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Development of an enantioselective three-dimensional HPLC system for the determination of alanine, valine, isoleucine, *allo*-isoleucine and leucine in human plasma and urine

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ABSTRACT

A three-dimensional (3D) HPLC system was designed/developed for the discriminative determination of aliphatic chiral amino acids, namely, alanine (Ala), valine (Val), isoleucine (Ile), *allo*-Ile (aIle) and leucine (Leu), in human physiological fluids. These aliphatic amino acid enantiomers are expected to be new physiologically active molecules and/or biomarkers in mammals. Among these aliphatic amino acids, the structural chain isomers of Leu (Ile/aIle/Leu) have similar chemical-physical properties, and the analytical method for these aliphatic amino acids is required to be highly enantio- and chemo-selective. In the present study, a reversed-phase column (Singularity RP18, first dimension) and a mixed-mode column (Singularity MX-103, second dimension) were utilized to separate these aliphatic amino acids as their scalemic mixtures, then chiral separations were performed using a Pirkle-type enantioselective column (Singularity CSP-001S) in the third dimension. By using the 3D-HPLC system, these aliphatic amino acid enantiomers were completely discriminated, and the analysis of these aliphatic chiral amino acids in the human plasma and urine was successfully carried out. The obtained amounts of the aliphatic amino acids (and %D value, the percentage of D-form to D + L forms) in the human plasma were 2.1 μM (0.5%) for D-Ala, and trace/not-detected for the other D-forms. In human urine, the values were 102.0 μM (24.7%) for D-Ala, 2.0 μM (3.4%) for D-Val, 2.3 μM (10.8%) for D-aIle (%D value of D-aIle was calculated using D-aIle and L-Ile) and 3.3 μM (5.5%) for D-Leu. The present 3D-HPLC system is a powerful and well validated tool for the simultaneous determination of aliphatic amino acid enantiomers, and further biological and clinical studies are expected.

1. Introduction

D-Amino acids are the enantiomers of L-amino acids, and it has been thought that the D-forms are useless substances in higher animals [1,2]. However, recent advances in analytical techniques have revealed the presence of D-amino acids in a variety of mammalian species [1–3]. Several D-amino acids were reported to have physiological functions in mammals, and also associated with diseases. Especially, D-aspartic acid (Asp) and D-serine (Ser) have been widely investigated [4–17]. D-Asp is localized in the endocrine tissues [4,5] and regulates hormonal synthesis and secretion [6,7]. D-Ser is present in the brain tissues, such as the cerebral cortex and hippocampus [8,9], and it was revealed to control neurotransmission [10]. Concerning the relation to diseases, the amount of D-Asp in the brain is reported to decrease with Alzheimer's disease [11], and the amount of D-Ser increases along with the progression of chronic kidney disease [12,13] and amyotrophic lateral sclerosis [14]. Besides D-Asp and Ser, several trace levels of the D-amino acids were found in mammals, and among them, aliphatic D-amino acids, namely, alanine (Ala), valine (Val), isoleucine (Ile), *allo*-Ile (aIle) and leucine (Leu) have frequently been found in several tissues and physiological fluids [18–26]. The structures of these five chiral amino acids are shown in Fig. 1. For instance, D-Ala is localized to the beta-cells in the Langerhans islets of the pancreas [18] and to the adrenocorticotropic hormone-producing cells in the anterior pituitary gland [19]. D-Ala is also found in the plasma [20,21], and the amount is related to kidney disfunction [12,13]. Concerning D-Leu, the presence

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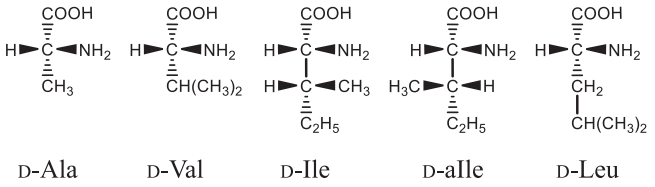

Fig. 1. Structures of D-Ala, Val, Ile, aIle and Leu.

in the plasma, urine and brain tissues has been revealed [20–24]. The other aliphatic D-amino acids, i.e., D-Val, Ile and aIle, have been found in the urine [24–26]. The chemical properties of these aliphatic D-amino acids and also the substrate specificity against the metabolizing enzyme (D-amino acid oxidase) are similar [27]. Therefore, these D-amino acids might still have unknown physiological functions and/or diagnostic significance in mammals, and their precise simultaneous determination in mammalian tissues and physiological fluids is highly expected. However, the simultaneous and enantioselective determination of these five aliphatic amino acids, especially the discrimination of Ile/aIle/Leu is difficult. Because Ile, aIle and Leu are isobaric structural isomers and have quite similar chemical properties, their separation is practically difficult using both current chromatographic and also mass spectrometric approaches. The enantiomers of these Leu structural isomers were occasionally found in the plasma of the patients with chronic kidney disease, and were suggested to have diagnostic values [12]. Therefore, development of a practically useful simultaneous and enantioselective analytical method for the determination of these five aliphatic amino acids to clarify their biological significance is expected.

For the determination of chiral aliphatic amino acids, several chromatographic approaches including LC-MS or LC-MS/MS [26,28–31] and two-dimensional (2D) HPLC [24,32] methods have been reported. For the LC-MS (or MS/MS) systems, chiral derivatization reagents [26,28,29] and chiral stationary phases [30,31] have been widely adopted. Concerning the chiral derivatization, a variety of reagents, such as (*R*)-4-nitrophenyl-*N*-[2-(diethylamino)-6,6-dimethyl-[1,1-biphenyl]-2-yl]carbamate hydrochloride ((*R*)-BiAC) [26,28] and N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (L-FDAA) [29] are utilized, and the diastereomeric amino acid derivatives are separated by a reversed-phase column. The aliphatic amino acid enantiomers were also determined using the LC-MS/MS systems with chiral stationary phases, and the native amino acids were separated by a Crownpak CR-I (+) column [30]. By using a Chiralpak QN-AX column, the amino acid enantiomers derivatized with 6-methoxyquinoline-4-carboxylic acid-*N*-succinimidylester (MQ-OSu) were separated [31]. As the 2D-HPLC methods, several achiral derivatization reagents, e.g., 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [24] and 5-(dimethylamino)naphthalene-1-sulfonyl chloride (Dns-Cl) [32], have been utilized. In these approaches, NBD or Dns derivatized amino acids were isolated on a reversed-phase column (first dimension), and chiral separations were carried out on the enantioselective columns, such as Chiralpak QN/QD-AX (second dimension). These LC-MS (or MS/MS) and 2D-HPLC approaches have been widely used for the selective analysis of D-amino acid in the biological samples containing uncountable interfering substances. However, the separation of the Ile/aIle/Leu structural isomers is always difficult by using both reversed-phase and enantioselective columns, and the simultaneous determination completely discriminating the Ile/aIle/Leu isomers has still not been accomplished even when adopting these LC-MS (or MS/MS) and 2D-HPLC methods, although these Leu analogs are expected to be new biomarkers and/or physiologically active substances in mammals.

To acquire higher chemoselectivity, three-dimensional (3D) HPLC combining the reversed-phase (1D), anion-exchange/mixed-mode (2D) and enantioselective (3D) columns is an effective and straightforward approach [5,13,33]. Using the 3D-HPLC method, structural chain isomers of Ile/aIle/Leu are expected to be separated as their D/L-mixtures in combination of the first and second dimensions, and the chiral separations are accomplished in the third dimension. In the present study, therefore, a novel 3D-HPLC method was designed and developed for the simultaneous determination of the five aliphatic chiral amino acids including Ile/aIle/Leu, and the method was applied to the enantioselective amino acid analysis in the human plasma and urine.

2. Experimental

2.1. Materials

D-Ala, L-Ala, DL-Ala, D-Leu and L-Leu were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). D-Ile, L-Ile, D-aIle and L-aIle were purchased from Merck (Darmstadt, Germany). DL-Ile and DL-aIle were products of Watanabe Chemical Industries (Hiroshima, Japan) and D-Val, L-Val and DL-Leu were from Nacalai Tesque (Kyoto, Japan). DL-Val and NBD-F were products of Tokyo Chemical Industry (Tokyo, Japan). Methanol (MeOH) of HPLC grade, boric acid, formic acid (FA) and trifluoroacetic acid (TFA) were purchased from FUJIFILM Wako Pure Chemical Corporation. Acetonitrile (MeCN) of HPLC grade was obtained from Nacalai Tesque. Water was purified using a Milli-Q Integral 3 system (Merck). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Sample preparation

The human blood and urine samples were obtained from 6 healthy volunteers (male, 21–24 years old, from whom informed consent was obtained) at 8 AM after one night of fasting. The human blood samples were collected in Venoject II blood collection tubes (EDTA II Na, Terumo, Tokyo, Japan), and centrifuged at 1200 × *g* at 4°C for 10 min to obtain the plasma. The acquired plasma and urine samples were stored at − 30°C until used. The experiments were approved by the review board of the Graduate School of Medical Science, Kyushu University (approval number 2019–182). To 20 μL of the human plasma, 20 μL of water and 360 μL of MeOH were added and thoroughly mixed, then the solution was centrifuged at 12,000 × *g* for 5 min to obtain the supernatant. A portion (10 μL) of the collected supernatant was dried under reduced pressure at 40°C for 10 min, and the residue was re-dissolved by water (10 μL). To the solution, 10 μL of 400 mM sodium borate buffer (pH 8.0) and 5 μL of 40 mM NBD-F/MeCN were added, and the mixed solution was heated at 60°C for 2 min. An aqueous 0.2% TFA solution (75 μL) was added to the reaction mixture, and the solution (10 μL) was injected into the 3D-HPLC system described in Section 2.3. The human urine sample was 10 times diluted with water before the derivatization, and a portion (10 μL) of the diluted urine sample was derivatized with NBD-F using the same procedure as that for the plasma sample.

2.3. 3D-HPLC conditions

For the 3D-HPLC analysis, a NANOSPACE SI-2 3202 degasser, 3101 and 3201 pumps, a 3023 auto sampler, 3004 and 3014 column ovens, two 3012 high-pressure valves and a 9986 multi-loop valve with four loops of 500 μL (Shiseido, Tokyo, Japan) were used. For the control of these valves, a Singularity valve controlling system was adopted and an EZChrom Elite was employed for the data processing. For detection, a Singularity LED-3 fluorescence detector was used. The flow diagram is shown in Fig. 2. In the first dimension, a Singularity RP18 column (1.0 mm i.d. × 250 mm, 3-μm particle size) was utilized for the reversed-phase separation of the NBD-amino acids at 45 °C. The mobile phase was an aqueous 24% MeCN 0.01% TFA solution, and the flow rate was 50 μL/min. The fractions of NBD-Ala (collected for 2 min, 100 μL), NBD-Val (3 min, 150 μL), NBD-Ile/aIle (NBD-Ile and aIle were collected together in the same loop for 6 min, 300 μL) and NBD-Leu (5 min, 250 μL) were automatically collected into the multi-loop valve and introduced into the mixed-mode column (having anion-exchange,

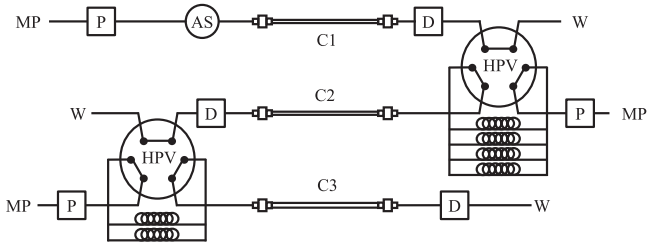

Fig. 2. Flow diagram of the 3D-HPLC system: C1, Singularity RP18 (1.0 mm i.d. × 250 mm); C2, Singularity MX-103 (1.0 mm i.d. × 250 mm); C3, Singularity CSP-001S (1.5 mm i.d. × 250 mm); AS, auto sampler; D, detector; HPV, high-pressure valve; MP, mobile phase; P, pump; W, waste.

hydrogen-bonding and π-π interactions) representing the second dimension. The mixed-mode column was a Singularity MX-103 (1.0 mm i.d. × 250 mm, 5-μm particle size [34]), and the column temperature was 25°C. The mobile phases were mixed solutions of MeCN-MeOH (50/50, v/v) containing 0.01% FA (for NBD-Leu), 0.02% FA (for NBD-Val and Ile/aIle) or 0.05% FA (for NBD-Ala), and the flow rate was 100 μL/min. The fractions of NBD-Ala (4 min, 400 μL), NBD-Val (4 min, 400 μL), NBD-Ile (4 min, 400 μL), NBD-aIle (3 min, 300 μL) and NBD-Leu (4 min, 400 μL) were again collected into a high-pressure valve (having two loops of 600 μL) and introduced into the enantioselective column representing the third dimension. For the enantiomer separations of the target NBD-amino acids, a Singularity CSP-001S column (1.5 mm i.d. × 250 mm, 5-μm particle size) was used at 25 °C. As for the mobile phases, mixed solutions of MeCN-MeOH (50/50, v/v) containing 0.06% FA (for NBD-Val, Ile, aIle and Leu) or 0.08% FA (for NBD-Ala) were utilized, and the flow rate was 200 μL/min. The NBD-amino acids were detected by the fluorescence emission at 530 nm with excitation at 470 nm. The design and development of Singularity instruments and columns used in the present study were conducted in our previous studies [5,16,34].

3. Results and discussion

3.1. Development of a 3D-HPLC system for the determination of aliphatic amino acid enantiomers

An on-line 3D-HPLC system was developed for the determination of Ala, Val, Ile, aIle and Leu in human physiological fluids (plasma and urine). Pre-column derivatization with NBD-F was carried out to improve the detection sensitivity of the amino acids, and a portion of the reaction mixture was subjected to the 3D-HPLC system. The 3D-HPLC system consisted of three different separation modes, i.e., reversed-phase separation (1D), mixed-mode separation (2D) and enantioselective separation (3D). In the first reversed-phase separation, the target amino acids were separated from other amino acids and intrinsic substances by the difference in their hydrophobicity, and the D- and L-forms of the target amino acids were collected as their scalemic mixtures. The fractions were introduced into the second dimension, where the target amino acids were further purified. After re-collecting the target amino acids, the fractions were introduced into the third dimension, and the amino acid enantiomers were separated. To develop the 3D-HPLC system, the analytical conditions of all three dimensions were investigated. For the first dimension, a Singularity RP18 column (1.0 × 250 mm) was adopted, and aqueous 0.01% TFA solutions containing various MeCN concentrations were tested as the mobile phase to separate the target NBD-amino acids. As a result, the target amino acids (NBD-Ala, Val, Ile, aIle and Leu) were separated within 90 min when using 24% MeCN 0.01% TFA in water. Under these conditions, NBD-Ala, Val and Leu were clearly separated, however, the separation of NBD-Ile and aIle was difficult. Therefore, the analytical conditions in the second dimension were investigated focusing on the separation of the NBD-Ile and NBD-aIle.

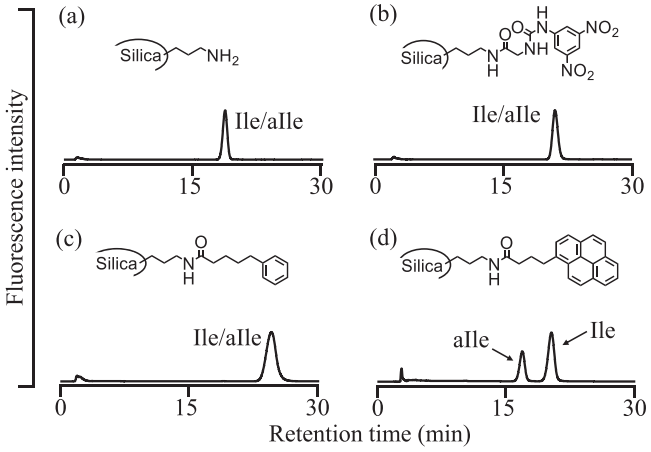

Fig. 3. Separations of NBD-Ile/aIle by using Singularity (a) AX, (b) MX-001, (c) MX-102 and (d) MX-103 columns.

In the second dimension, an anion-exchange column (Singularity AX, 1.0 × 150 mm) and the originally designed/developed mixed-mode columns (Singularity MX-001, MX-102 and MX-103, 1.0 × 250 mm) were tested as the stationary phases. For all the columns, mixed solutions of MeCN/MeOH (50/50, v/v) containing FA were adopted as the mobile phases and the separation of NBD-Ile and aIle was evaluated. The concentration of FA was adjusted to retain NBD-Ile around 20 min, and 0.02% FA was used for AX, MX-102 and MX-103, while 0.03% FA was used for MX-001. The obtained chromatograms are shown in Fig. 3. Using the Singularity AX, MX-001 and MX-102 columns, NBD-Ile and NBD-aIle were not separated at all (Fig. 3a–c). On the other hand, the two diastereomeric Ile analogs were completely separated by a Singularity MX-103 column with a resolution (*Rs*) value of 2.7 (Fig. 3d). Therefore, a Singularity MX-103 column was selected as the stationary phase for the 2D, and the mobile phases for the other three target NBD-amino acids were investigated to have the appropriate retention around 20 min. As a result, the mobile phases were determined as follows: mixed solutions of MeCN/MeOH (50/50, v/v) containing 0.05% FA for NBD-Ala, 0.02% FA for NBD-Val and 0.01% FA for NBD-Leu. For the third dimension, a Singularity CSP-001S column (1.5 × 250 mm) was selected as the stationary phase, and mixed solutions of MeCN/MeOH (50/50, v/v) containing FA were adopted as the mobile phases. The FA concentration was modulated to have sufficient chiral recognitions (*Rs* values were around 2.0 or higher) within 30 min, and 0.08% FA was used for NBD-Ala and 0.06% FA was used for the other four NBD-amino acids. The selected conditions for all the dimensions were integrated, and an on-line 3D-HPLC system was developed. Fig. 4 shows the chromatograms obtained for all the five target NBD-amino acids using the present 3D-HPLC system. The five amino acids were separated/purified in the first/second dimensions, followed by the enantiomer separations in the third dimension with the total analysis time of 300 min. Because Ile and aIle were separated in the second dimension, using the 3D-HPLC system was essential for the determination of all target chiral amino acids separately. The *Rs* values of the enantiomers obtained in the third dimension were 3.1 for Ala, 2.2 for Val, 1.9 for Ile, 2.7 for aIle and 4.8 for Leu. The fraction volumes injected from first dimension to second dimension were 100–300 μL, and those injected from second dimension to third dimension were 300–400 μL. These fraction volumes are large considering the size of the columns integrated in the second and third dimensions. However, mobile phase compatibility was well regulated and no peak broadening due to the volume overload effect was observed as shown in Fig. 4.

The developed 3D-HPLC system was validated by the calibration curves, intra-day precision and inter-day precision using the standard amino acid enantiomers. The precision and accuracy were also checked using human plasma and urine samples. Table 1 shows the calibration

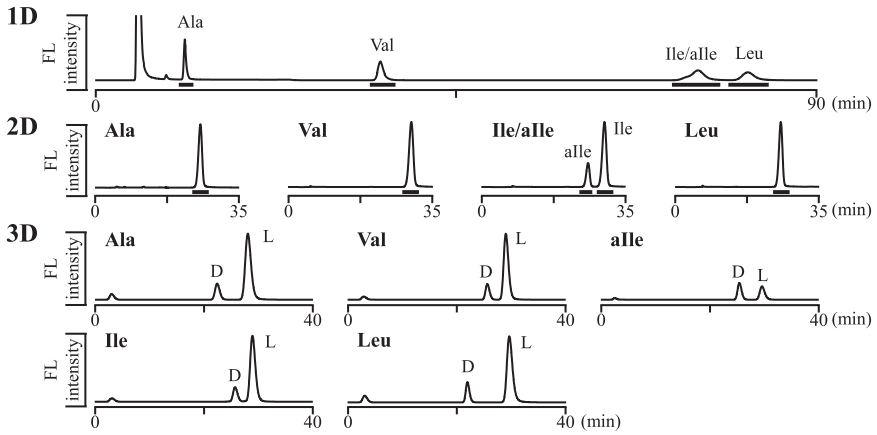

Fig. 4. 3D-HPLC separations of target amino acid enantiomers. The injection amounts were 2 pmol for D-Ala, D-Val, D-aIle, L-aIle, D-Ile and D-Leu, and 10 pmol for L-Ala, L-Val, L-Ile and L-Leu.

curves and precision of the standard amino acid enantiomers. The calibration ranges were 0.02–20 pmol (injection amounts) for D-Ala, D-Val, D-Ile, D-aIle, L-aIle and D-Leu, and 0.1–100 pmol for the proteinogenic L-Ala, L-Val, L-Ile and L-Leu. The amounts of amino acids were the injection amounts to the first dimension, and the fractions were collected quantitatively between the first/second and second/third dimensions. Therefore, the target chiral amino acids could be determined directly in the third dimension. All of the calibration curves showed satisfactory linearity with correlation coefficients (r^2) higher than 0.9999. For the precision, the intra-day precision and inter-day precision were checked by using the standard amino acid solutions containing 0.05 and 5 pmol of D-Ala, D-Val, D-Ile, D-aIle, L-aIle and D-Leu; 0.25 and 25 pmol of L-Ala, L-Val, L-Ile and L-Leu. The obtained RSD values of the intra-day precision were 0.86–2.72% and those of the inter-day precision were 0.46–3.32% (n = 4). The precision and accuracy checked by using the human plasma and urine are summarized in Table 2. To evaluate the precision for the human physiological fluids, the intrinsic amounts of the chiral amino acids were used, and the RSD values of the intra-day precision and inter-day precision acquired for human plasma were 0.70–1.90% and 1.90–3.57%, respectively (n = 4). In the urine, the RSD values of the intra-day precision were 0.90–2.71% and those of the inter-day precision were 1.46–3.43% (n = 4). Regarding the accuracy, the standard amino acids were added to the human plasma and urine, and the obtained accuracy values were 103.3–114.9% and 95.0–104.0%, respectively. These results indicated that the developed 3D-HPLC system is applicable to the enantioselective determination of the five target amino acids in the human plasma and urine.

Until now, a variety of reports has been published for the simultaneous determination of aliphatic D-amino acids. LC-MS and LC-MS/MS techniques are useful for the purpose, and several methods using chiral derivatization reagents [26,28,29] and chiral stationary phases [30,31] have been developed. As the chiral derivatization reagents, a number of reagents including (*R*)-BiAC [26,28] and L-FDAA [29] were utilized. By these methods, the aliphatic amino acid enantiomers were separated as their diastereomeric derivatives mainly by the reversed-phase column; a YMC Triart Phenyl column for (*R*)-BiAC, and an Agilent Zorbax SB-C_3 column for L-FDAA. The analysis time of the (*R*)-BiAC method was 15 min, and that of the L-FDAA approach was 45 min. However, L-Ile and L-aIle were not separated by the (*R*)-BiAC method, and the separations of D-Ile/D-Leu and L-Ile/L-Leu were not achieved by the L-FDAA method. Concerning the LC-MS/MS methods using the chiral stationary phases, several enantioselective columns (e.g., Chiralpak QN/QD-AX [31] and Crownpak CR-I(+) columns [30]) with/without achiral pre-column derivatization have also been reported. By using the Chiralpak QN/QD-AX columns, aliphatic amino acids (derivatized with MQ-OSu) were enantio-separated within 15 min, however, the separations of D-Ile/D-aIle and L-Ile/L-aIle were insufficient. By using the Crownpak CR-I(+) column, the enantiomeric pairs of underivatized aliphatic amino acids were separated within 5 min, however, the discrimination of D-Ile and D-aIle was not accomplished. 2D-HPLC systems combining two different separation modes have also been reported for the simultaneous determination of aliphatic amino acid enantiomers. Until now, 2D-HPLC methods adopting NBD-F [24] and Dns-Cl [32] as the achiral fluorescence derivatization reagents were reported. Using

Table 1
Method validation for 3D-HPLC determination of standard target amino acid enantiomers.

Amino acids	Calibration curve			Precision (RSD, %)			
	Range (pmol/inj)	Equation	r^2	Intra-day		Inter-day	
				Low	High	Low	High
D-Ala	0.02–20	y = 12.9x + 0.2	0.9999	1.94	1.85	1.32	2.90
L-Ala	0.1–100	y = 10.7x + 0.7	1.0000	2.01	2.06	2.21	2.92
D-Val	0.02–20	y = 20.6x + 0.3	0.9999	1.07	1.94	0.65	0.46
L-Val	0.1–100	y = 17.3x + 1.5	0.9999	0.86	2.10	1.25	0.70
D-Ile	0.02–20	y = 15.4x − 0.5	1.0000	2.29	2.72	1.48	2.66
L-Ile	0.1–100	y = 13.9x − 2.0	1.0000	2.30	2.60	1.47	2.68
D-aIle	0.02–20	y = 17.7x − 0.3	1.0000	1.71	1.19	1.33	3.32
L-aIle	0.02–20	y = 14.1x − 0.3	1.0000	1.69	0.92	1.38	3.20
D-Leu	0.02–20	y = 15.5x − 0.2	1.0000	1.79	1.84	2.15	1.64
L-Leu	0.1–100	y = 10.2x − 1.1	1.0000	1.50	1.77	2.12	1.73

Equations were made where x was the injection amount of the amino acid enantiomers (pmol) and y was the peak height (mV). Injection amounts of D-Ala, D-Val, D-Ile, D-aIle, L-aIle and D-Leu were 0.02, 0.05, 0.2, 0.5, 2, 5 and 20 pmol. Those of L-Ala, L-Val, L-Ile and L-Leu were 0.1, 0.25, 1, 2.5, 10, 25 and 100 pmol. For the precision, 0.05 and 5 pmol of D-Ala, D-Val, D-Ile, D-aIle, L-aIle and D-Leu, 0.25 and 25 pmol of L-Ala, L-Val, L-Ile and L-Leu were injected (n = 4).

Table 2
Precision and accuracy of target amino acid enantiomers in human physiological fluids.

Amino acids	Human plasma			Human urine		
	Precision (RSD, %)		Accuracy (%)	Precision (RSD, %)		Accuracy (%)
	Intra-day	Inter-day		Intra-day	Inter-day	
D-Ala	1.83	2.25	103.3	1.60	2.61	101.2
L-Ala	1.77	2.06	112.9	1.49	2.64	100.3
D-Val	–	–	107.2	0.93	2.40	95.0
L-Val	1.00	3.30	111.7	0.90	2.29	100.9
D-Ile	–	–	105.7	–	–	95.0
L-Ile	1.89	1.90	107.5	1.02	1.46	96.8
D-aIle	–	–	110.9	1.70	2.69	97.6
L-aIle	0.70	2.60	112.0	2.71	3.43	96.5
D-Leu	–	–	111.6	0.98	2.69	99.7
L-Leu	1.90	3.57	114.9	0.95	2.77	104.0

For the precision of human plasma and urine, the intrinsic amino acids were determined. For the accuracy, 0.5 pmol (per injection) of D-Ala, D-Val, D-Ile, D-aIle, L-aIle and D-Leu and 15 pmol of L-Ala, L-Val, L-Ile and L-Leu were added to human plasma, and 10 pmol of D-Ala, 30 pmol of L-Ala, 1 pmol of D-Val, D-Ile, D-aIle, L-aIle and D-Leu and 5 pmol of L-Val, L-Ile and L-Leu were added to human urine.

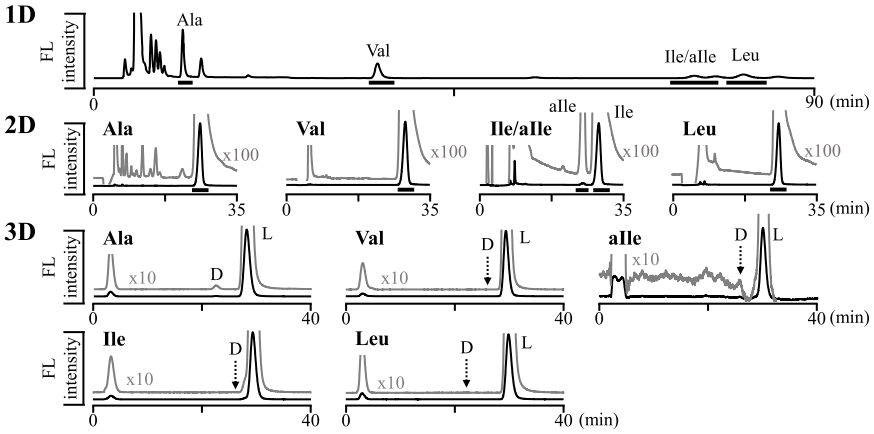

Fig. 5. 3D-HPLC determination of target amino acid enantiomers in human plasma.

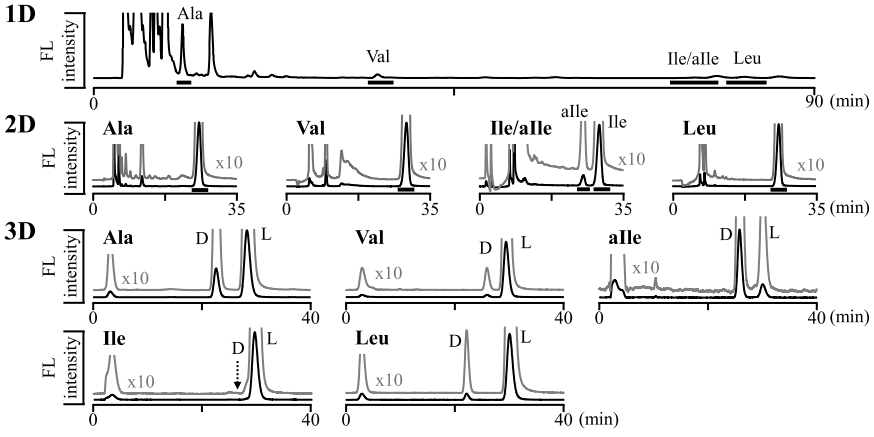

Fig. 6. 3D-HPLC determination of target amino acid enantiomers in human urine.

these methods, the NBD or Dns derivatized amino acids were separated in the first dimension as their D/L-mixtures by the reversed-phase column (a Capcell pak C18 MG II column for the NBD-amino acids and a GraceSmart RP18 column for the Dns-amino acids), and the enantiomers were separated in the second dimension by the chiral stationary phase (Chiralpak QN/QD-AX columns). However, by using these methods, the complete separations of NBD-Ile/aIle, NBD-Ile/Leu, Dns-Ile/Leu and Dns-aIle/Leu were not accomplished in the first dimension, and the precise determination of the Ile/aIle/Leu enantiomers was practically difficult. In the present study, an additional mixed-mode separation (2D) was introduced between the reversed-phase (1D) and enantioselective (3D) separations to acquire higher chemoselectivity. By using a mixed-mode Singularity MX-103 column [34], the separation of the NBD-Ile/aIle derivatives was achieved. The developed 3D-HPLC system enables the complete separation of the five target aliphatic NBD-amino acids in the first and second dimensions as their D + L mixtures, and their enantiomers were separated in the third dimension. Sufficient validation results were also obtained using both standard amino acids and human physiological fluids. These results indicated that the developed 3D-HPLC method is practically useful for

Table 3
Amounts of target amino acid enantiomers in human physiological fluids.

Amino acids		Plasma		Urine	
		μM	%D	μM	%D
Ala	D	2.1 ± 0.5	0.5 ± 0.1	102.0 ± 29.2	24.7 ± 5.1
	L	451.7 ± 58.3		287.7 ± 38.0	
Val	D	n.d.	–	2.0 ± 0.5	3.4 ± 0.9
	L	336.0 ± 43.7		57.4 ± 8.7	
Ile	D	n.d.	–	tr.	–
	L	99.3 ± 13.2		18.0 ± 2.5	
aIle	D	n.d.	–	2.3 ± 0.6	10.8 ± 2.4[a]
	L	2.9 ± 0.4		0.6 ± 0.1	
Leu	D	tr.	–	3.3 ± 1.3	5.5 ± 2.2
	L	203.5 ± 25.4		51.1 ± 6.0	

Values represent means ± SE of six plasma and urine samples. %D = D/(D + L) × 100. n.d.: Not detected. tr.: Trace.
[a] The %D value of aIle was calculated as follows; %D-aIle = D-aIle/(D-aIle + L-Ile) × 100.

the precise determination of the target aliphatic amino acid enantiomers in biological samples, and was applied to the analyses of human plasma and urine in Section 3.2.

3.2. Determination of aliphatic amino acid enantiomers in human physiological fluids

The developed 3D-HPLC system was applied to the analysis of the Ala, Val, Ile, aIle and Leu enantiomers in human plasma and urine samples (healthy male, 21–24 years old, sample information was shown in Section 2.2.). The obtained chromatograms are shown in Figs. 5 and 6, respectively. For the 3D-HPLC analysis of the real samples, the fractions of the target NBD-amino acids were collected at the same retention times as those of the standard NBD-amino acids. In the first and second dimensions, many unknown peaks were observed (e.g. a peak eluted between Ile/aIle and Leu in the first dimension). However, the five target NBD-amino acids were separated from these unknown compounds by the reversed-phase and mixed-mode columns, and the determination of the target enantiomers was successfully carried out in the third dimension. The amounts of the amino acid enantiomers and their %D values (%D = D/(D + L) × 100) are summarized in Table 3. Concerning D-aIle, the percentage of the D-form was calculated against L-Ile (%D-aIle = D-aIle/(D-aIle + L-Ile) × 100), because it is considered that D-aIle is produced from L-Ile in living organisms via the stereoconversion of the α-carbon. For the human plasma, the amount of D-Ala was 2.1 ± 0.5 μM and the %D value was 0.5 ± 0.1%. The peaks of the other target D-amino acids were not detected. For the human urine, all of the target D-amino acids, except for D-Ile, could be determined, and a trace level of the D-Ile peak was observed. The concentrations (and %D values) of D-Ala, D-Val, D-aIle and D-Leu were 102.0 ± 29.2 μM (24.7 ± 5.1%), 2.0 ± 0.5 μM (3.4 ± 0.9%), 2.3 ± 0.6 μM (10.8 ± 2.4%) and 3.3 ± 1.3 μM (5.5 ± 2.2%), respectively.

To confirm these results, the tested six plasma and urine samples were mixed, and the amounts of the D-amino acids (and %D values) were compared using different analytical conditions. For the analysis, the mobile phase conditions (especially the composition of organic solvents) in the third dimension were changed. For the mobile phases in the third dimension, MeCN/MeOH (25/75, v/v) containing 0.06–0.09% FA were utilized. In the plasma, the obtained D-Ala amount was 1.4 μM (0.50%), and the peaks of the other target D-amino acids were not observed. For the human urine, the amounts (and %D values) were 96.8 μM (26.3%) for Ala, 1.9 μM (3.2%) for Val, 2.2 μM (11.6%) for aIle and 2.7 μM (5.7%) for Leu, and a trace level of D-Ile was detected. These results were quite consistent with those obtained using the standard analytical conditions adopting mobile phases containing MeCN/MeOH (50/50, v/v), indicating that the amounts of the target amino acid enantiomers in the human plasma and urine were correctly determined by the present 3D-HPLC system.

Until now, the presence of several aliphatic D-amino acids has been reported in the human plasma and urine. For the plasma, the concentrations of D-Ala have been reported as 0.3–1.8 μM, and the %D values were 0.2–0.5% [21,35,36]. The amounts of the other D-amino acids were reported to be extremely low. In the present study, the D-Ala amount (and %D value) was 2.1 μM (0.5%), and trace/not-detected for the other aliphatic D-amino acids. Concerning aIle, only the L-forms were determined. These aliphatic D-amino acid amounts obtained in the present study were consistent with those in the previous reports. For the urine, the amounts of the D-amino acids have been reported to be 2.9–84.5 μM for Ala, 0–3.7 μM for Val and 0–1.4 μM for Leu, and the reported %D values of Ala, Val and Leu were 5.7–39.4%, 0–4.2% and 1.6–3.6%, respectively [24,25,36–38]. The amounts of these D-amino acids obtained in the present study were 102.0 μM for D-Ala, 2.0 μM for D-Val and 3.3 μM for D-Leu, and the %D values were 24.7%, 3.4% and 5.5%, respectively. Additionally, the D- and L-forms of aIle were clearly determined and the concentrations were 2.3 μM and 0.6 μM, respectively. These values of Ala, Val and Leu were in good agreement with those previously reported, and this is the first report showing the discriminative determination of D/L-aIle in human urine to the best of our knowledge. In the present study, the simultaneous and enantioselective determination of five target aliphatic amino acids was accomplished for the first time in human plasma and urine without interference by intrinsic substances. These results indicated that the selectivity of the present 3D-HPLC system was sufficient for the enantioselective determination of five aliphatic amino acids, and further investigations are expected.

4. Conclusion

In the present study, a 3D-HPLC system was developed for the enantioselective determination of aliphatic amino acids (Ala, Val, aIle, Ile and Leu). By using this system, these aliphatic amino acid enantiomers were completely separated as their NBD derivatives, and good validation results were obtained. The developed 3D-HPLC system was applied to the analyzes of human plasma and urine, and D-Ala was determined in both matrices. The other D-amino acids were trace or not detected in the plasma, however, in the urine, D-Val, D-aIle and D-Leu were quantified, and a trace amount of D-Ile was observed. To the best our knowledge, this is the first report showing the simultaneous and precise determination of these five aliphatic amino acids including Ile/aIle/Leu in human physiological fluids. These results indicated that the present 3D-HPLC is a useful approach for the analysis of these aliphatic amino acid enantiomers in biological samples, and would be applicable to various matrices due to the high enantio- and chemo-selectivity. Further investigations using the tissue and clinical samples to clarify the distributions, physiological significance and relation to diseases are expected.

CRediT authorship contribution statement

Yuri Nagata: Investigation, Writing – original draft. **Takeyuki Akita:** Investigation. **Chiharu Ishii:** Investigation. **Mai Oyaide:** Investigation. **Masashi Mita:** Investigation. **Tomomi Ide:** Investigation. **Kenji Hamase:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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