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Automated and simultaneous two-dimensional micro-high-performance liquid chromatographic determination of proline and hydroxyproline enantiomers in mammals☆

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ABSTRACT

A fully automated 2D-HPLC system employing a microbore-ODS column and a narrowbore-enantioselective column has been developed for the simultaneous enantiomer determination of proline, *trans*-4-hydroxyproline and *cis*-4-hydroxyproline in mammals. As a first dimension, a monolithic ODS column of 0.53 mm i.d. showed 3–6 times larger theoretical plate numbers than those of particle-packed ODS columns, and the best enantioseparations were obtained by a Chiralpak QN-2-AX column of 1.5 mm i.d. in the second dimension (separation factors: 1.44–1.83). The R.S.D. values for within-day and day-to-day precisions were less than 5.8%, and the lower limits of quantitation for the D-enantiomers were 1 fmol. The present method was successfully applied to the determination of proline and hydroxyproline enantiomers in the serum and collagen-rich skin tissue. Small amounts of D-proline were found both in the serum (1.57 ± 0.19 nmol/mL) and in the skin (0.093 ± 0.015 nmol/mg protein), while the amounts of D-hydroxyproline were smaller than the lower limit of quantitation.

1. Introduction

Most of the proteinogenic and non-proteinogenic amino acids have a chiral center at the alpha position, and only L-enantiomers were thought to exist in higher animals such as mammals. However, recent studies revealed that several D-amino acids are present widely in mammals, and some of them have also been reported to play important physiological roles [1–3]. Especially, a relatively large amount of D-serine (D-Ser) was found in the forebrain and has been thought to activate the neurotransmission on the *N*-methyl-D-aspartate (NMDA) receptors [4–7]. D-Aspartic acid (D-Asp) has been reported to regulate the synthesis and/or secretion of specific hormones such as prolactin and testosterone in various neuroendocrine and endocrine organs [8–11]. With the progress of analytical technologies, several D-amino acids of small amount, such as D-alanine [12,13], branched aliphatic D-amino acids [14,15], D-proline (D-Pro) [16–18] and D-threonine [19], have also been found in mammals, and the tissue distribution and alteration of their amounts have partially been revealed. These D-amino acids are also thought to be candidates for novel physiologically active substances or biomarkers [1], and further studies using sensitive and reliable analytical methods are highly recommended.

Concerning D-Pro, a small amount of this D-amino acid in free form was found in a variety of mouse tissues, and was also excreted in the urine in a relatively large amount [20]. Because several reports show the nephrotoxicity and neurotoxicity of D-Pro orally or intraventricularly administered to rats and chicks [21–23], elucidation of the origin of intrinsic free D-Pro as well as the relationships between metabolically relevant compounds are highly expected. In mammals, a large part of Pro is present as amino acid residues in collagens, which are one of the most abundant proteins distributed ubiquitously in the entire body. About a quarter of the amino acid residues in mammalian collagens is Pro, and nearly 40% of these Pro residues are enzymatically metabolized to 4-hydroxyproline (Hyp) [24,25]. These Pro and Hyp form a part of a free amino acid pool via the degradation reactions mainly by collagenase and protease through the metabolic turnover of collagen proteins. Therefore, for elucidating the origin, metabolism and physiological function of free D-Pro in mammals, collagens are one of the important candidates having connection to the formation of the D-form, and the

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enantiomers of both Pro and a metabolically relevant analog, Hyp, should be considered.

The aim of the present study is to establish a simultaneous analytical method for the determination of Pro and Hyp enantiomers, and to clarify the intrinsic amounts of the individual enantiomers in mammalian samples including collagen-rich tissues. The present investigation is useful for obtaining fundamental information to elucidate the origins and physiological meanings of Pro and Hyp enantiomers. Several approaches have been reported to determine D/L-Pro in mammals, e.g., a reversed-phase HPLC method combined with 2D-TLC [16], a 2D-HPLC method using a reversed-phase column and a cyclodextrin-bonded enantioselective column [17], and a GC–MS method employing a chiral capillary column [26]. By using these methods, the amounts of free D-Pro in the blood, urine, liver and kidney have been determined [16,17,26,27]. However, their applications were limited to abundant physiological fluids or large organs, because the amounts of D-Pro in mammalian tissues are too small in most cases. Concerning the determination of Hyp enantiomers in biological samples, an HPLC-MS method has been reported [28] to determine three Hyp isomers (*trans*-4-hydroxy-L-Pro, *cis*-4-hydroxy-L-Pro and *cis*-4-hydroxy-D-Pro) in the type I collagen hydrolysate. However, to our knowledge, there is no report on a two-dimensional method for the simultaneous determination of small amounts of D/L-Pro and all four isomers of 4-Hyp in mammalian tissues. In order to determine small amounts of D-Pro in mammalian tissues, we have already reported a highly sensitive and selective 2D-HPLC system combining a micro-reversed-phase column and an enantioselective column [18]. D-Pro was determined after pre-column fluorescence derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and the amounts of free D-Pro in seven brain tissues, eleven peripheral tissues, blood and urine of rats were clarified [18,20]. This 2D-HPLC method has sufficient sensitivity and selectivity for the determination of small amounts of D-amino acid in real biological samples, and the same concept is thought to be advantageous to establish a method for the simultaneous determination of Pro and Hyp enantiomers in mammals.

In the present investigation, a highly sensitive and selective 2D-HPLC system has been established for the simultaneous determination of the enantiomer pairs of Pro, *trans*-Hyp and *cis*-Hyp in mammalian samples. This system employs a program-controlled column switching valve combining a monolithic microbore-reversed-phase column and a narrowbore-enantioselective column, and a fully automated analysis could be performed without any complicated operations. By using this system, the amounts of six enantiomers of Pro and Hyp in mouse serum and collagen-rich skin tissue have been determined.

2. Experimental

2.1. Chemicals

trans-4-Hydroxy-L-proline (*trans*-L-Hyp), *cis*-4-hydroxy-L-proline (*cis*-L-Hyp) and *cis*-4-hydroxy-D-proline (*cis*-D-Hyp) were purchased from Sigma–Aldrich (Steinheim, Germany), and *trans*-4-hydroxy-D-proline (*trans*-D-Hyp) was obtained from Bachem (Bubendorf, Switzerland). Enantiomers of Pro, methanol (MeOH) of HPLC grade, trifluoroacetic acid (TFA) and citric acid monohydrate were from Wako (Osaka, Japan). The derivatizing reagent NBD-F and acetonitrile (MeCN) of HPLC grade were obtained from Tokyo Kasei (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Water was purified using a Milli-Q gradient A 10 system (Millipore, Bedford, MA, USA). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Animals

Male HR-1 hairless mice (20-week-old, specific-pathogen-free) were obtained from Hoshino (Yashio, Japan). They were housed under a 12-h light/12-h dark cycle (lights on at 7:00 am) and given free access to food (type NMF, Oriental Yeast, Tokyo, Japan) and water. All experiments were performed with the permission (A19-060-0) of the animal care and use committee of Kyushu University, and were approved by the animal research committee of the Shiseido Research Center in accordance with the National Research Council Guide (1996).

2.3. Sample preparation procedure

The mice were anesthetized with diethyl ether and euthanized by exsanguination from the carotid artery, and the skin was quickly excised. The blood collected from the carotid artery was stored at room temperature for 30 min and was centrifuged at 4500 × *g* at 4 °C for 15 min to obtain the serum. The skin tissue and serum were stored at −80 °C until use. The skin was cut into a square shape (1.5 cm × 1.5 cm) and frozen at −198 °C with liquid nitrogen, crushed into smaller pieces at −198 °C using a Cryo-press® (Microtec, Funabashi, Japan). Then, stirred in 500 μL of 10 mM Na-phosphate buffer (pH 8.0), and centrifuged at 4500 × *g* for 10 min to obtain the supernatants. To this buffer extract of the skin or to the serum, 20× volumes of MeOH were added and mixed for 2 min. The mixtures were centrifuged at 4500 × *g* for 10 min, and the obtained supernatants (20 μL for the skin, 10 μL for the serum) were evaporated to dryness under reduced pressure at 40 °C. To the residue, 30 μL of 200 mM Na-borate buffer (pH 8.0) and 5 μL of 40 mM NBD-F in dry MeCN were added and heated at 60 °C for 2 min. To the reaction mixture, 90 μL of 2% (v/v) TFA in water was added to terminate the reaction, and 2 μL was then subjected to the HPLC.

2.4. HPLC system for the determination of Pro and Hyp enantiomers

The HPLC system (NANOSPACE SI-2 series, Shiseido, Tokyo, Japan) consisted of a type 3202 degasser, three 3101 pumps, a 3033 auto sampler, a 3014 column oven, two 3013 fluorescence detectors and a 3011 column selection unit with a loop of 150 μL. A data processing program, EZChrom Elite Client/Server (Version 3.1.6, Scientific Software, Pleasanton, CA, USA), was used to monitor the detector response. The analytical columns tested for the reversed-phase separation were CAPCELL PAK C_{18} MGII S5 (150 mm × 0.5 mm i.d., particle size 5 μm, Shiseido), CAPCELL PAK C_{18} MGII S3 (150 mm × 0.5 mm i.d., particle size 3 μm, Shiseido) and a monolithic ODS column (500 mm × 0.53 mm i.d., prepared in a fused silica capillary, provided from Shiseido), maintained at 40 °C. A gradient elution was performed for the reversed-phase separation using two mobile phases. Mobile phase A was MeCN-TFA-water (2:0.02:98, v/v/v), and mobile phase B was MeCN-TFA-water (22:0.02:78, v/v/v). The gradient conditions were as follows; isocratic elution with 10% mobile phase B (0–18 min), linear gradient from 10 to 30% mobile phase B (18–23 min), isocratic elution with 30% mobile phase B (23–55 min). The flow rate was 40 μL/min.

For the enantiomer separations, Chiralpak QN-1/QD-1-AX, prototype QN-2/QD-2-AX (150 mm × 1.5 mm i.d., Chiral Technologies Europe, Illkirch, France), Sumichiral OA-2500S and OA-2500R (150 mm × 1.5 mm i.d., Sumika Chemical Analysis Service, Osaka, Japan) columns were tested. The mobile phase for the QN and QD type columns was 10 mM citric acid in a mixed solution of MeOH–MeCN (50:50, v/v), and that for OA type columns was 5 mM

citric acid in MeOH. The flow rate was 200 μL/min. Fluorescence detection was carried out at 530 nm with excitation at 470 nm.

3. Results and discussion

3.1. Two-dimensional HPLC separation of Pro and Hyp enantiomers as their NBD-derivatives

For the determination of Pro and Hyp enantiomers in mammals, a two-dimensional HPLC system employing a microbore-reversed-phase column and a narrowbore-enantioselective column has been established. Because Hyp has *cis*/*trans*-isomers due to the two chiral centers, all of the six enantiomers shown in Fig. 1 should be separately determined in the present study. After derivatization of these amino acids with NBD-F, the NBD-derivatives of Pro, *trans*-Hyp and *cis*-Hyp were isolated as the respective D and L mixtures in the first dimension using a microbore-reversed-phase column. For the second dimension, each fraction of NBD-amino acid was automatically introduced to a narrowbore-enantioselective column using a program-controlled column switching valve, and the D and L enantiomers were separated and determined.

The reversed-phase separation (first dimension) of NBD-Pro, NBD-*trans*-Hyp and NBD-*cis*-Hyp was tested using several microbore-ODS columns including packed columns with 3 or 5 μm particles and monolithic columns. Fig. 2 shows the results obtained by the particle-packed columns of 150 mm length (a) and (b),

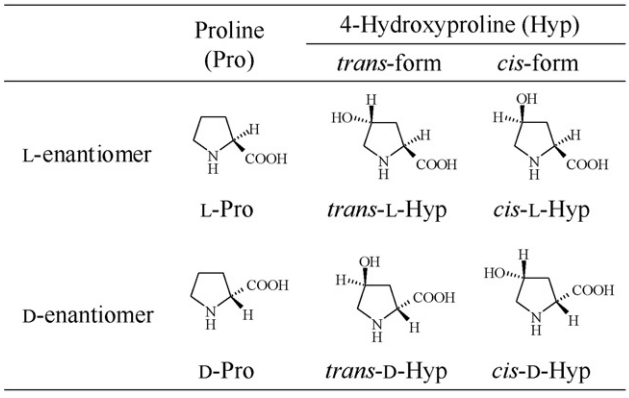

Fig. 1. Structures of Pro analogs investigated in the present study.

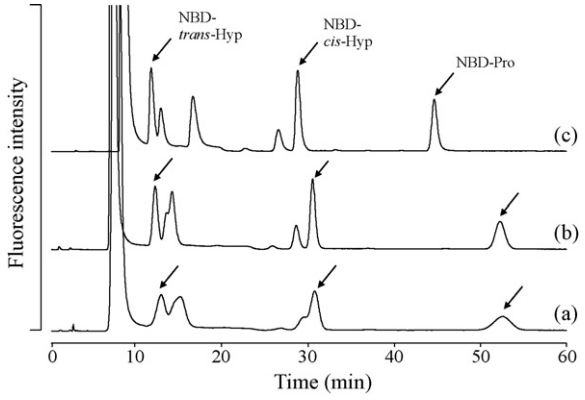

Fig. 2. Separation of NBD-*trans*-Hyp, NBD-*cis*-Hyp and NBD-Pro using microbore-reversed-phase columns. (a) CAPCELL PAK C_{18} MGII S5 (150 mm × 0.50 mm i.d., particle size 5 μm), (b) CAPCELL PAK C_{18} MGII S3 (150 mm × 0.50 mm i.d., particle size 3 μm) and (c) monolithic column (500 mm × 0.53 mm i.d.). A gradient elution using aqueous MeCN solutions containing 0.02% TFA was performed. The detailed HPLC conditions are described in Section 2.4.

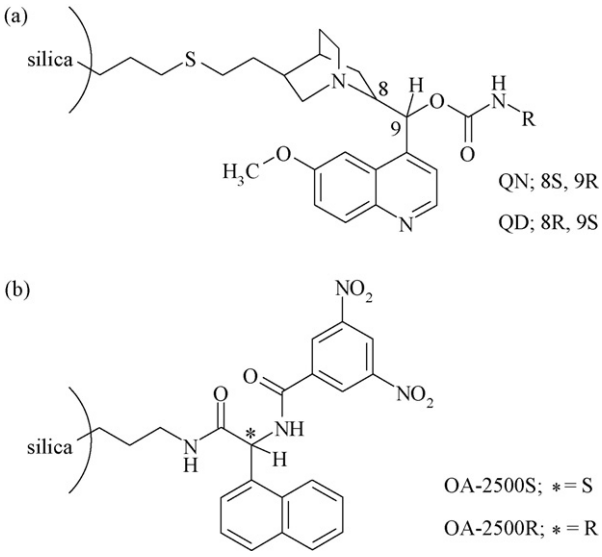

Fig. 3. Structures of stationary phases of enantioselective columns tested. (a) QN and QD type columns; Chiralpak QN-1-AX and QD-1-AX (R = *tert*-butyl), prototype QN-2-AX and QD-2-AX (R = diisopropylphenyl). (b) Sumichiral OA-2500S and OA-2500R.

and by a monolithic column of 500 mm length (c). The mobile phase compositions were determined by investigating the concentrations of MeCN (2–10%, v/v) and trifluoroacetic acid (TFA, 0.01–0.05%, v/v) to obtain sufficient separations of the NBD-Pro analogs and the reagent peaks. The gradient conditions were also optimized to achieve adequate time windows for the successive two-dimensional enantiomer separations; the selected conditions are described in Section 2.4. The column pressures throughout the separations were around 10, 24 and 11 MPa for the 5-μm particle-packed column, the 3-μm particle-packed column and the monolithic column, respectively, and the monolithic ODS column gave theoretical plate numbers (N = 22,278 for NBD-Pro) 2–7 times greater than those of packed ODS columns (N = 2939 (5 μm) and 9616 (3 μm) for NBD-Pro). Because of the high resolution and the low column pressure, the monolithic ODS column was selected for the first dimension, micro-reversed-phase separation of NBD-Pro analogs.

For the second dimension, the enantiomer separations of NBD-Pro analogs were investigated using six narrowbore-enantioselective columns. As shown in Fig. 3, the QN and QD type stationary phases have quinine and quinidine modified moieties as the enantioselective group, and the OA-2500 type has a naphthylglycine moiety. Acid concentration and composition of organic solvents in the mobile phase were adjusted for each column to obtain retention times of around 10 min for all NBD-Pro analogs. As a result, 10 mM citric acid in a mixed solution of MeOH-MeCN (50:50, v/v) was used for QN/QD type columns, and 5 mM citric acid in MeOH was used for OA type columns. QN/QD type columns are the stronger anion exchange type enantioselective columns, therefore, a stronger mobile phase should be used. Retention times of the enantiomers, resolutions and separation factors are summarized in Table 1. Among the six columns tested, the best separations were obtained using a QN-2-AX column for all enantiomer pairs, and the column was selected as the stationary phase of the second dimension. Whereas, by using the pseudo enantiomer stationary phase, QD-2-AX, the retention orders of the enantiomers were reversed. Therefore, these two enantioselective columns are useful to confirm the quantitative values of target D-amino acids in real biological samples.

Table 1
Enantiomer separations of Pro and Hyp as their NBD derivatives using six narrowbore-enantioselective columns

Column	t_0 (min)	*trans*-Hyp				*cis*-Hyp				Pro			
		Retention time (min)		Rs	α	Retention time (min)		Rs	α	Retention time (min)		Rs	α
		L	D			L	D			L	D		
QN-1-AX	1.01	9.59	11.81	2.69	1.26	7.45	8.75	2.09	1.20	7.03	7.62	0.97	1.10
QD-1-AX	0.99	10.80	10.28	0.40	1.06	8.31		n.s.	n.s.	7.44		n.s.	n.s.
QN-2-AX	1.00	7.57	12.68	6.17	1.83	6.35	9.69	5.04	1.65	6.14	8.28	3.51	1.44
QD-2-AX	1.00	11.66	8.71	3.44	1.38	8.95	7.23	2.44	1.28	7.86	6.70	1.70	1.20
OA-2500S	1.03	9.70	10.81	1.35	1.13	7.71	8.53	1.09	1.12	8.55	9.45	1.11	1.12
OA-2500R	1.04	10.45	9.45	0.91	1.12	8.33	7.59	0.69	1.11	9.05	8.25	0.75	1.11

Mobile phase for the QN and QD type columns was 10 mM citric acid in the mixed solution of MeCN–MeOH (50:50, v/v), and that for OA type columns was 5 mM citric acid in MeOH. Flow rate was 200 μL/min. n.s.: Not separated. For each column, t_0 was determined by injecting the mobile phase of the first dimension. The enantiomers eluted faster are indicated by underlines.

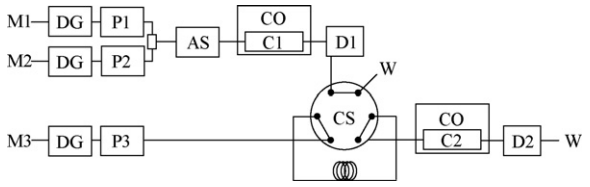

Fig. 4. Flow diagram of the 2D-HPLC system for the determination of Pro and Hyp enantiomers. M, mobile phase; DG, degasser; P, pump; AS, auto sampler; C, column; CO, column oven; D, fluorescence detector; CS, column switching valve; W, waste.

According to these results, a monolithic ODS column (500 mm × 0.53 mm i.d.) and a Chiralpak QN-2-AX column (150 mm × 1.5 mm i.d.) were combined via a program-controlled column switching valve, and a fully automated 2D-HPLC system has been established. Fig. 4 illustrates the flow diagram of the 2D-HPLC system, and the obtained chromatograms are shown in Fig. 5. The fractions of NBD-*trans*-Hyp, NBD-*cis*-Hyp and NBD-Pro were successively introduced to the enantioselective column (*trans*-Hyp and *cis*-Hyp fractions were collected in the loop for 110 s; the Pro fraction was collected for 180 s and immediately introduced to the enantioselective column), and the 2D determination of all enantiomers could be completed within 60 min.

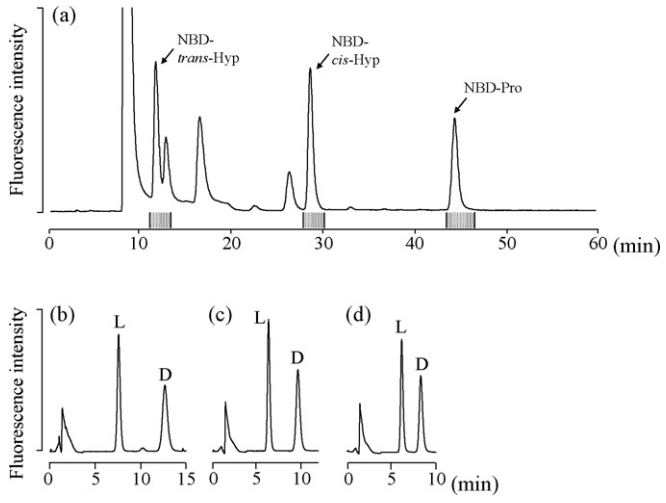

Fig. 5. Reversed-phase separation using a monolithic ODS column (a) and the simultaneous enantiomer separation of (b) NBD-*trans*-Hyp, (c) NBD-*cis*-Hyp and (d) NBD-Pro using a QN-2-AX column. The fractions indicated by gray bars (73 μL (110 s) for NBD-*trans*-Hyp and NBD-*cis*-Hyp, 120 μL (180 s) for NBD-Pro) are collected on-line to a loop and automatically introduced to the enantioselective column. A gradient elution using aqueous MeCN solutions containing 0.02% TFA was performed for the micro-reversed phase separation, and 10 mM citric acid in a mixed solution of MeOH-MeCN (50:50, v/v) was used as a mobile phase for the enantiomer separation. The detailed HPLC conditions are described in Section 2.4.

3.2. Validation of the method

The present method was validated by checking the calibration lines and the precisions of peak heights using both the standard amino acids and those in mouse serum. The calibration lines for *trans*-L-Hyp and L-Pro were constructed from 10 fmol to 5 pmol, and those for the other analogs (*trans*-D-Hyp, *cis*-L-Hyp, *cis*-D-Hyp and D-Pro) were from 1 to 500 fmol; because relatively large amounts of *trans*-L-Hyp and L-Pro are observed in mammalian samples and the amounts of other Pro analogs are extremely small. The results are summarized in Table 2. Calibration curves for standard amino acids are linear with correlation coefficients of over 0.9999, and the lower limits of calibration range of *trans*-D-Hyp, *cis*-L-Hyp, *cis*-D-Hyp and D-Pro in the present study are 1 fmol (S/N = 4.9–10.3). Calibration curves for amino acids spiked into the mouse serum were also constructed, and the obtained slopes were almost the same as those for the corresponding standard amino acids.

Within-day and day-to-day precisions of the peak heights were also evaluated using the standard amino acids and those in mouse serum. Precisions for standard amino acids were determined using 10 fmol (injection amount) of D-amino acids and *cis*-L-Hyp, and 100 fmol of *trans*-L-Hyp and L-Pro (Table 2). In contrast, the precisions for amino acids in mouse serum ($n = 4$) were evaluated using intrinsic amounts of *trans*-L-Hyp, L-Pro, D-Pro, and 10 fmol of other amino acids spiked into the serum. As shown in Table 2, good R.S.D. values were obtained both for the standard amino acids and for those in mouse serum (0.982–5.80%). These results clearly indicate that the present method was applicable to the quantification of Pro analogs in biological samples.

Until now, a simple 1D-HPLC UV/MS method for the simultaneous determination of *trans*-L-Hyp, *cis*-L-Hyp and *cis*-D-Hyp has been reported using the derivatization with N^2-(5-fluoro-2,4-dinitrophenyl)-L-valine amide (FDVA) [28]. The method has calibration ranges of 5–500 pmol, and was successfully applied to detect *trans*-L-Hyp and *cis*-D-Hyp in the protein hydrolysate. Concerning the determination of Pro enantiomers in mammals, a variety of methods has already been reported. Nagata et al. reported an HPLC method following 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) derivatization and preparative 2D-TLC, which enables the determination of D-Pro in human plasma [16]. Brückner et al. reported the amounts of several amino acid enantiomers including D-Pro in human urine and blood using a GC–MS method employing a chiral capillary column after purification of the amino acids with a cation exchange column [26]. However, for the precise determination of small amounts of amino acid enantiomers in biological samples, the use of a selective two-dimensional method is highly recommended. For the 2D-HPLC methods, the combination of a reversed-phase column and an enantioselective column [17,18] is commonly accepted, and these

Table 2
Calibration curves and precisions of the present method

Amino acid	Calibration curve					Precision (R.S.D.%)			
	Standard amino acid			Spiked amino acid into mouse serum		Standard amino acid[b]		Amino acid in mouse serum[c]	
	Calibration range (pmol)	Equation[a]	Correlation coefficient	Equation[a]	Correlation coefficient	Within-day	Day-to-day	Within-day	Day-to-day
trans-L-Hyp	0.01–5	$y = 56.4x + 0.06$	0.99999	$y = 54.0x + 10.6$	0.99991	2.17	2.82	3.16	5.39
trans-D-Hyp	0.001–0.5	$y = 32.3x - 0.01$	0.99999	$y = 30.1x + 0.03$	0.99995	1.22	1.89	0.982	2.27
cis-L-Hyp	0.001–0.5	$y = 59.7x + 0.00$	0.99999	$y = 51.7x + 0.04$	0.99992	2.09	2.43	3.20	4.41
cis-D-Hyp	0.001–0.5	$y = 39.0x + 0.00$	0.99998	$y = 34.0x + 0.04$	0.99994	0.632	3.39	3.01	5.80
L-Pro	0.01–5	$y = 52.0x + 0.42$	0.99996	$y = 52.5x + 30.7$	0.99985	2.00	3.17	3.58	3.78
D-Pro	0.001–0.5	$y = 36.0x + 0.00$	0.99999	$y = 34.1x + 0.22$	0.99989	1.70	2.28	3.85	2.64

[a] Equations were created where x is the amount of amino acids added (injection amount, pmol), and y is the fluorescence intensities (peak height, mV). Injection amounts (or spiked amounts into the mouse serum) of all D-amino acids and *cis*-L-Hyp are 0.001, 0.005, 0.01, 0.05, 0.1 and 0.5 pmol. Those for *trans*-L-Hyp and L-Pro are 0.01, 0.05, 0.1, 0.5, 1 and 5 pmol.
[b] Precisions ($n = 4$) were determined using 0.01 pmol (injection amount) of D-amino acids and *cis*-L-Hyp, and 0.1 pmol of the other L-amino acids.
[c] Precisions ($n = 4$) were determined using intrinsic amounts of *trans*-L-Hyp, L-Pro and D-Pro in mouse serum. Concerning the other amino acids, 0.01 pmol (injection amount) were spiked into the serum.

Fig. 6. Determination of Pro and Hyp enantiomers in mouse serum. Reversed phase separation using a monolithic ODS column (a) and the enantiomer separations of (b) NBD-*trans*-Hyp, (c) NBD-*cis*-Hyp and (d) NBD-Pro using QN-2-AX as an enantioselective column. The enantiomer separation of NBD-Pro using QD-2-AX as an enantioselective column is also shown (e). *Not detected. The fractions indicated by gray bars (73 μL (110 s) for NBD-*trans*-Hyp and NBD-*cis*-Hyp, 120 μL (180 s) for NBD-Pro) are collected on-line to a loop and automatically introduced to the enantioselective column. A gradient elution using aqueous MeCN solutions containing 0.02% TFA was performed for the micro-reversed phase separation, and 10 mM citric acid in a mixed solution of MeOH–MeCN (50:50, v/v) was used as a mobile phase for the enantiomer separation. The detailed HPLC conditions are described in Section 2.4.

methods could successfully be applied to determine small amounts of D-Pro in mammalian tissues as shown in our previous reports [18,20]. This 2D-HPLC concept is also practically useful for the simultaneous determination of Pro and Hyp enantiomers, and in the present study, we have established a micro-2D-HPLC method combining a monolithic ODS column (500 mm × 0.53 mm i.d.) and a narrowbore-enantioselective column (150 mm × 1.5 mm i.d.). Concerning the sensitivity, LOQ values for Pro analogs of already reported methods using FDAA and FDVA were 5 and 20 pmol, respectively [16,28]. Those of our previous 2D-HPLC systems for various D-amino acids were 1–5 fmol [12,15,18,20]. Therefore, the present system has a sensitivity of the highest level for the determination of D-amino acids in mammals, and enables the selective 2D-determination of all six enantiomers of Pro and Hyp in mammalian tissues for the first time. In addition, the present system also has an advantage in using a small volume of the mobile phases, and a fully automated analysis could be performed by employing a program-controlled column switching valve.

3.3. Determination of six enantiomers of Pro and Hyp in mouse serum and skin

Using the 2D-HPLC system described in Sections 3.1 and 3.2, six enantiomers of Pro and Hyp in free form were simultaneously determined in mouse serum and skin. The obtained chromatograms for mouse serum are shown in Fig. 6(a–d), in which the presence of *trans*-L-Hyp and D/L-Pro were clearly demonstrated. In contrast, the amounts of *trans*-D-Hyp and *cis*-D/L-Hyp were smaller than the lower limits of detection. The amounts of all six enantiomers in the serum and in the skin are summarized in Table 3. The amino acid amounts in the skin were corrected by the amounts of proteins in the skin extracts due to the difficulty in measuring the accurate tissue weight (the protein amounts were fluorometrically determined using Qubit with Quant-iT Protein Assay Kits, Invitro-

Table 3
Amounts of Pro and Hyp enantiomers in mouse serum and skin

Amino acid	Serum (nmol/mL)	Skin (nmol/mg protein)
D-Pro	1.57 ± 0.19	0.093 ± 0.015
L-Pro	160.89 ± 15.96	215.99 ± 29.68
trans-D-Hyp	n.d.	n.d.
trans-L-Hyp	19.92 ± 2.01	7.249 ± 0.715
cis-D-Hyp	n.d.	n.d.
cis-L-Hyp	n.d.	n.d.

Values represent mean ± S.E. of four mice; n.d.: smaller than the limit of detection.

gen, Paisley, UK). The amounts of D-Pro both in the serum and in the skin were confirmed using a QD-2-AX column for the enantioseparation, by which the elution order of Pro enantiomers is reversed. Enantiomer separation of NBD-Pro in mouse serum using QD-2-AX is shown in Fig. 6(e), and the amounts of D-Pro in the serum and in the skin determined using this enantioselective column were 1.78 ± 0.20 nmol/mL and 0.125 ± 0.015 nmol/mg protein, respectively. The amounts of D-Pro determined using these two enantioselective columns are almost the same, which indicates the high reliability of the present method for the determination of small amounts of D-Pro in biological samples.

Until now, the amounts of free D-Pro and L-Pro in mouse blood were determined to be about 1 and 100 nmol/mL, respectively [18,20], and the amount of free *trans*-Hyp (determined as a mixture of L- and D-forms) in rat serum was reported to be ca. 30 nmol/mL [29]. These values were consistent with the values determined in the present investigation. Concerning skin tissues, the amount of free Pro in rat skin was reported to be 1.35 μmol/g of wet tissue [30]. Considering that 1 g of skin tissue contains about 2 mg of proteins, the present results are acceptable. In contrast, the amounts of free D-Pro in mouse skin were investigated for the first time to our knowledge. The ratio of the D form (D/(D + L) × 100) of Pro in the skin was 0.048%, which was much lower than that in the serum (0.98%). This is attributed to an extremely small amount of D-Pro and an abundant amount of L-Pro in the skin. These results clearly demonstrate that the present 2D-HPLC system enables the determination of extremely small amounts of the D-enantiomer in the presence of various interfering substances and abundant L-enantiomers.

4. Conclusion

In the present investigation, we established a validated 2D-HPLC method for the determination of Pro and Hyp enantiomers in mammals. The present method has sufficient selectivity and sensitivity to determine small amounts of D-Pro and Hyp enantiomers in the presence of abundant L-Pro and a variety of interfering substances in real biological samples, and D-Pro in the serum and in the collagen-rich skin tissue was clearly determined. The enantiomer ratios of Pro and Hyp are expected to be the candidate biomarkers for skin conditions, and further studies using skin tissues under various physiological conditions and diseases are highly recommended.

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