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Determination of DL-amino acids, derivatized with R(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole, in nail of diabetic patients by UPLC–ESI-TOF-MS☆

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ABSTRACT

The resolution of free DL-amino acids in human nail was carried out by combination of the *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [*R*(−)-DBD-PyNCS] derivatives and UPLC–ESI-TOF-MS. The reaction of the reagent with amino acids effectively proceeds at 55 °C for 20 min in the presence of 1% triethylamine (TEA) to produce the corresponding diastereomers. Each pair of the resulting derivatives was efficiently separated by a gradient program (a mixture of H_2O and CH_3CN containing 0.1% formic acid (HCOOH) or 5 mM CH_3COONH_4 and CH_3CN) using a reversed-phase ACQUITY UPLC™ BEH C_{18} (1.7 μm, 100 mm × 2.1 mm i.d.) column and sensitively detected by TOF-MS. The detection limits (S/N = 3) of the TOF-MS were 1.0–750 fmol, respectively. A good linearity was achieved from the calibration curves, which was obtained by plotting the peak area ratios of the analytes relative to the internal standard (IS), i.e., 6-aminohexanoic acid, versus the injected amounts of each amino acid ($r^2 > 0.996$), and the intra-day and inter-day assay precisions were less than 8.93%. The derivatives of the free DL-amino acids in human nail were successfully identified by the proposed procedure. As we know, for the first time, these five kinds of D-amino acids, which were D-Ala, D-Val, D-Pro, D-Ile and D-Leu, were found from human nail samples. Fifteen kinds of L-amino acids were also recognized from human nails. Using these methods, the amounts of DL-amino acids in the nails of healthy volunteers and diabetic patients were determined. When comparing the index from diabetic patients to those from healthy volunteers, there is no significant difference in the content of the L-amino acids in the nails. However, a statistically significant ($P < 0.01$) correlation was observed between the D/L-amino acid concentration ratios (Ala, Val, Ile, Leu). Therefore, because the proposed method provides a good mass accuracy and the trace detection of the DL-amino acids in human nails, this analytical technique could be a noninvasive technique to assist in the diagnosis and assessment of disease activity in diabetic patients.

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1. Introduction

Recent studies have shown an unexpectedly wide distribution of free D-amino acids in a variety of organisms [1–3]. In particular, free D-Asp, D-Ser and D-Ala are found in mammalian tissues, and their physiological roles are being investigated. D-Asp is observed in various endocrine and neuroendocrine tissues and regulates the hormonal synthesis and secretion in tissues [4–6]. D-Ser is also localized to the frontal brain areas and regulates *N*-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission [7–14]. In addition, D-Ala is localized in the insulin-secreting beta-cells in the pancreas, and insulin are typical regulatory hormones of blood glucose. D-Ala is suggested to have some functional relationships to blood glucose level regulation in mammals [15–17]. Along with the elucidation of their distributions, origins and physiological functions, the D-amino acids have been recognized as candidates of

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novel physiologically active substances and the marker molecules of diseases. Therefore, the high sensitive detection and accurate identification of the DL-amino acids are becoming more important for the study of their biochemical roles.

Tissues and blood samples have been extensively investigated for the DL-amino acid assay in biological specimens. The inherent problems of blood, such as the fluctuation in its composition during the day, and its analysis should be considered. Hygienic practice during its collection and handling is also another consideration. In contrast, the human nail is relatively clean and the sampling is easy. Nail analysis provides one important means for determining the individual past history of long-term chemical exposures, because many substances have been detected in the nail [18,19]. Many studies concerning nail analysis have dealt with drugs of abuse, such as cocaine, itraconazole and amphetamines [19]. Because of the stability of drugs in nails, it is very useful to analyze the drugs when doing post mortem investigations, especially when it is impossible to perform other tests because of the lack of common body fluids or when decomposition of the remains can produce false results [20,21]. In the last decade, interest in nail analysis has gradually shifted to other drug species, e.g., doping agents and therapeutic drugs. According to recent reports, human nails may be used to obtain physiologic information, and may serve as the noninvasive bio sample for diagnosis of the chronic disease. Certain kinds of endogenous biogenic amino acids have been detected in the human nail [22]. However, a method for the determination of D-amino acids of the human nails has not been reported.

As described in a previous paper [23], the separations of each pair of DL-amino acids after labeling with the *R*(−)-DBD-PyNCS are possible by UPLC–ESI-TOF-MS. In addition, we described the determination of DL-amino acids in human nails [23]. The aim of the present study is to inspect the usefulness of the human nail as a new noninvasive biological sample for diagnosis of the chronic disease. Therefore, this paper describes the resolution of DL-amino acid labeled with *R*(−)-DBD-PyNCS and the application to the determination of DL-amino acids in the nail from diabetic patients.

2. Experimental

2.1. Materials and reagents

The amino acid enantiomers were obtained from Sigma (St. Louis, MO, USA), Tokyo Kasei (Tokyo, Japan) and Wako (Osaka, Japan). 6-Aminohexanoic acid (Tokyo Kasei, Japan) was used as the internal standard (IS). 4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole, [*R*(−)-DBD-PyNCS and *S*(+)-DBD-PyNCS] was purchased from the Tokyo Kasei Co. (Tokyo, Japan). Triethylamine (TEA), trifluoroacetic acid (TFA), ammonium acetate (CH_3COONH_4), formic acid (FA), hydrochloric acid (HCl), sodium dodecylsulfate (SDS), methanol (CH_3OH), and acetonitrile (CH_3CN) were of special reagent grade (Wako Pure Chemicals, Osaka, Japan). All other chemicals were of analytical reagent grade and were used without further purification. Deionized and distilled water (H_2O) was used throughout the study (Aquarius pwu-200 automatic water distillation apparatus, Advantec, Tokyo, Japan).

2.2. UPLC–ESI-TOF-MS

The UPLC–ESI-TOF-MS systems consisted of an ACQUITY™ Ultra Performance Liquid Chromatography and Micromass LCT Premier™ XE Mass Spectrometer (high sensitivity orthogonal time-of-flight instrument; Waters, Milford, USA). An ACQUITY UPLC™ BEH C_{18} column (1.7 μm, 100 mm × 2.1 mm i.d., Waters) was used as the analytical column. The column was maintained at 40 °C. The flow rate of the mobile phase was 0.4 mL/min. The TOF-MS was operated in the positive and negative ion modes using an electrospray-ionization source (ESI). The optimized conditions for the UPLC separation and TOF-MS detections are shown in Table 1.

Table 1
UPLC–ESI-TOF-MS conditions.

UPLC (Waters, Milford, USA)	
Column	ACQUITY UPLC™ BEH C_{18} (1.7 μm, 100 mm × 2.1 mm i.d.)
Mobile phase A_1	0.1% HCOOH in H_2O
Mobile phase B_1	0.1% HCOOH in CH_3CN
Gradient	B_1% = 20–20–23–45% (0–2–10–20 min)
Mobile phase A_2	5 mM CH_3COONH_4
Mobile phase B_2	CH_3CN
Gradient	B_2% = 14–14–21–21% (0–17–17–35 min)
Column temperature	40 °C
Flow rate	0.4 mL/min
Injection volume	2 μL
TOF-MS (Micromass LCT Premier™ XE Mass Spectrometer)	
Polarity	ESI^+ (V mode) or ESI
Capillary voltage	3000 V
Sample cone voltage	10 V
Desolvation gas flow	700 L/h
Cone gas flow	50 L/h
Source temperature	120 °C
Desolvation temperature	300 °C
MS range	100–1000 *m*/*z*

2.3. Derivatizing the DL-amino acids with DBD-PyNCS

A 100 μL volume of the reagent [5 mM *R*,(*S*)-DBD-PyNCS] in acetonitrile and 20 μL of amino acid enantiomers (5 μM of each enantiomer) in acetonitrile–water (1:1), 20 μL of 6-aminohexanoic acid (IS) (5 μM), and 100 μL of acetonitrile containing 3% TEA were mixed in a 1.5-mL mini-vial. The vials were tightly capped and heated at 55 °C for 20 min utilizing a dry heat block. The reaction mixture was adequately diluted with acetonitrile and then 2 μL of the solution was injected into the UPLC–ESI-TOF-MS.

2.4. Human nail samples

We obtained nail from 20 diabetic patients (age: 40–64; 10 men and 10 women) and 20 healthy volunteers (age: 33–69; 10 men and 10 women) treated at the Fengxian Branch of Shanghai Sixth People's Hospital from January 2009 to February 2010. All patients provided written informed consent before entry into the study. The nail samples were rinsed with 1 mL of 0.1% SDS for 1 min by ultrasonication. The procedure was repeated another two times. After rinsing, the SDS on the nail samples was removed by three washings with distilled water. The nails were then dried in a desiccator under reduced pressure. The dried nails were crushed into a powder using a Shake Master (Bio Medical Science, Tokyo, Japan).

2.5. Determination of DL-amino acids in human nail

Twenty microliters of 5 μM IS in water and 980 μL of MeOH were added to 5.0 mg of the crushed nail samples. The mixture was kept at 50 °C for 12 h to extract the amino acids, vortex-mixed for 30 s and centrifuged at 3000 × *g* for 10 min. After the extraction, the nail samples were washed with MeOH (200 μL, two times), and all the supernatant fluids were collected and dried under a gentle stream of nitrogen gas. The resulting residues were redissolved in 100 μL of 3% TEA and reacted with 100 μL of 5 mM *R*(−)-DBD-PyNCS in acetonitrile at 55 °C for 20 min. Each 2 μL portion of the reaction mixture was then subjected to the UPLC–ESI-TOF-MS system.

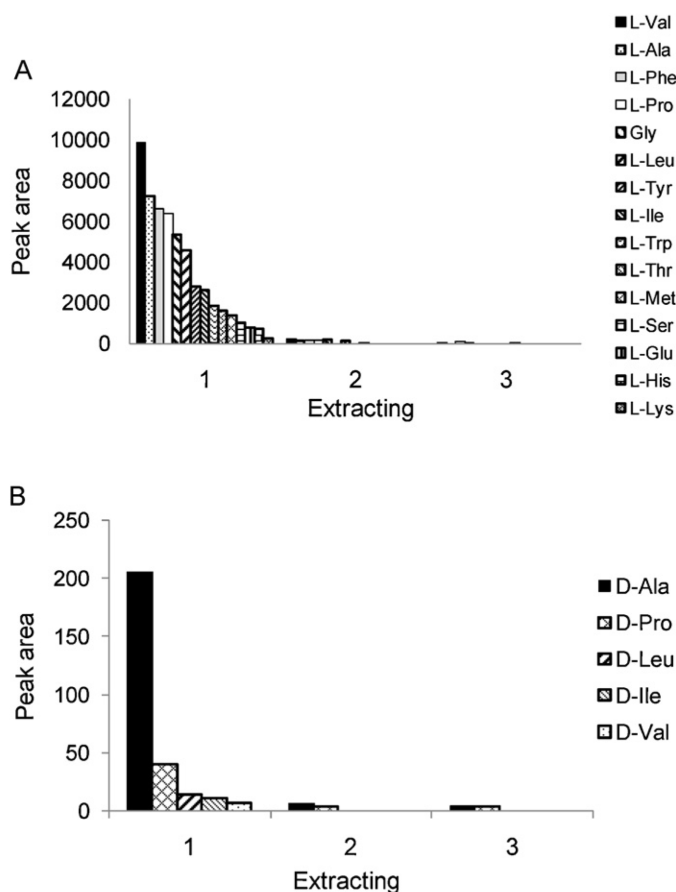

Fig. 1. Remaining DL-amino acids after extracting with the methanol solution at 50 °C for 12 h. (A) L-Amino acids; (B) D-amino acids. The UPLC–ESI-TOF-MS conditions are described in Table 1.

2.6. Validation of the method

2.6.1. Calibration curve preparation

Twenty microliters of the DL-amino acids in water (each 3 μM-120 μM) was mixed with 20 μL of 5 μM IS in water. The solution was reacted at 55 °C for 20 min with 100 μL of 5 mM *R*(−)-DBD-PyNCS and 100 μL of 3% TEA. After the reaction, 2 μL each of the solutions was subjected to the UPLC–ESI-TOF-MS system. The amounts corresponding to an injection of 2 μL were 0.5–20 pmol. The calibration curves were obtained by plotting the peak area ratios of the analytes relative to the IS versus the injected amounts of the DL-amino acids. The precision (CV, %) for each concentration was also calculated from five replicated determinations.

2.6.2. Accuracy and precision of intra-day and inter-day assays

The accuracy (%) and precision (CV) based on the intra-day and inter-day assays were determined using the standard DL-amino acids described in Section 2.6.1. These parameters were evaluated using three different concentrations in the range of 0.5–20 pmol for the DL-amino acids. The determinations were repeated three times within one day and between days. The 20 μL of DL-amino acids mixtures was reacted with *R*(−)-DBD-PyNCS and then subjected to UPLC–TOF-MS, as described in Section 2.6.1. The accuracy (%) at each concentration was calculated from the calibration curves obtained from Section 2.6.1. The precision (CV, %) for each concentration was also calculated from the SD values for three replicated determinations.

2.6.3. Determining DL-amino acids spiked into human nail

Twenty microliters of DL-amino acids in acetonitrile–water (1:1), 20 μL of 5 μM IS in water, and 960 μL of MeOH were poured into glass vials containing 2 mg of nail ($n = 3$). The mixture was kept at 50 °C for 12 h to extract the amino acids, vortex-mixed for 30 s and centrifuged at 3000 × *g* for 10 min. After the extraction, the nail samples were washed with MeOH (200 μL, two times), and all the supernatant fluids were collected and dried under a gentle stream of nitrogen gas. The resulting residues were redissolved in 100 μL of 3% TEA and reacted with 100 μL of 5 mM *R*(−)-DBD-PyNCS in acetonitrile at 55 °C for 20 min, and determined with UPLC–ESI-TOF-MS, as described in Section 2.5. The recovery (%) and precision (CV, %) of the three concentration sets ($n = 3$) were calculated from the calibration curve obtained by the method described in Section 2.6.1.

The intra-day and inter-day precisions ($n = 3$) were evaluated from the CV (%) value as an index. For the determination of the intra-day precision, the DL-amino acids in the human nails were immediately determined after derivatization by UPLC–ESI-TOF-MS. On the other hand, the determination of the inter-day precision was performed after 24 h from the derivatization by UPLC–ESI-TOF-MS.

2.6.4. Limit of detection

The limit of detection (LOD) was defined as the calculated concentration at a signal-to-noise ratio of 3 (S/N = 3). The standard solutions of the DL-amino acids were diluted to a series of concentrations (6 nM–4.5 μM). Each 20 μL solution was reacted with *R*(−)-DBD-PyNCS and then subjected to the UPLC–ESI-TOF-MS system, as described in Section 2.6.1. The limits of detection of each of the DL-amino acids were calculated from a comparison of the noise level and the peak height on the suitable mass chromatogram which had detected the target DL-amino acids.

2.7. Statistical analysis

The statistical analyses were performed using the Welch's *t*-test or Mann–Whitney's *U*-test. A *P* value of <0.05 (0.01) was considered statistically significant.

3. Results and discussion

3.1. Optimization of separation and detection conditions

The optimal derivatization conditions of the DL-amino acids were reported in a previous paper [24]. The proposed derivatization conditions at 55 °C for 20 min in aqueous acetonitrile containing 1% TEA as the base catalyst were also adopted in the present research. In a previous study, the DL-amino acids in food samples were successfully determined by LC separation and FL detection [25,26]. However, the determination in complex matrices, such as plasma and nails, seems to be fairly difficult by the FL detection. Indeed, the determination of several DL-amino acids in human nails by the FL detection was interfered by any of the peaks based upon the endogenous substances. Although the interference seemed to be evitable by optimization of the elution conditions, the determination with a short run time failed. Furthermore, no structural information can be obtained from the FL detection. On the other hand, MS has recently become a popular technique for the determination of trace quantities of chemicals in real samples, such as blood and urine. Among the various types of available MS instruments, ESI-TOF-MS is recommended for the selective determination of target compounds because of its excellent accuracy and the precision of the resulting *m*/*z* values. Thus, the simultaneous determination of DL-amino acids by UPLC–ESI-TOF-MS was attempted in this study. The derivatives of 17 DL-amino acids were well separated

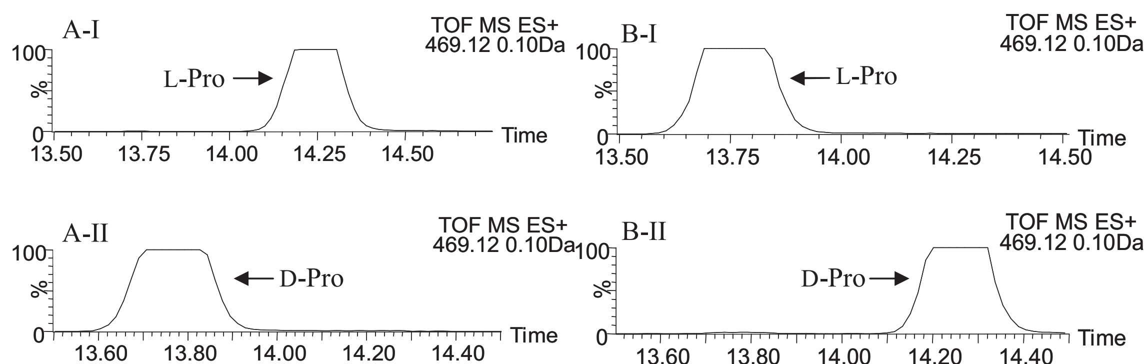

Fig. 2. Mass chromatograms of the diastereomers obtained from the reaction of each enantiomer of proline with *R*(−)- and *S*(+)-DBD-PyNCS. A-I–II, *R*(−)-DBD-PyNCS; B-I–II, *S*(+)-DBD-PyNCS. The UPLC–ESI-TOF-MS conditions are described in Table 1.

using both elution systems; i.e., the gradient elution with water-acetonitrile containing 0.1% FA, and the gradient elution with 5 mM ammonium acetate buffer (pH 6.7) 0.1% FA acetonitrile [23].

The retention factor (k), separation factor (α) and resolution (Rs) of the DL-amino acid derivatives are listed in Table 2. A relatively good separation was obtained for the hydrophobic amino acids, such as Ala,Val, Ile, Leu, Pro, Tyr, Trp and Phe. The D-amino acids, except for His, Lys and Arg, were eluted consistently faster than those of the L-enantiomers. The elution order is advantageous because L-amino acids are the dominant components in real samples. Of course, the opposite elution order was observed with the resulting derivatives of *S*(+)-DBD-PyNCS as the tagging reagent.

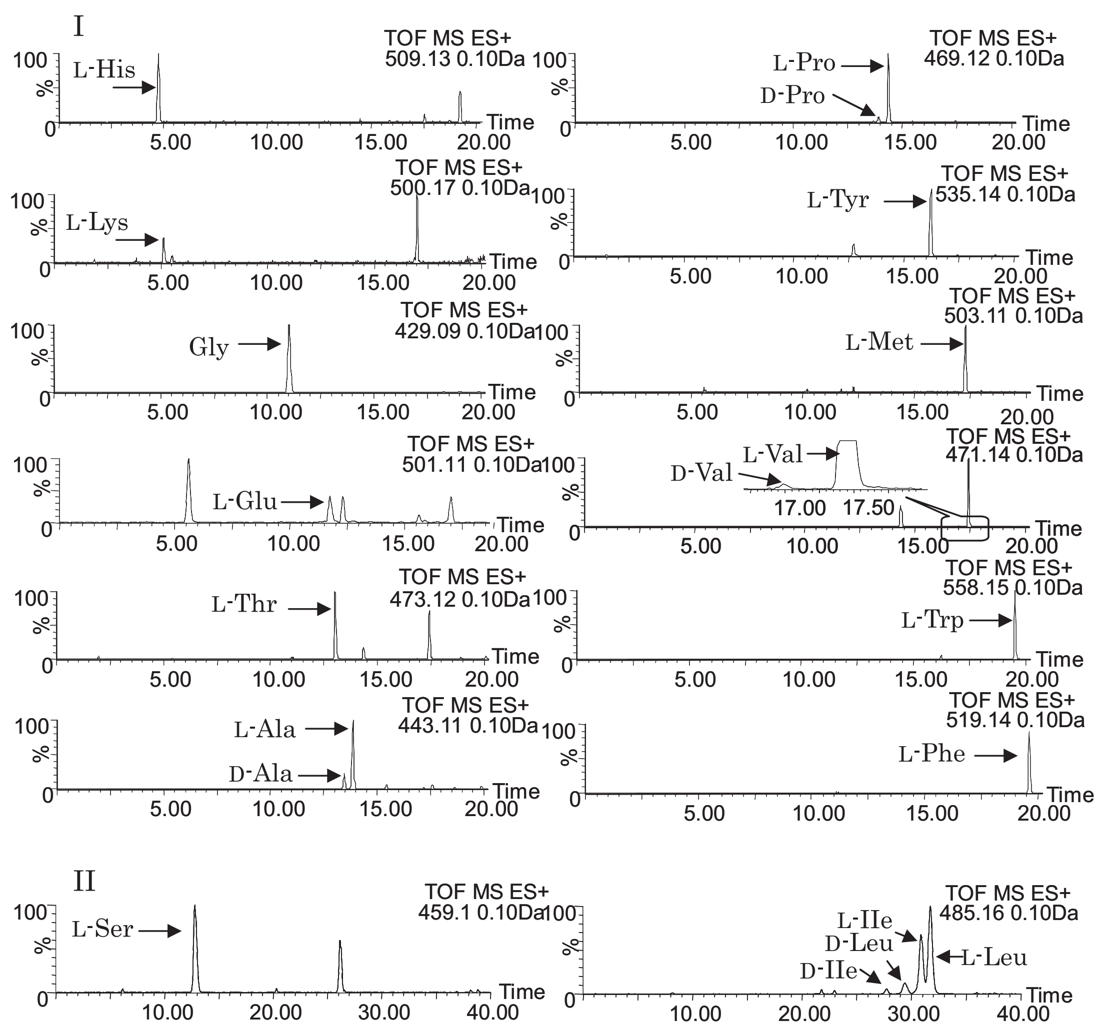

Fig. 3. Mass chromatograms obtained from the DL-amino acids in nails from healthy volunteers by the UPLC–ESI-TOF-MS. (I) Mobile phase A = 0.1% HCOOH in H_2O, B = 0.1% HCOOH in CH_3CN a linear gradient from B 20–20–23–45% (0–2–10–20 min); (II) A = 5 mM CH_3COONH_4, B = CH_3CN a linear gradient B 14–14–21–21% (0–17–17–35 min). The other UPLC–ESI-TOF-MS conditions are described in Table 1.

Table 2
Separatability of diastereomers derived from chiral reagents.

Amino acids	R(−)-DBD-PyNCS					S(+)-DBD-PyNCS				
	k(D)	k(L)	a	Rs	Elution	k(D)	k(L)	a	Rs	Elution
His	6.80	6.23	1.09	1.20	a	6.02	6.83	1.14	1.72	a
Lys	7.42	6.97	1.06	0.92	a	6.97	7.49	1.08	1.10	a
Arg	8.38	7.94	1.06	0.94	a	7.98	8.35	1.05	0.66	a
Gly		15.9			a		15.9			a
Glu	16.3	16.8	1.03	1.48	a	16.8	16.3	1.03	1.26	a
Asp	17.8	18.3	1.02	1.53	a	18.3	17.9	1.02	1.09	a
Thr	18.2	19.0	1.05	3.34	a	19.1	18.2	1.05	3.43	a
Ala	19.7	20.3	1.03	2.29	a	20.3	19.7	1.03	2.53	a
Pro	20.4	21.1	1.04	2.97	a	21.1	20.4	1.03	2.99	a
Tyr	23.5	24.0	1.02	2.52	a	24.0	23.5	1.02	2 21	a
Met	25.0	25.6	1.02	3.38	a	25.6	25.0	1.02	2.90	a
Val	25.1	25.8	1.03	3.73	a	25.8	25.2	1.03	3.17	a
Trp	28.1	29.1	1.04	5.19	a	29.0	28.1	1.03	5.03	a
Phe	28.3	29.2	1.03	4.54	a	29.2	28.3	1.03	4.71	a
Asn	17.0	15.8	1.08	1.22	b	15.6	16.8	1.08	1.38	b
Ser	19.5	18.4	1.06	1.30	b	18.2	19.1	1.05	1.17	b
He	41.4	45.9	1.11	4.80	b	45.6	41.2	1.11	5.08	b
Leu	43.2	47.0	1.09	3.49	b	46.8	43.0	1.09	4.43	b

a, $A_1 = 0.1\%$ HCOOH in H_2O, $B_1 = 0.1\%$ HCOOH in CH_3CN, Bconc. 20–20–23–45% (0–2–10–20 min); b. $A_2 = 5$ mM CH_3COONH_4, $B_2 = CH_3CN$, Bconc. 14–14–21–21% (0–17–17–35 min). $k = (t - t_0)/t_0$; $a = (t_{R2} - t_0)/(t_{R1} - t_0)$; $Rs = 1.18(t_{R2} - t_{R1})/(W_{0.5h1} + W_{0.5h2})$.

Based upon these observations, the UPLC–ESI-TOF-MS system was adopted for the simultaneous determination of the DL-amino acids after labeling with R(−)-DBD-PyNCS.

3.2. Validation of the proposed method

Table 3 shows the calibration curves of the DL-amino acid. The calibration curves were obtained by plotting the peak area ratios of the DL-amino acids relative to the IS versus the injected amounts of the DL-amino acids (0.5–20 pmol, $r^2 > 0.996$). The calibration curves were obtained from five different concentrations. The determination at each concentration was repeated five times. A good calibration curve was obtained for each DL-amino acid. The CV values for each injected amount were in the range of 0.17–9.86% ($n = 5$). The detection limits (S/N = 3) in the MS were 1.0–750 fmol. To evaluate the present method, the accuracy (%) and the precision (CV) were determined. The accuracies (%) and precisions (CVs, %) for three different concentrations were evaluated using the intra-day and inter-day assays. As shown in Table 4, the accuracies of the intra-day and inter-day determinations were 93.85–110.0% and 91.33–111.3%, respectively. The CVs of the intra-day and inter-day determinations were 0.960–5.07% and 0.706–8.93%, respectively.

To evaluate the present method, the recovery (%) and the precision (CV) were determined. As shown in Table 5, the recoveries (%) of the DL-amino acids spiked in the human nail were 94.13–112.3%. Furthermore, the intra-day and inter-day precisions (CV) were within 0.180–9.79% and 0.42–14.7%, respectively. A good linearity, sensitivity, and precision demonstrated that the present method is applicable for human nail analyses.

3.3. Extraction of DL-amino acids in the human nails

The extraction of a nail sample is essential for human nail analysis since the sample is not a fluid, but a solid sample. The extraction solvent of the amino acids was studied. Water-soluble solvents such as MeOH, CH_3CN and (a mixture of MeOH and CH_3CN) containing acids (HCl and TFA) and an alkaline NaOH solution, were tried as the extraction solution at 4 °C, 50 °C, and 60 °C. Among the tested solutions, the amino acids were efficiently extracted by the MeOH at 50 °C for 12 h. As shown in Fig. 1., the remaining amounts of the DL-amino acids decreased with the extraction number, and

Table 3
Calibration curves of amino acids by the proposed method.

Amino acids	Calibration range (pmol)	Linear equation	Linearity (R^2)	CV (%) ($n = 5$)	Detection limit (fmol)
L-His	0.5–8.0	$y = 0.2574x + 0.0399$	0.9980	2.72–5.57	30.0
L-Lys	0.5–8.0	$y = 0.2293x + 0.0291$	0.9992	2.32–8.25	10.0
Gly	0.5–8.0	$y = 0.261x + 0.07$	0.9981	1.47–6.47	5.00
L-Glu	0.5–8.0	$y = 0.0685x + 0.0067$	0.9981	2.17–9.22	150
L-Thr	0.5–8.0	$y = 0.092x + 0.0169$	0.9977	2.01–8.82	60.0
L-Ala	0.5–8.0	$y = 0.1414x + 0.2973$	0.9979	0.99–3.44	1.00
L-Pro	0.5–8.0	$y = 2.6589x + 0.1272$	0.9995	1.43–6.32	1.50
L-Tyr	0.5–8.0	$y = 0.2271x + 0.0146$	0.9990	1.38–7.81	30.0
L-Met	0.5–8.0	$y = 0.1788x + 0.013$	0.9989	1.53–7.23	30.0
L-Val	0.5–8.0	$y = 0.2744x + 0.0713$	0.9979	0.17–5.43	20.0
L-Trp	0.5–8.0	$y = 0.7536x + 0.0415$	0.9996	1.43–5.67	20.0
L-Phe	0.5–8.0	$y = 0.2966x + 0.016$	0.9990	1.28–8.87	30.0
L-Ser	3.0–20	$y = 0.0323x - 0.0241$	0.9996	1.37–5.05	750
L-Ile	3.0–20	$y = 0.0895x + 0.0058$	0.9987	2.54–9.86	500
L-Leu	3.0–20	$y = 0.049x + 0.0072$	0.9961	0.87–9.62	750
D-Ala	0.5–8.0	$y = 0.1425x + 0.0311$	0.9976	1.26–5.94	50.0
D-Pro	0.5–8.0	$y = 2.4401x + 0.1285$	0.9987	2.90–6.32	10.0
D-Val	0.5–8.0	$y = 0.2139x + 0.0307$	0.9982	1.11–7.43	30.0
D-Ile	3.0–20	$y = 0.1477x - 0.0186$	0.9989	1.61–8.90	300
D-Leu	3.0–20	$y = 0.1633x - 0.0169$	0.9995	0.55–9.81	300

Table 4
Accuracy and precision of the proposed method by intra-day and inter-day assays.

Amino acids	Amount (nmol/mL)	Intra-day assay			Inter-day assay		
		Mean ± SD	CV (%) (n = 3)	Accuracy (%)	Mean ± SD	CV (%) (n = 3)	Accuracy (%)
L-His	0.5	0.488 ± 0.0047	0.96	97.60	0.485 ± 0.0054	1.11	97.00
	1.5	1.64 ± 0.068	4.13	109.3	1.48 ± 0.13	8.92	98.62
	3.0	2.86 ± 0.072	2.51	95.33	2.79 ± 0.14	4.96	93.00
L-Lys	0.5	0.471 ± 0.016	3.34	94.16	0.462 ± 0.010	2.20	92.41
	1.5	1.50 ± 0.044	2.94	99.71	1.43 ± 0.10	7.30	95.40
	3.0	2.82 ± 0.096	3.42	93.85	2.74 ± 0.16	5.74	91.41
Gly	0.5	0.515 ± 0.026	5.07	103.0	0.545 ± 0.014	2.56	109.1
	1.5	1.57 ± 0.058	3.69	104.7	1.52 ± 0.073	4.82	101.3
	3.0	2.86 ± 0.043	1.50	95.33	2.89 ± 0.077	2.66	96.33
L-Glu	0.5	0.484 ± 0.021	4.40	96.79	0.493 ± 0.026	5.18	98.60
	1.5	1.62 ± 0.039	2.43	108.0	1.54 ± 0.094	6.08	102.7
	3.0	2.89 ± 0.068	2.36	96.33	2.93 ± 0.092	3.14	97.67
L-Thr	0.5	0.506 ± 0.0088	1.75	101.2	0.557 ± 0.038	6.83	111.3
	1.5	1.65 ± 0.036	2.15	110.0	1.59 ± 0.050	3.12	106.0
	3.0	2.89 ± 0.046	1.60	96.33	2.89 ± 0.050	1.73	96.33
L-Ala	0.5	0.537 ± 0.027	5.05	108.8	0.515 ± 0.041	7.95	103.0
	1.5	1.56 ± 0.017	1.10	104.0	1.54 ± 0.030	1.97	102.7
	3.0	2.93 ± 0.063	2.16	97.67	2.92 ± 0.089	3.04	97.33
L-Pro	1.0	1.02 ± 0.015	1.43	102.0	0.937 ± 0.059	6.32	93.75
	3.0	3.02 ± 0.14	4.79	100.6	2.89 ± 0.12	4.18	96.19
	6.0	6.12 ± 0.15	2.48	102.0	5.67 ± 0.29	5.05	94.48
L-Tyr	0.5	0.505 ± 0.020	4.05	101.0	0.535 ± 0.044	8.28	107.0
	1.5	1.63 ± 0.070	4.28	108.7	1.61 ± 0.044	2.74	107.3
	3.0	2.90 ± 0.14	4.98	96.67	3.06 ± 0.081	2.65	102.0
L-Met	0.5	0.501 ± 0.015	2.91	100.2	0.495 ± 0.010	2.07	99.00
	1.5	1.62 ± 0.035	2.17	108.0	1.63 ± 0.030	1.84	108.7
	3.0	3.00 ± 0.042	1.38	100.0	3.00 ± 0.073	2.45	100.0
L-Val	0.5	0.479 ± 0.019	4.06	95.80	0.553 ± 0.036	6.56	110.6
	1.5	1.63 ± 0.024	1.47	108.7	1.64 ± 0.018	1.08	109.3
	3.0	3.02 ± 0.10	3.45	100.7	3.00 ± 0.057	1.92	100.0
L-Trp	0.5	0.499 ± 0.024	4.79	99.80	0.512 ± 0.035	6.92	102.4
	1.5	1.59 ± 0.051	3.18	106.0	1.60 ± 0.046	2.89	106.7
	3.0	2.98 ± 0.034	1.14	99.33	3.07 ± 0.11	3.49	102.3
L-Phe	0.5	0.497 ± 0.021	4.16	99.40	0.529 ± 0.047	8.93	105.7
	1.5	1.61 ± 0.056	3.47	107.3	1.60 ± 0.051	3.17	106.7
	3.0	2.98 ± 0.042	1.42	99.33	3.05 ± 0.11	3.65	101.7
D-Ala	0.5	0.522 ± 0.016	3.06	104.4	0.514 ± 0.0036	0.706	102.8
	1.5	1.63 ± 0.073	4.47	108.8	1.67 ± 0.067	4.01	111.1
	3.0	3.11 ± 0.10	3.24	103.7	3.03 ± 0.060	1.98	101.0
D-Pro	1.0	1.03 ± 0.052	5.07	102.5	0.922 ± 0.058	6.32	92.27
	3.0	3.00 ± 0.15	5.07	100.2	2.75 ± 0.12	4.18	91.55
	6.0	6.05 ± 0.18	2.90	100.9	5.48 ± 0.28	5.05	91.33
D-Val	0.5	0.541 ± 0.0081	1.50	108.2	0.539 ± 0.0082	1.52	107.8
	1.5	1.59 ± 0.028	1.78	106.0	1.67 ± 0.097	5.78	111.3
	3.0	3.12 ± 0.069	2.20	104.0	3.10 ± 0.022	0.708	103.3
L-Ser	2.5	2.46 ± 0.10	4.21	98.36	2.62 ± 0.095	3.64	104.8
	4.0	4.06 ± 0.20	4.82	101.5	4.20 ± 0.30	7.08	105.0
	5.0	5.30 ± 0.27	5.02	106.0	5.40 ± 0.37	6.94	108.0
L-Ile	2.5	2.63 ± 0.086	3.27	105.2	2.63 ± 0.068	2.59	105.2
	4.0	4.12 ± 0.21	4.98	102.9	4.31 ± 0.37	8.60	107.7
	5.0	5.49 ± 0.20	3.72	109.8	5.32 ± 0.29	5.54	106.4
L-Leu	2.5	2.61 ± 0.12	4.75	104.4	2.66 ± 0.11	4.15	106.4
	4.0	4.08 ± 0.19	4.69	101.9	4.39 ± 0.39	8.89	109.8
	5.0	5.34 ± 0.21	3.98	106.8	5.36 ± 0.36	6.77	107.2
D-Ile	2.5	2.61 ± 0.058	2.22	104.4	2.59 ± 0.040	1.53	103.6
	4.0	4.09 ± 0.20	4.82	102.3	4.12 ± 0.22	5.30	103.0
	5.0	5.40 ± 0.27	5.00	108.1	5.56 ± 0.44	7.98	111.2
D-Leu	2.5	2.51 ± 0.077	3.08	100.4	2.61 ± 0.077	2.95	104.4
	4.0	4.14 ± 0.21	5.00	103.5	4.16 ± 0.24	5.70	104.0
	5.0	5.40 ± 0.26	4.83	107.9	5.46 ± 0.42	7.65	109.2

SD, standard deviation; CV, coefficient of variation.

DL-amino acids of human nail almost extracted after one extraction.

The most important risk involved in using chiral derivatization reagents to determine optical isomers is racemization during the derivatization reaction and extraction process. Therefore, it is essential that the reaction and extraction proceed without racemization. Thus, the possibility of racemization was investigated. Fig. 2 shows the mass chromatograms of the diastereomers derived from the reactions of each enantiomer of standard D,L-Pro after extractant MeOH at 50 °C for 12 h with *R*(−)-and *S*(+)-DBD-PyNCS. Sole peak appeared on each mass chromatogram. The other also produced single peak on the mass chromatograms obtained from the reaction with each enantiomer of the amino acids. Therefore, the racemization does not occur during a derivatization reaction. Based upon these observations, MeOH extraction at 50 °C for 12 h was performed for the sample preparation in this study.

Table 5
Determination of DL-amino acids spiked into human nail ($n = 3$).

Amino acids	Spiked amount (pmol/mg)	Detection amount (pmol/mg)	Intra-day CV (%)	Inter-day CV (%)	Recovery (%)	Mean recovery (%)
L-His	50	55.9 ± 5.47	9.79	7.56	112	
	100	109 ± 3.97	3.65	3.81	109	105.6
	200	192 ± 6.52	3.40	0.42	95.9	
L-Lys	50	57.4 ± 3.02	5.27	3.46	115	
	100	111 ± 3.84	3.46	1.30	111	105.3
	200	180 ± 9.16	5.10	7.25	89.9	
Gly	50	58.1 ± 4.49	7.73	11.0	116	
	100	95.9 ± 9.21	9.60	5.47	95.9	108.9
	200	181 ± 7.04	3.88	6.37	90.7	
L-Glu	50	57.6 ± 3.67	6.36	1.43	115	
	100	lll. ± 14.4	5.37	12.9	111	103.6
	200	170 ± 11.8	6.94	12.6	84.8	
L-Thr	50	58.0 ± 3.19	5.50	2.67	116	
	100	114 ± 5.70	4.99	1.52	114	112.3
	200	213 ± 2.23	1.05	6.43	107	
L-Ala	50	53.5 ± 1.40	2.62	6.27	107	
	100	97.5 ± 4.94	5.07	2.74	97.5	101.0
	200	197 ± 13.3	6.74	3.88	98.6	
L-Pro	50	61.6 ± 1.17	1.90	1.78	123	
	100	113 ± 6.10	5.41	5.78	113	110.3
	200	190 ± 3.62	1.91	6.64	95.0	
L-Tyr	50	58.5 ± 2.09	3.57	3.58	117	
	100	118 ± 4.77	4.05	3.54	118	108.2
	200	179 ± 4.04	2.26	4.61	89.7	
L-Met	50	55.7 ± 3.12	5.60	8.49	111	
	100	113 ± 3.65	3.24	3.07	113	110.3
	200	213 ± 6.92	3.25	4.00	107	
L-Val	50	57.4 ± 4.18	7.28	2.27	115	
	100	120 ± 0.215	0.180	1.79	120	109.5
	200	187 ± 12.5	6.68	7.01	93.5	
L-Trp	50	58.2 ± 1.81	3.10	1.33	116	
	100	115 ± 0.669	0.583	2.37	115	109.8
	200	197 ± 1.97	1.00	2.38	98.5	
L-Phe	50	58.0 ± 1.70	2.94	3.28	116	
	100	104 ± 2.11	2.03	3.55	104	105.8
	200	195 ± 1.79	0.920	1.56	97.5	
L-Ser	50	59.6 ± 4.34	7.28	7.83	119	
	100	98.7 ± 4.06	4.11	2.31	98.7	102.7
	200	181 ± 7.36	4.07	3.29	90.5	
L-Ile	50	56.8 ± 1.61	2.83	14.7	114	
	100	105 ± 0.741	0.709	3.18	104	105.2
	200	196 ± 1.02	0.522	4.23	97.7	
L-Leu	50	59.0 ± 1.40	2.38	3.02	118	
	100	109 ± 1.25	1.14	5.22	109	108.6
	200	198 ± 2.25	1.14	4.10	98.7	
D-Ala	1	0.910 ± 0.0557	6.11	3.93	91.0	
	5	6.00 ± 0.0809	1.35	13.1	120	103.3
	10	9.90 ± 0.720	7.27	10.2	99.0	
D-Pro	1	0.981 ± 0.0434	4.43	4.31	98.1	
	5	5.30 ± 0.190	3.58	7.14	106	103.0
	10	10.5 ± 0.625	5.93	3.37	105	
D-Val	1	0.989 ± 0.0706	0.594	7.13	98.9	
	5	5.76 ± 0.217	3.77	4.83	115	102.2
	10	9.26 ± 0.430	4.64	4.14	92.6	
D-Ile	1	0.814 ± 0.0192	2.37	2.91	81.3	
	5	5.36 ± 0.179	3.33	2.52	107	94.13
	10	9.41 ± 0.179	1.91	2.77	94.1	
D-Leu	1	0.804 ± 0.0106	1.32	6.49	80.4	
	5	6.13 ± 0.419	6.84	3.78	123	99.37
	10	9.47 ± 0.368	3.89	1.06	94.7	

3.4. Determination of free DL-amino acids in the nails of diabetic patients and healthy volunteers

The extracted DL-amino acids from the nails of diabetic patients (age: 40–64; 10 men and 10 women) and healthy volunteers (age: 33–69; 10 men and 10 women) were then labeled with *R*(−)-DBD-PyNCS. Fig. 3 shows the typical mass chromatograms obtained from the DL-amino acids in a nail from a healthy volunteer by the UPLC–ESI-TOF-MS. The peaks corresponding to the DL-amino acid derivatives were completely separated without any interference from the endogenous substances in the nails. Furthermore, a rapid separation within 20 min and 35 min was performed by the combination of the anti-pressurized column and the UPLC instrument. Of course, the structures of the derivatives were identified from a comparison of the positive and negative ion mode MS of the authentic DL-amino acids. These five kinds of D-amino acids, i.e., D-Ala, D-Val, D-Pro, D-Ile and D-Leu, were detected from the human nail samples. Fifteen kinds of L-amino acids were also identified from the human nails.

Using these methods, the amounts of DL-amino acids in the nails of healthy volunteer and diabetic patient nails were determined. Table 6 shows the concentration of the D,L-amino

Table 6
Amounts of D,L-amino acids in the nails of healthy volunteers and diabetic patients (10 healthy men, 10 healthy women and 10 diabetic men, 10 diabetic women).

Amino acids	Mean ± SD (pmol/mg nail)					
	HM	HW	DM	DW	HV	DP
L-His	137.2 ± 79.50	181.9 ± 75.40	258.2 ± 215.4	366.5 ± 223.3	159.6 ± 78.83	312.3 ± 220.6
L-Lys	68.19 ± 22.16	64.20 ± 43.18	98.11 ± 97.28	76.55 ± 71.98	66.19 ± 33.46	87.33 ± 84.02
Gly	396.1 ± 145.9	248.0 ± 143.3	423.1 ± 485.7	512.1 ± 398.4	322.1 ± 160.0	467.6 ± 434.7
L-Glu	575.0 ± 294.5	457.4 ± 175.5	591.2 ± 392.9	725.4 ± 321.6	516.2 ± 243.5	650.9 ± 360.8
L-Thr	221.1 ± 63.46	230.3 ± 87.62	222.9 ± 220.1	417.7 ± 266.7	225.7 ± 74.61	325.4 ± 259.0
L-Ala	387.2 ± 117.3	314.8 ± 133.7	345.6 ± 355.5	504.8 ± 292.6	351.0 ± 127.9	425.2 ± 327.1
L-Pro	306.0 ± 133.2	221.9 ± 78.50	287.0 ± 323.6	451.5 ± 270.6	264.0 ± 114.8	369.2 ± 302.3
L-Tyr	200.6 ± 44.66	216 ± 87.09	343.8 ± 147.2	363.3 ± 280.3	208.3 ± 67.82	353.0 ± 214.1
L-Met	1284 ± 612.5	1193 ± 768.7	1290 ± 1114	1187 ± 1104	1238 ± 675.9	1242 ± 1070
L-Val	406.2 ± 105.0	433.2 ± 126.5	521.1 ± 270.5	597.8 ± 357.1	419.7 ± 114.0	559.4 ± 310.8
L-Trp	282.3 ± 194.7	406.9 ± 250.1	998.7 ± 649.1	1210 ± 1091	334.6 ± 227.3	1104 ± 880.3
L-Phe	181.0 ± 49.45	185.0 ± 55.43	266.7 ± 94.61	259.9 ± 139.0	183.0 ± 51.17	263.3 ± 115.8
L-Ser	138.6 ± 81.71	130.6 ± 94.64	179.4 ± 315.1	199.3 ± 185.8	134.6 ± 86.15	188.8 ± 255.1
L-Ile	308.7 ± 143.2	314.3 ± 141.2	379.1 ± 377.9	480.9 ± 410.2	311.5 ± 138.4	427.3 ± 385.9
L-Leu	893.8 ± 438.3	893.5 ± 421.1	1037 ± 1020	1349 ± 1105	893.7 ± 418.3	1185 ± 1043
D-Ala	10.68 ± 9.332	3.202 ± 2.261	30.52 ± 25.57	22.59 ± 20.57	6.942 ± 7.642	26.55 ± 22.95
D-Pro	1.513 ± 1.293	1.077 ± 0.6462	4.770 ± 5.495	2.035 ± 2.636	1.295 ± 1.020	3.402 ± 4.423
D-Val	0.2291 ± 0.1585	0.2835 ± 0.2093	8.526 ± 12.93	1.727 ± 2.498	0.2578 ± 0.1840	5.126 ± 9.710
D-Ile	0.2169 ± 0.2054	0.1499 ± 0.1275	3.835 ± 5.170	0.7248 ± 0.6187	0.1893 ± 0.1760	2.362 ± 4.010
D-Leu	3.005 ± 1.469	2.093 ± 0.8350	25.45 ± 21.20	8.177 ± 8.320	2.549 ± 1.254	17.27 ± 18.28

HM, healthy men; HW, healthy women; DM, diabetic men; DW, diabetic women; HV, healthy volunteers; DP, diabetic patients.

acids in the healthy volunteers and diabetic patients. The mean amounts of 159.6(L-His), 66.19(L-Lys), 322.1(Gly), 516.2(L-Glu), 225.7(L-Thr), 351.0(L-Ala), 264.0(L-Pro), 208.3(L-Tyr), 1238(L-Met), 419.7(L-Val), 334.6(L-Trp), 183.0(L-Phe), 134.6(L-Ser), 311.5(L-Ile), 893.7(L-Leu), 6.942(D-Ala), 1.295(D-Pro), 0.2578(D-Val), 0.1893(D-Ile) and 2.549(D-Leu) pmol in 1 mg of nail in the healthy men ($n=10$) and women ($n=10$), were calculated from each calibration curve. On the other hand the diabetic patient amounts were 312.3(L-His), 87.33(L-Lys), 467.6(Gly), 650.9(L-Glu), 325.4(L-Thr), 425.2(L-Ala), 369.2(L-Pro), 353.0(L-Tyr), 1242(L-Met), 559.4(L-Val), 1104(L-Trp), 263.3(L-Phe), 188.8(L-Ser), 427.3(L-Ile), 1185(L-Leu), 26.55(D-Ala), 3.402(D-Pro), 5.126(D-Val), 2.362(D-Ile) and 17.27(D-Leu) pmol in 1 mg of nail, respectively. The L-amino acid concentrations were similar in the healthy volunteers and diabetic patients. Furthermore, in the diabetic patients, the L-amino acid concentrations were not statistically different from the healthy volunteers. When the healthy persons were compared with the diabetic patients, D-amino acid concentrations were higher in diabetic patients than in healthy volunteers.

Fig. 4 shows a statistical analysis of the D/L-amino acid concentration ratios in healthy volunteers ($n=20$) and the diabetic patients ($n=20$). Significant differences ($P<0.01$) were observed between the Ala, Val, Ile and Leu in the healthy volunteers and diabetic patients. Furthermore, Fig. 5 shows a comparison of the concentration ratios of the men and women separate D/L-amino acid in the nail of healthy volunteers and the diabetic patients. When comparing the D/L-amino acid concentration ratios, significant differences were observed between the Ala ($P<0.01$), Val ($P<0.05$), Leu ($P<0.05$) in the men and women. Pro and Ile were statistically significant in the men, but not statistically significant in the women. A strong correlation was observed between the ratios of D/L-amino acid concentration. Although the biochemical mechanisms responsible for these peculiar diabetic D/L-amino acids profiles are unclear, the D/L-amino acid concentration ratios might serve as a potential marker for diabetes. This analytical technique could be a noninvasive procedure to assist in the diagnosis and assessment of disease activity in diabetic patients.

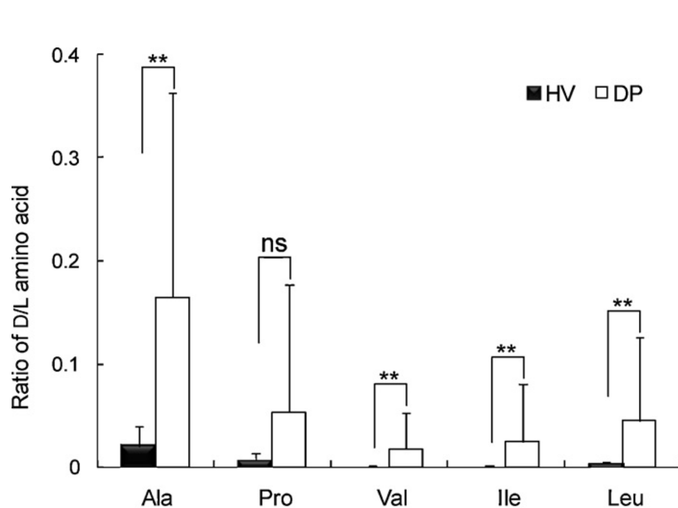

Fig. 4. Statistical analysis of D/L-amino acid ratios in the healthy volunteers ($n=20$) and the diabetic patients ($n=20$). The UPLC–ESI-TOF-MS conditions are described in Table 1. HV, healthy volunteers; DP, diabetic patients. (**$P<0.01$); ns: not significant.

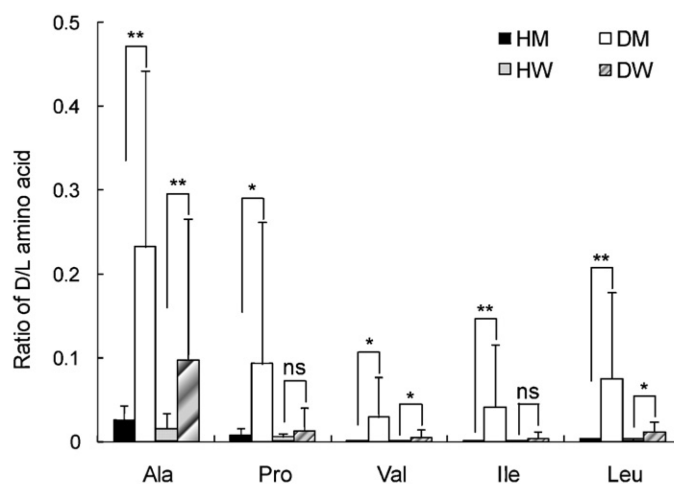

Fig. 5. Statistical analysis of D/L-amino acid ratios of men ($n=10$) and women ($n=10$) in the nails of healthy volunteers and the diabetic patients. HM, healthy men; DM, diabetic men; HW, healthy women; DW, diabetic women. The UPLC–ESI-TOF-MS conditions are described in Table 1. (*$P<0.05$; **$P<0.01$); ns: not significant.

4. Conclusion

The resolution of free DL-amino acids in the human nail was carried out by the combination of the *R*(−)-DBD-PyNCS and UPLC–ESI-TOF-MS. Because the proposed method provides the trace detection of DL-amino acids in human nails, this study was useful to detect D-Ala, D-Pro, D-Val, D-Ile and D-Leu from the nails of diabetic patients and healthy volunteers. Fifteen kinds of L-amino acids were also recognized from human nails. There was no significant difference in the content of the L-amino acids in the nail. However, a statistically significant ($P < 0.01$) and strong correlation was observed between the D/L-amino acids concentrations ratios (Ala, Val, Ile, Leu). Although the biochemical mechanisms responsible for these peculiar diabetic D/L-amino acids profiles are unclear, the D/L-amino acid concentration ratios may be useful as potential diagnostic markers in patients with diabetes. Fingernails grow at a rate of approximately 0.1–0.15 mm per day, and reflect long-term chronic concerns with little fluctuation, whereas blood and urine reflect the acute status. This study may be the starting point for additional studies in the field of DL-amino acid analyses in human nails.

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