was effective in minimizing the variation of D-amino acid ratios, and could be applied for profiling D-amino acids because of its high repeatability.

[**Key words:** D-Amino acids analysis; Liquid chromatography-tandem mass spectrometry; Automatic switching valve; High repeatability; Rat urine]

D-Amino acids, the enantiomers of L-amino acids, have attracted considerable attention as bioactive compounds in mammals, including humans (1). It has been suggested that D-serine might have an important role in cognitive function, such as learning and memory, because it acts as a co-agonist at the *N*-methyl-D-aspartate (NMDA) receptor (2). Additionally, it has been reported that D-aspartic acid is involved in many physiological activities, such as negative regulation for melatonin synthesis (3), regulation of prolactin release (4), stimulation of testosterone synthesis (5), and biosynthesis of NMDA as a precursor (6). Some recent studies have addressed the significance of D-amino acids as a potential biomarker for disease diagnosis. The levels of D-serine and D-asparagine in human plasma are closely associated with the progress of chronic kidney disease (7). In addition, increase in D-serine levels was observed in the spinal cords of amyotrophic lateral sclerosis (ALS) patients (8). While several studies have focused on the bioactivity or clinical utility of D-amino acids, little effort has been expended on comprehensive analyses of D-amino acids owing to associated difficulties.

The analytical challenges of D-amino acids arise from their low natural abundance and the need for separation from L-amino acids with high sensitivity and selectivity. In order to overcome these hurdles, several methods have been developed for D-amino acid analysis. Some of them utilize enzyme specificity by indirectly detecting α-keto acids (9,10) or hydrogen peroxide (11) catalyzed by D-amino acid oxidase. While enzymatic assays have some advantages in terms of simplicity and rapidness, simultaneous detection of different D-amino acids in a mixture is technically difficult because of the limitation of substrate specificity (12). For chromatographic analytical techniques, various separation modes have been suggested to achieve desirable enantiomeric resolution of chiral amino acids. Many reports have described enantioseparation techniques based on thin layer chromatography (TLC) (13,14), gas chromatography (GC) (15,16), and high performance liquid chromatography (HPLC) (17–19). Chromatographic techniques have been applied for various biological samples, such as urine (20,21), plasma (7,22), and brain (23–25), as expectations of D-amino acids as physiologically active substances and novel biomarkers have increased.

Recently, an analytical method was developed for the enantioseparation of chiral amino acids using a combination of crown ether-based chiral column (CROWNPAK CR-I(+)) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (26). The method analyzed proteinogenic amino acids, except for proline, without the need for derivatization. The system also enabled elution of all targeted amino acids within eight minutes and was able to detect them with high sensitivity. This method demonstrated several outstanding features among reported chromatographic techniques, but there were two problems for practical applications, especially for biological specimens. First, L-glutamine and D-lysine could not be discriminated because of co-elution and exact equal multiple reaction monitoring (MRM) transition. Secondly, targeted amino acids could be co-eluted with some impurities in real samples despite careful pretreatment. These problems

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could trigger inaccurate identification and integration of detected peaks. In order to avoid these issues, another crown ether column (CROWNPAK CR-I(−)), which had a complementary structure of the main column (CR-I(+)) and can reverse the elution order of chiral amino acids, was utilized. When targeted amino acids co-eluted with other targeted compounds or impurities in CR-I(+), the data obtained by CR-I(−) was used. In the previous method (26), the two chiral columns had to be changed manually, and this could lead to a large intra- and inter-batch analytical error.

Therefore, the aim of this study was to establish a method for chiral amino acid analysis with high repeatability in biological samples, and was achieved by installing two automatic switching valves in the conventional LC-MS/MS method. The advantage of the switching valves was that they allowed continuous analysis of samples by operating two columns (CR-I(+) and CR-I(−)) without any manual column exchange process. Introduction of two switching valves enabled successive analysis of the two columns, which could minimize the analytical variation. In this study, we have developed an LC-MS/MS method-based automatic switching valve system. The newly constructed and previous methods were used to conduct analyses of 40 rat urine samples and the performances of both methods were compared.

MATERIALS AND METHODS

Chemicals and 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). L-Alanine, DL-cysteine hydrochloride monohydrate (DL-Cys), DL-*allo*-threonine (DL-*allo*-Thr), ethanol for LC/MS (EtOH), and 0.1 mol/L hydrochloric acid for volumetric analysis were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Methanol-Plus for LC/MS (MeOH), acetonitrile-Plus for LC/MS (ACN), and trifluoroacetic acid for HPLC (TFA) were purchased 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) was received from Santa Cruz Biotechnology (Dallas, TX, USA).

Preparation of standard solutions For the preparation of 20 μmol/mL standard solutions, each amino acid standard was dissolved in 50% MeOH (MeOH/water = 1/1, v/v), 50% MeOH-0.02 mol/L-HCl (for DL-Glu, DL-His and DL-Trp), or 50% MeOH-0.05 mol/L-HCl (for DL-Asp and DL-Tyr). Eighteen DL-amino acid standard solutions (20 μmol/mL) were mixed to obtain mixture solutions with concentrations 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 nmol/mL. A solution of DL-Ala-d_4 (20 μmol/mL) was prepared as described above and used as an internal standard (IS).

Animals Ten male, four weeks-old Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). They were housed individually in conventional cages in a temperature- and humidity-controlled room (22−26°C and 40−70%) under a 12-h light/dark cycle (light on 8:00 to 20:00). The animals had free access to pelleted feed (CE-2, Oriental Yeast Co., Ltd., Tokyo, Japan) and water. All procedures were approved by the Sumitomo Dainippon Pharmaceutical Committee on Animal Research.

Rat urine sample Male Sprague-Dawley rats (6 weeks old) were randomly divided into two groups: the first, in which rats were subtotal nephrectomized (n = 5), the second, in which the animals were sham-operated (n = 5). The subtotal nephrectomized rats underwent two-step surgical resection of five-sixths kidney, in a first-step a two-thirds of the left kidney was dissected and after 1 week, the right kidney was completely removed. Sham-operated rats experienced incision and suturation of the abdominal skin. One week after the operation, urine was collected in the individual metabolic cages once a week, up to four weeks. Four urine samples were obtained from each rat, and therefore 40 samples were applied to the analysis. The urine was frozen at −80°C until the analysis.

Sample pretreatment Sample extraction was performed according to the previously reported extraction method (27). Urine samples were prepared in the following manner. One hundred microliter of the 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 vortexing and centrifugation at 10,000 rpm for 10 min at 4°C. Then, 360 μL of the 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 under the same conditions as described above, 50 μL of the supernatant was diluted with 200 μL of ACN/EtOH (4/1, v/v). The sample tube was yet again vortexed and centrifuged under the same conditions, and approximately 100 μL of the supernatant was transferred to a vial and subjected to LC-MS/MS analysis.

LC-MS/MS analysis LC-MS/MS analysis was performed using the Nexera HPLC System (Shimadzu Corporation, Kyoto, Japan) connected to LCMS-8060 (Shimadzu), and two flow-switching valves FCV-32AH (Shimadzu) were installed. 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 autosampler and oven temperatures were maintained at 4°C and 25°C, respectively. The mobile phase consisted of a mixture of ACN, EtOH, water, and TFA (80/15/5/0.5, v/v/v/v) and the flow rate was set to 0.4 mL/min in the isocratic condition. The triple quadrupole mass spectrometer was coupled with a dual ion source for electrospray ionization and atmospheric pressure chemical ionization. Column elutes were analyzed in positive ionization mode with 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. Triple quadrupole mass spectrometer operated in the MRM mode. Protonated molecules $[M+H]^+$ were chosen as precursor ions, and the MRM transitions and parameters were optimized for all target compounds (Table S1). Compound identities were verified by comparing their precursor and product masses and retention times with standard solutions. MRM transitions of the targeted amino acids were monitored only around the expected retention times (±0.75 min). Data acquisition and processing were performed using LabSolutions (Shimadzu).

RESULTS AND DISCUSSION

Development of LC-MS/MS method-based automatic switching valve system For successive analysis using CROWNPAK CR-I(+) and CR-I(−) chiral columns, two switching valves were installed to the conventional analytical system. Initially, pump A-delivered mobile phase and injected samples were loaded on the CR-I(+) column (Fig. 1A). The two valves were automatically switched after all targeted chiral amino acids had eluted from CR-I(+) (Fig. 1B). The same samples were then injected and separated on CR-I(−) as well. This method required two injections in one analysis, unlike the conventional method. In this system, eighteen chiral proteinogenic amino acids were successfully separated on both CR-I(+) and CR-I(−) columns within 20 min (Figs. 2A and S1). As seen in Fig. 2A, the peak shapes were similar between the separations of both columns. In addition, it was possible to confirm whether the elution order of enantiomers was reversed in the two columns by analyzing the DL-Ala solution at different concentration between the D- and L-forms (L-Ala: 50 nmol/mL, D-Ala: 10 nmol/mL) (Fig. 2B). The D-amino acid ratio (ratios of a D-amino acid to the total free L- and D-amino acids) calculated from the area values of each peak were 0.174 and 0.177, obtained from the separation on CR-I(+) and CR-I(−), respectively. This result supported the understanding that the combination of CR-I(+) and CR-I(−) reversed the elution order of targeted chiral amino acids with similar peak shapes.

Next, the LC-MS/MS method-based automatic switching valve system was evaluated for retention time (RT), linearity of the dilution curve, practicality of the linear range, and limit of detection (LOD) (Table 1). Eighteen DL-amino acid mixtures were diluted at concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 nmol/mL. Dilution curves were obtained by plotting the peak area values of the targeted amino acids divided by the peak area of the IS. The evaluation results of D-Lys and L-Gln could not be acquired from the analysis using CR-I(+) because MS/MS detected their peaks as identical compounds. Linearity was evaluated by the coefficient of determination (R^2). The R^2 values were over 0.99 over a wide range of the dilution curves, which indicated good correlation of the data. The LODs were expressed as the concentration that had a signal-to-noise ratio of 3:1. Furthermore, it was confirmed that this method was comparable to the conventional one (26) in terms of the linearity, linear range, and LODs. Repeatability was also evaluated by relative standard deviation

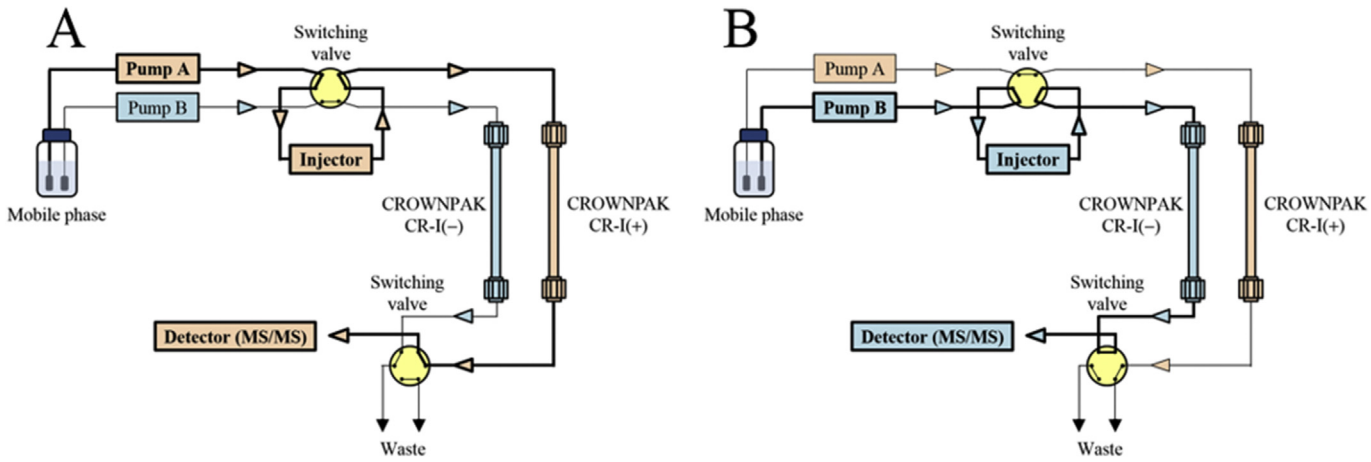

FIG. 1. Schematic diagram of LC-MS/MS-based automatic switching valve system. (A) Pump A-delivered mobile phase and samples were separated on the CROWNPAK CR-I(+) column. (B) Pump B-delivered mobile phase and samples were separated on the CROWNPAK CR-I(−) column.

(RSD) of the peak areas obtained from three successive analyses. All targeted D-amino acids, except for D-Ala, D-Ser and D-*allo*-Thr, showed RSDs below 15% at 0.05 nmol/mL, and D-Ala, D-Ser and D-*allo*-Thr exhibited RSDs below 15% at 0.5 nmol/mL. This result indicated the LC-MS/MS method-based automatic switching valve system had high repeatability. In addition, D-amino acid ratios were calculated from the analysis of standard samples (Fig. S2). These ratios were almost same as theoretical value (0.5), and they were not huge difference between CROWNPAK CR-I(+) and CR-I(−).

Several methods have been reported for determining D-amino acids in biological samples using LC-MS/MS (12,28,29). Although some of them are over 10 times more sensitive than the newly developed method, they require derivatization in order to achieve the detection of trace D-amino acids. The derivatization procedure may cause undesirable side reactions, formation of decomposition products, and racemization (30). Some methods also require a couple of hours for one analysis. On the other hand, the present method enabled trace level detection of D-amino acids without derivatization, and it was possible to simultaneously analyze eighteen chiral proteinogenic amino acids within 20 min. With these advantages, the method demonstrated a great potential for profiling D-amino acids.

Application for rat urine samples In order to verify the applicability of the new LC-MS/MS system, the method was applied to biological samples. Urine samples from five-sixths nephrectomy rats were used as biological samples. Five-sixths nephrectomy rat is known as a model of renal failure (31,32). Forty urine samples were obtained from the nephrectomized and sham-operated rats, and pretreated to remove both proteins and lipids by liquid–liquid extraction. The peak area values were divided by that of the IS to correct injection errors. Consequently, 26 amino acids (D,L-Ala, D,L-Arg, D,L-Asn, D,L-Gln, D,L-Glu, D,L-His, D,L-Lys, D,L-Ser, D,L-Val, L-Asp, L-Ile, L-Leu, L-Met, L-Phe, L-Thr, L-Trp, L-Tyr) were detected out of

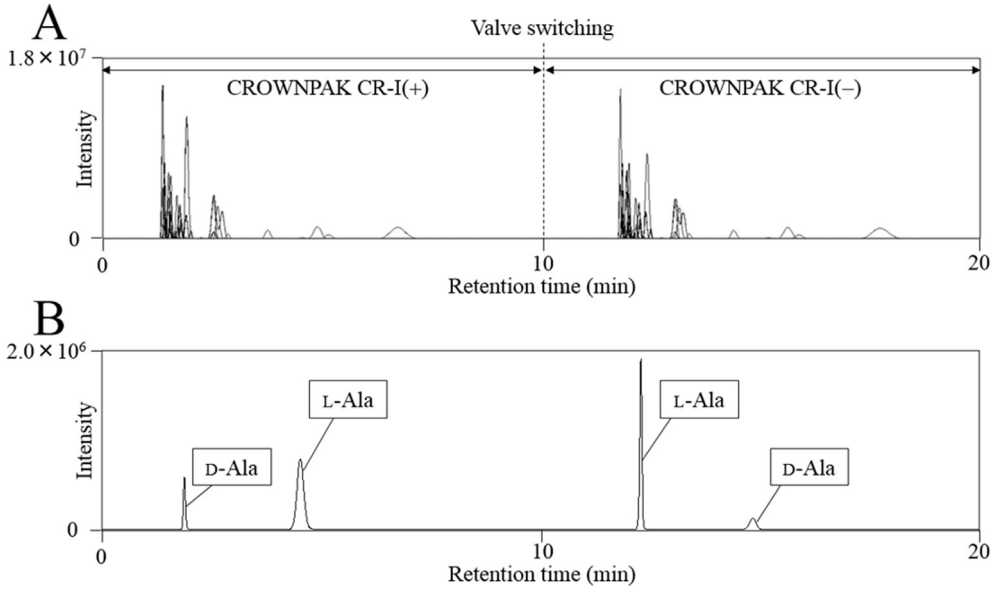

FIG. 2. Chromatograms of standard solutions obtained by LC-MS/MS method-based automatic switching valve system. Samples were injected twice (0 and 10 min) and separated on the CROWNPAK CR-I(+) (0–10 min) and CR-I(−) (10–20 min) columns, respectively. (A) Chromatogram of 18 chiral amino acids (10 nmol/mL). (B) Extracted ion chromatogram of D- and L-Ala (D-Ala: 10 nmol/mL, L-Ala: 50 nmol/mL). Ala was monitored with the MRM transition of *m/z* 90.10 to 44.10.

TABLE 1. Evaluation results of the LC-MS/MS method-based automatic switching valve system.

	RT (min)	Range (nmol/mL)	R^2 value	LOD (nmol/mL)
CROWNPAK CR-I(+)				
D-Ala	1.89	0.5–100	1.0000	0.5
D-Arg	1.39	0.005–10	0.9995	0.005
D-Asn	1.54	0.01–10	0.9951	0.01
D-Asp	1.72	0.05–50	0.9950	0.05
D-Cys	1.65	0.005–100	0.9983	0.005
D-Gln	1.52	0.005–50	0.9953	0.005
D-Glu	1.71	0.005–50	1.0000	0.005
D-His	1.39	0.005–50	0.9923	0.005
D-Ile + D-*allo*-Ile	1.43	0.005–50	0.9937	0.005
D-Leu	1.56	0.01–100	0.9988	0.01
D-Lys	–	–	–	–
D-Met	1.73	0.005–100	0.9997	0.005
D-Phe	1.56	0.01–50	0.9987	0.01
D-Ser	1.69	0.5–100	0.9904	0.5
D-Thr + D-*allo*-Thr	1.47	0.05–100	0.9965	0.05
D-Trp	1.57	0.005–50	0.9944	0.005
D-Tyr	1.56	0.01–10	0.9947	0.01
D-Val	1.44	0.01–100	0.9993	0.01
L-Ala	4.56	0.5–100	0.9993	0.5
L-Arg	1.93	0.005–10	0.9999	0.005
L-Asn	1.80	0.005–100	0.9984	0.005
L-Asp	2.56	0.05–100	0.9927	0.05
L-Cys	2.87	0.01–100	0.9999	0.01
L-Gln	–	–	–	–
L-Glu	4.90	0.05–100	0.9995	0.05
L-His	1.52	0.005–50	0.9998	0.005
L-Ile	1.92	0.01–100	0.9980	0.01
L-*allo*-Ile	1.78	0.01–50	1.0000	0.01
L-Leu	3.77	0.05–100	0.9992	0.05
L-Lys	6.73	0.05–100	0.9991	0.05
L-Met	5.15	0.05–100	0.9993	0.05
L-Phe	2.64	0.05–100	0.9906	0.05
L-Ser	2.27	0.5–100	0.9999	0.5
L-Thr	2.03	0.1–100	1.0000	0.1
L-*allo*-Thr	1.66	0.1–100	0.9993	0.1
L-Trp	2.53	0.005–50	0.9996	0.005
L-Tyr	2.53	0.05–100	0.9924	0.05
L-Val	1.80	0.005–100	1.0000	0.005
CROWNPAK CR-I(−)				
D-Ala	15.15	0.5–100	1.0000	0.5
D-Arg	12.39	0.05–50	0.9912	0.05
D-Asn	12.23	0.05–100	0.9938	0.05
D-Asp	13.03	0.05–50	0.9939	0.05
D-Cys	13.35	0.05–100	0.9997	0.05
D-Gln	–	–	–	–
D-Glu	15.59	0.05–100	1.0000	0.05
D-His	11.93	0.005–50	0.9980	0.005
D-Ile	12.36	0.01–100	0.9936	0.01
D-*allo*-Ile	12.21	0.005–50	0.9991	0.005
D-Leu	14.36	0.05–100	1.0000	0.05
D-Lys	17.69	0.05–100	0.9999	0.05
D-Met	15.83	0.05–100	1.0000	0.05
D-Phe	13.12	0.05–50	0.9998	0.05
D-Ser	12.72	0.5–100	0.9992	0.5
D-Thr	12.47	0.1–100	0.9974	0.1
D-*allo*-Thr	12.08	0.1–100	0.9978	0.1
D-Trp	13.02	0.005–50	0.9982	0.005
D-Tyr	13.03	0.05–50	0.9944	0.05
D-Val	12.23	0.01–100	0.9988	0.01
L-Ala	12.32	0.5–100	0.9989	0.5
L-Arg	11.79	0.005–10	0.9975	0.005
L-Asn	11.94	0.005–10	0.9967	0.005
L-Asp	12.14	0.01–50	0.9920	0.01
L-Cys	12.07	0.005–100	0.9951	0.005
L-Gln	11.92	0.005–10	0.9989	0.005
L-Glu	12.13	0.005–50	0.9998	0.005

TABLE 1 (*continued*)

	RT (min)	Range (nmol/mL)	R^2 value	LOD (nmol/mL)
L-His	11.78	0.005–10	0.9979	0.005
L-Ile + L-*allo*-Ile	11.83	0.005–10	0.9999	0.005
L-Leu	11.98	0.005–100	0.9962	0.005
L-Lys	–	–	–	–
L-Met	12.15	0.005–100	0.9942	0.005
L-Phe	11.97	0.01–50	0.9970	0.01
L-Ser	12.1	0.5–50	0.9948	0.5
L-Thr + L-*allo*-Thr	11.87	0.01–100	0.9933	0.01
L-Trp	11.98	0.005–10	0.9998	0.005
L-Tyr	11.97	0.005–10	0.9976	0.005
L-Val	11.84	0.005–100	0.9970	0.005

thirty eight chiral proteinogenic amino acids in rat urine. Although L-Gln and D-Lys were detected as an identical compound by separation on CROWNPAK CR-I(+), they could be successfully separated by CR-I(−) (Fig. 3A). In addition, D-Val co-eluted with impurity compounds when separated by CR-I(+), but was detected as a single peak by using CR-I(−) and appeared in the reverse elution order (Fig. 3B). L-Ser also eluted with other compounds in CR-I(+), but could be separated by using CR-I(−). Next, in order to confirm the feasibility of the analysis, forty rat urine samples were analyzed three times using the LC-MS/MS method-based automatic switching valve system. In this experiment, D-amino acid ratios were calculated because the value was not affected by the level of urine concentration. As a result, nine D-amino acid ratios (Fig. S3A) and RSDs of three replicates were obtained by using the new system (Fig. 4A, Table S2). The same samples were also applied to the conventional LC-MS/MS method, which required manual column switching, and nine D-amino acid ratios (Fig. S3B) and RSDs of three replicates (Fig. 4B, Table S3) were acquired. These results showed that RSDs of three replicate were quite different between new and conventional method, while the average values were nearly same. As we described earlier, L-Gln, D-Lys, L-Ser, and D-Val were not fully separated by CR-I(+), and therefore these D-amino acid ratios were calculated by combining the peak area values obtained from CR-I(+) and CR-I(−) analysis. All RSDs were below 10% using the new system, except for the D-Ser ratios, and the RSDs of D-Ser ratios were also below 13%. On the other hand, one-tenth (36/360) of all RSDs were higher than 15% using the conventional LC-MS/MS method. The result of D-Gln, D-Ser, and D-Val ratios, which were calculated by combining the result of two columns, showed especially high RSDs. The two following reasons can be considered. First, in the conventional method, the column had to be exchanged manually after all samples were analyzed, which led to a huge time difference between the analysis using two columns. This gap will probably cause high RSDs, because the mobile phase has high volatility and its composition can easily change. Secondly, washing time might be not enough in the conventional method. In case of the constructed method, one column is in washing operation while analysis using the other column. This operation may reduce the carryover problem. Thus, the newly developed method showed high repeatability because of the successive analysis of two columns using automatic switching valves and enough washing time.

In summary, an automatic switching valve system was applied in the LC-MS/MS method for D-amino acid analysis. It was also confirmed that the method was able to analyze biological samples (rat urine) with high repeatability as compared to the conventional protocol. D-Amino acids have attracted attention as bioactive

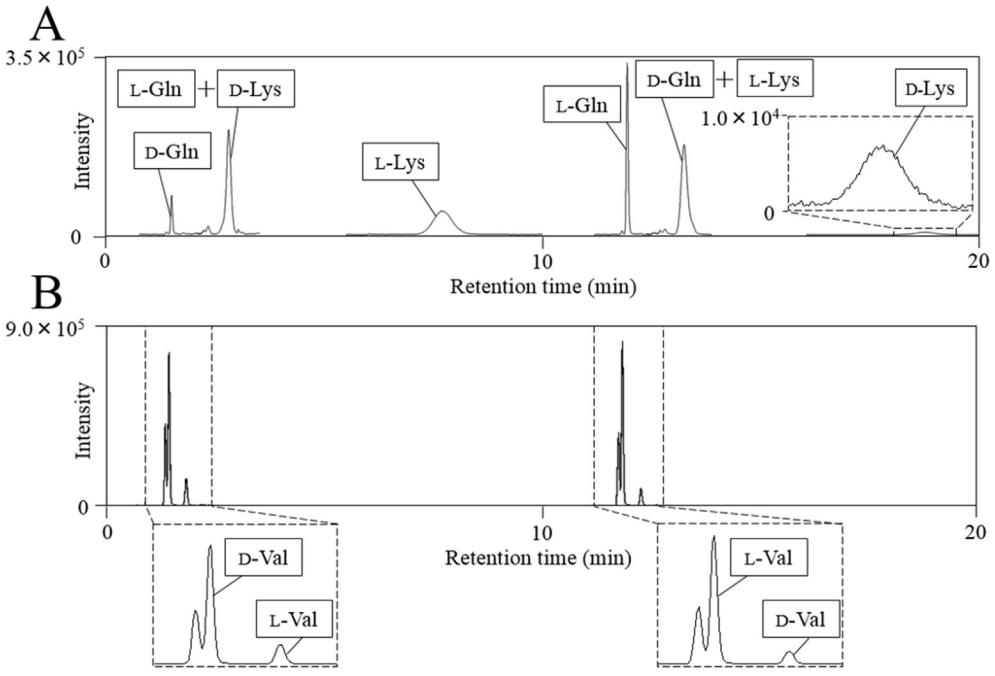

FIG. 3. Representative chromatograms of sham-operated rat urine sample. (A) Extracted ion chromatogram of DL-Gln and DL-Lys. In this figure, Gln and Lys were monitored with the MRM transition of *m/z* 147.10 to 84.10. (B) Extracted ion chromatogram of D- and L-Val. Val was monitored with the MRM transition of *m/z* 118.10 to 72.05.

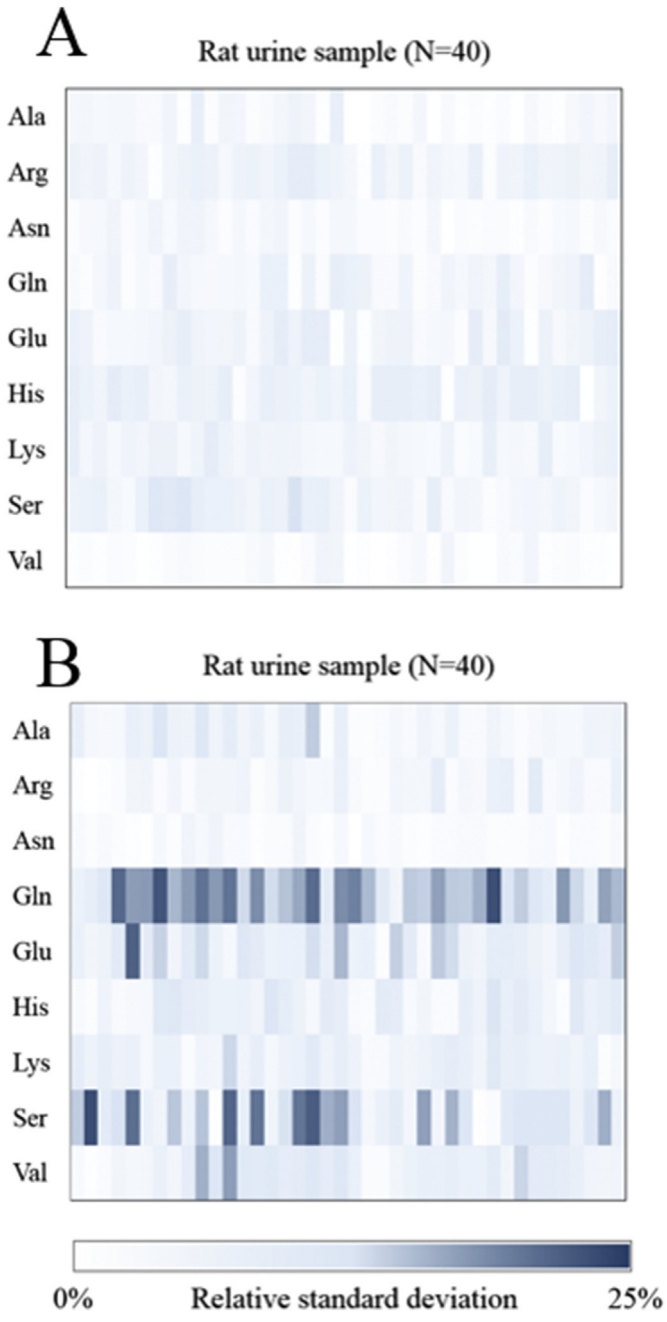

FIG. 4. Heat maps based on the RSDs of D-amino acid ratios (ratios of a D-amino acid to the total free L- and D-amino acid). Deep blue color indicates the highest RSD (25%) and white color shows the lowest RSD (0%). (A) RSDs of three replicates obtained from the LC-MS/MS-based automatic switching valve system analysis. (B) RSDs of three replicates obtained from the analysis of the previous method.

compounds or biomarker, but limited D-amino acids have been focused on owing to difficulty and complexity of analysis. Since automatic valve switching system can analyze biological samples with high repeatability while maintaining high sensitivity and high-throughput, it is a useful technique for D-amino acid profiling in biospecimens. Because of its great features, the proposed method would advance bioactivity-related or biomarker research of D-amino acids.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.06.005.

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References

1. **Miyoshi, Y., Koga, R., Oyama, T., Han, H., Ueno, K., Masuyama, K., Itoh, Y., and Hamase, K.:** HPLC analysis of naturally occurring free D-amino acids in mammals, J. Pharm. Biomed. Anal., **69**, 42–49 (2012).
2. **Oliet, S. H. R. and Mothet, J.-P.:** Regulation of *N*-methyl-D-aspartate receptors by astrocytic D-serine, Neuroscience, **158**, 275–283 (2009).
3. **Ishio, S., Yamada, H., Hayashi, M., Yatsushiro, S., Noumi, T., Yamaguchi, A., and Moriyama, Y.:** d-Aspartate modulates melatonin synthesis in rat pinealocytes, Neurosci. Lett., **249**, 143–146 (1998).
4. **D'Aniello, G., Tolino, A., D'Aniello, A., Errico, F., Fisher, G. H., and Di Fiore, M. M.:** The role of D-aspartic acid and *N*-methyl-D-aspartic acid in the regulation of prolactin release, Endocrinology, **141**, 3862–3870 (2000).
5. **Nagata, Y., Homma, H., Lee, J.-A., and Imai, K.:** D-Aspartate stimulation of testosterone synthesis in rat Leydig cells, FEBS Lett., **444**, 160–164 (1999).
6. **D'aniello, A., Di Fiore, M. M., Fisher, G. H., Milone, A., Seleni, A., D'aniello, S., Perna, A. F., and Ingrosso, D.:** Occurrence of d-aspartic acid and *N*-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release, FASEB J., **14**, 699–714 (2000).
7. **Kimura, T., Hamase, K., Miyoshi, Y., Yamamoto, R., Yasuda, K., Mita, M., Rakugi, H., Hayashi, T., and Isaka, Y.:** Chiral amino acid metabolomics for novel biomarker screening in the prognosis of chronic kidney disease, Sci. Rep., **6**, 26137 (2016).
8. **Sasabe, J., Chiba, T., Yamada, M., Okamoto, K., Nishimoto, I., Matsuoka, M., and Aiso, S.:** D-Serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis, EMBO J., **26**, 4149–4159 (2007).
9. **Nagata, Y., Akino, T., and Ohno, K.:** Microdetermination of serum D-amino acids, Anal. Biochem., **150**, 238–242 (1985).
10. **Oguri, S., Nomura, M., and Fujita, Y.:** A new strategy for the selective determination of D-amino acids: enzymatic and chemical modifications for precolumn derivatization, J. Chromatogr. A, **1078**, 51–58 (2005).
11. **Pernot, P., Mothet, J.-P., Schuvailo, O., Soldatkin, A., Pollegioni, L., Pilone, M., Adeline, M.-T., Raymond Cespuglio, A., and Marinesco, S.:** Characterization of a yeast D-amino acid oxidase microbiosensor for D-serine detection in the central nervous system, Anal. Chem., **80**, 1589–1597 (2008).
12. **Visser, W. F., Verhoeven-Duif, N. M., Ophoff, R., Bakker, S., Klomp, L. W., Berger, R., and de Koning, T. J.:** A sensitive and simple ultra-high-performance-liquid chromatography–tandem mass spectrometry based method for the quantification of D-amino acids in body fluids, J. Chromatogr. A, **1218**, 7130–7136 (2011).
13. **Ruterbories, K. J. and Nurok, D.:** Thin-layer chromatographic separation of diastereomeric amino acid derivatives prepared with marfey's reagent, Anal. Chem., **59**, 2735–2736 (1987).
14. **Bhushan, R., Brückner, H., Kumar, V., and Gupta, D.:** Indirect TLC resolution of amino acid enantiomers after derivatization with Marfey's reagent and its chiral variants, J. Planar Chromatogr. Mod. TLC, **20**, 165–171 (2007).
15. **Hasegawa, H., Matsukawa, T., Shinohara, Y., and Hashimoto, T.:** Simultaneous determination of the enantiomers of leucine and [$^2H_7$]leucine in plasma by capillary gas chromatography–mass spectrometry, J. Chromatogr. B Biomed. Sci. Appl., **735**, 141–149 (1999).
16. **Bertrand, M., Chabin, A., Brack, A., and Westall, F.:** Separation of amino acid enantiomers VIA chiral derivatization and non-chiral gas chromatography, J. Chromatogr. A, **1180**, 131–137 (2008).
17. **Péter, A., Vékes, E., and Török, G.:** Application of (*S*)-*N*-(4-nitrophenoxycarbonyl) phenylalanine methoxyethyl ester as a new chiral derivatizing agent for proteinogenic amino acid analysis by high-performance liquid chromatography, Chromatographia, **52**, 821–826 (2000).
18. **Fuchs, S. A., de Sain-van der Velden, M. G. M., de Barse, M. M. J., Roeleveld, M. W., Hendriks, M., Dorland, L., Klomp, L. W. J., Berger, R., and de Koning, T. J.:** Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: potential confounders and age-dependent ranges, Clin. Chem., **54**, 1443–1450 (2008).
19. **Bhushan, R. and Tanwar, S.:** Reversed-phase liquid chromatographic determination of enantiomers of atenolol in rat plasma using derivatization with Marfey's reagent, Biomed. Chromatogr., **23**, 787–791 (2009).
20. **Armstrong, D. W., Duncan, J. D., and Lee, S. H.:** Evaluation of D-amino acid levels in human urine and in commercial L-amino acid samples, Amino Acids, **1**, 97–106 (1991).
21. **Brückner, H., Haasmann, S., and Friedrich, A.:** Quantification of D-amino acids in human urine using GC-MS and HPLC, Amino Acids, **6**, 205–211 (1994).

22. **Nagata, Y., Masui, R., and Akino, T.:** The presence of free D-serine, D-alanine and D-proline in human plasma, Experientia, **48**, 986–988 (1992).
23. **Hashimoto, A., Nishikawa, T., Oka, T., Takahashi, K., and Hayashi, T.:** Determination of free amino acid enantiomers in rat brain and serum by high-performance liquid chromatography after derivatization with *N-tert.*-butyloxycarbonyl-L-cysteine and *o*-phthaldialdehyde, J. Chromatogr. B Biomed. Sci. Appl., **582**, 41–48 (1992).
24. **Hashimoto, A., Nishikawa, T., Hayashi, T., Fujii, N., Harada, K., Oka, T., and Takahashi, K.:** The presence of free D-serine in rat brain, FEBS Lett., **296**, 33–36 (1992).
25. **Miyoshi, Y., Okamura, T., Konno, R., Kasai, N., Tojo, Y., and Zaitsu, K.:** Simultaneous two-dimensional HPLC determination of free D-serine and D-alanine in the brain and periphery of mutant rats lacking D-amino-acid oxidase, J. Chromatogr. B, **879**, 3184–3189 (2011).
26. **Nakano, Y., Konya, Y., Taniguchi, M., and Fukusaki, E.:** Development of a liquid chromatography-tandem mass spectrometry method for quantitative analysis of trace D-amino acids, J. Biosci. Bioeng., **123**, 134–138 (2017).
27. **Konya, Y., Taniguchi, M., and Fukusaki, E.:** Novel high-throughput and widely-targeted liquid chromatography–time of flight mass spectrometry method for D-amino acids in foods, J. Biosci. Bioeng., **123**, 126–133 (2017).
28. **Reischl, R. J., Hartmanova, L., Carrozzo, M., Huszar, M., Frühauf, P., and Lindner, W.:** Chemoselective and enantioselective analysis of proteinogenic amino acids utilizing N-derivatization and 1-D enantioselective anion-exchange chromatography in combination with tandem mass spectrometric detection, J. Chromatogr. A, **1218**, 8379–8387 (2011).
29. **Ishii, C., Akita, T., Mita, M., Ide, T., and Hamase, K.:** Development of an online two-dimensional high-performance liquid chromatographic system in combination with tandem mass spectrometric detection for enantiomeric analysis of free amino acids in human physiological fluid, J. Chromatogr. A, **1570**, 91–98 (2018).
30. **Gübitz, G. and Schmid, M. G.:** Chiral separation by chromatographic and electromigration techniques. A review, Biopharm. Drug Dispos., **22**, 291–336 (2001).
31. **Barata, K., Yoshida, M., Hokao, R., and Maekawa, A.:** Sequential alterations in clinical biochemical indicators of renal function in 5/6 nephrectomized rats. Basic study for renal toxicity using 5/6 nephrectomized rats, J. Toxicol. Sci., **23**, 433–442 (1998).
32. **Tsujimura, T., Nagamine, J., Sugaru, E., Ono-Kishino, M., Tokunaga, T., Kitoh, M., Nagata, R., Nakagawa, T., and Taiji, M.:** Combination therapy with SMP-534 and an angiotensin-converting enzyme inhibitor provides additional renoprotection in 5/6 nephrectomized rats, Biol. Pharm. Bull., **32**, 1991–1996 (2009).