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The determination of 2β-(3-hydroxypropoxy)-1α,25-dihydroxy vitamin D_3 (ED-71) in human serum by high-performance liquid chromatography–electrospray tandem mass spectrometry

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Abstract

A liquid chromatography–electrospray ionization tandem mass spectrometric (LC/ESI–MS/MS) method for the determination of 2β-(3-hydroxypropoxy)-1α,25-dihydroxy vitamin D_3 (ED-71) in human serum has been developed. ED-71 in human serum was extracted using two solid-phase extraction steps on Bond Elut C18 and NH2 cartridge. The separation of ED-71 and preED-71 isomer was attained by LC using 2 mmol/L ammonium acetate–methanol (15:85, v/v) as a mobile phase on a Symmetry C18 column (5 μm, 150 mm × 2.1 mm i.d.). ESI–MS/MS analysis was operated using selected reaction monitoring (SRM) in positive ion mode. The method achieved a lower limit of quantitation of 25 pg/mL. The calibration curve (25–3200 pg/mL) gave acceptable linearity ($r > 0.9964$). Intra-assay precision ranged from 2.3 to 9.7%. Inter-assay precision ranged from 1.0 to 3.4%. The accuracy was within 90.8–107.0%. This highly sensitive and reproducible method is able to determine only biologically active ED-71 by separating it from preED-71, which is considered to be applicable for the determination of serum samples from pharmacokinetic studies in human.

Keywords: 2β-(3-Hydroxypropoxy)-1α,25-dihydroxy vitamin D_3 (ED-71); preED-71; LC/ESI–MS/MS; Human serum

1. Introduction

2β-(3-Hydroxypropoxy)-1α,25-dihydroxy vitamin D_3 (ED-71) is a synthetic analog of 1α,25-dihydroxy vitamin D_3 ($1\alpha,25(OH)_2D_3$) substituted with a hydroxypropoxy group at the 2β-position of the A-Ring [1–3]. It is now well recognized that $1\alpha,25(OH)_2D_3$ is not only the biologically active metabolite for calcium homeostasis [4], but also plays an important role in differentiation inducing activity [5] and regulating immunological function [6]. At present, $1\alpha OHD_3$ as prodrug of $1\alpha,25(OH)_2D_3$ has been clinically used for the treatment of rickets, hypovitaminosis, hypocalcemia, chronic renal failure, and osteoporosis [7,8]. ED-71 was developed as a candidate for the treatment of osteoporosis with less calcemic activity than $1\alpha OHD_3$ and $1\alpha,25(OH)_2D_3$ [9–14]. ED-71 has unique properties that its binding capacity to Vitamin D binding protein (DBP) is twice as strong and the affinity to Vitamin D receptor (VDR) is eight times weaker compared with those of $1\alpha,25(OH)_2D_3$ [9]. It is considered that ED-71 has a long half-life (approximately 70 h in rat) by the higher affinity to DBP.

$1\alpha,25(OH)_2D_3$ and its related analogs exhibit therapeutic effects at very low concentrations and are used as low-dose oral drugs. Therefore, a sensitive and reliable method for the determination in biological fluids is required. For the determination of Vitamin D analogs, bioassays such as competitive protein binding assays [15,16], radioimmunoassay (RIA) [17–19], and radioreceptor assays (RRA) [20–22] are currently used. The bioassay is highly sensitive, but it of-

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ten shows poor specificity and accuracy owing to the interference by endogenous Vitamin D metabolites, and requires extensive sample clean-up. Further, high-performance liquid chromatography (HPLC) with electrochemical detection [23] and fluorometric detection [24] employing chemical derivatization is also difficult to apply to routine analysis of biological samples at low concentrations because these methods show poor sensitivity or require complicated pretreatment.

Liquid chromatography–mass spectrometry (LC/MS) is now a powerful tool for the determination of Vitamin D compounds in biological samples. Electrospray ionization (ESI) in conjunction with atmospheric pressure chemical ionization (APCI) is especially considered to be a sensitive and convenient method [25–29]. In a previous paper, we reported the method for the determination of 22-oxa-1α-25-dihydroxy vitamin D_3 (OCT) in human plasma, employing LC/ESI–MS/MS [25]. Vitamin D analogs are normally in thermal equilibrium with their previtamin D isomers. The equilibrium of the reaction between Vitamin D_3 and previtamin D_3 greatly inclines toward Vitamin D_3 under normal conditions without biological fluids (neutral pH, room temperature). In addition, it was reported that the previtamin D form of 1α,25-$(OH)_2D_3$ lost most of its biological activity in vitro and in vivo [30]. However, the isomeric conversion into previtamin D after administration of Vitamin D has not been well understood. Further, there is the possibility of causing overestimation in the determination of Vitamin D using LC/MS/MS because previtamin D isomer has the same molecular weight as Vitamin D. Therefore, the determination method of Vitamin D analogs should be a method in which they are separated from previtamin D isomer. Separation of Vitamin D and previtamin D has not been examined in previously reported methods [25–29]. The present paper describes the development of the determination of ED-71 in human serum for clinical pharmacokinetic study using LC/ESI–MS/MS. This method is very specific, rapid and reliable compared with commonly used bioassays and can completely separate ED-71 from preED-71.

2. Experimental

2.1. Materials and chemicals

ED-71 [2β-(hydroxypropoxy)-1α,25-dihydroxy vitamin D_3], [26,27-methyl-D6] ED-71 (used as internal standard, D6-ED-71) and preED-71 (Fig. 1) were synthesized at Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Bond Elut C18 cartridge (200 mg, 3cc), Bond Elut C8 cartridge (200 mg, 3cc), and Bond Elut NH2 cartridge (100 mg, 1cc) were obtained from Varian Inc. (Harbor City, CA). Drug free human serum (Consera) was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Bovine thymus 1α,25$(OH)_2D_3$ receptor was purchased from Yamasa Corp. (Chiba, Japan).

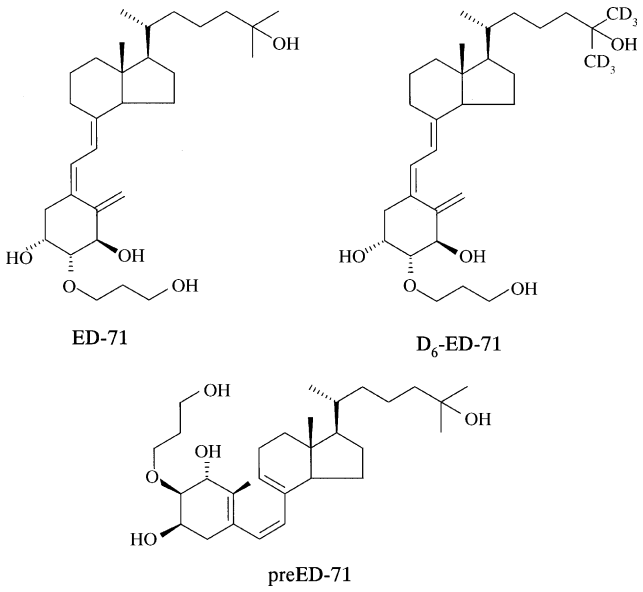

Fig. 1. Chemical structures of ED-71, D6-ED-71 and preED-71.

All other chemicals and solvents were of analytical reagent grade.

2.2. Apparatus

LC/ESI–MS/MS was performed using a Quattro LC (Micromass, Manchester, UK) equipped with an Alliance 2790 Separation Module (Waters Corp., Milford, MA). The ESI was operated in the positive ion mode at 2.8 kV for the capillary voltage. The cone voltage was set at 15 V. The neblizer gas (nitrogen gas) flow setting was 60 L/h at 110 °C and desolvation gas (nitrogen gas) flow setting was 600 L/h at 350 °C. Argon was used as collision gas and gas cell pressure was maintained at 8.0×10^{-4} to 9.0×10^{-4} mbar for SRM. A collision energy of 10 eV was used for ED-71 and D6-ED-71. The analytical chromatography of ED-71 was performed on a Symmetry C18 column (5 μm, 150 mm × 2.1 mm i.d., Waters Corp., Milford, MA) with 2 mmol/L ammonium acetate–methanol (15:85) as a mobile phase at a flow rate of 0.2 mL/min.

A TSKgel ODS-120T (5 μm, 250 mm × 4.6 mm i.d., Tosoh Corp., Tokyo, Japan) was also used for the purification of ED-71 in HPLC-RRA with acetonitrile–water (60:40) as a mobile phase at a flow rate of 1 mL/min.

2.3. Preparation of serum samples

A stock solution of ED-71 in ethanol was prepared at 100 μg/mL and was further diluted with ethanol to obtain the working solutions ranging from 1.25 to 800 ng/mL. For the calibration study, human serum (1 mL) was spiked with each working solution (20 μL). Final concentrations of calibration standards were 25.0, 50.0, 100, 200, 400, 800, 1600, and 3200 pg/mL, respectively. In addition, 0 pg/mL of cali-

bration blank was prepared by adding ethanol (20 μL) instead of the working solution to human serum (1 mL). Quality control (QC) samples used for accuracy, precision, recovery, and stability tests were prepared by diluting the working solutions with human serum at 25, 50, 400, and 2400 pg/mL. To measure the recovery of preED-71, the serum sample spiked with preED-71 was prepared at 2400 pg/mL.

2.4. Pretreatment of serum samples

After adding D6-ED-71 solution (800 ng/mL, 20 μL), serum samples (1 mL) were acidified with 1 mol/L hydrochloric acid (200 μL) and loaded on a Bond Elut C18 cartridge that was successively washed with methanol (2 mL), water (2 mL), and 1 mol/L hydrochloric acid (2 mL). After washing with water (2 mL) and 50% methanol (2 mL), ED-71 and D6-ED-71 were eluted with methanol (3 mL). Then, the eluate was evaporated under a nitrogen gas stream at 40 °C. The residue was dissolved in *n*-hexane–isopropanol (90:10, 500 μL) and applied to a Bond Elut NH2 that was successively washed with *n*-hexane–isopropanol (60:40, 1 mL) and *n*-hexane–isopropanol (90:10, 1 mL). After washing with *n*-hexane–isopropanol (90:10, 1 mL), ED-71 and D6-ED-71 were eluted with *n*-hexane–isopropanol (60:40, 3 mL). Then the eluate was evaporated under a nitrogen gas stream at 40 °C. The residue was reconstituted in 2 mmol/L ammonium acetate–methanol (15:85, 50 μL) and a 30 μL aliquot of the solution was then subjected to the LC/ESI–MS/MS analysis.

2.5. Specificity

Human blank serums from five healthy subjects were extracted as described above. Chromatograms were compared with calibration blank (0 pg/mL) and calibration standard (25 pg/mL) and confirmed that was no interference peak at the retention time of ED-71 and D6-ED-71.

2.6. Precision and accuracy of assay

The intra-assay precision and accuracy were evaluated by analyzing multiple replicates ($n = 5$) of QC samples at 25, 50, 400, and 2400 pg/mL on the same day. The inter-assay precision and accuracy were evaluated by analyzing these QC samples on three days.

2.7. Recovery test

After extraction of blank serum ($n = 3$) as described above, the working solutions and D6-ED-71 solution were added to extracts of blank serum by the concentration corresponding to each QC level. Extraction recovery of ED-71 and D6-ED-71 was calculated from the ratio between the mean peak area of 50, 400 and 2400 pg/mL of QC samples ($n = 5$) versus the mean peak area of blank serum added ED-71 and D6-ED-71 after extraction. Similarly, the recovery of pre-ED-71 was also evaluated.

2.8. Stability test

2.8.1. Freeze/thaw stability

The freeze/thaw stability of ED-71 in human serum was determined using QC samples at 50 and 2400 pg/mL. Samples were frozen at −20 °C and thawed at room temperature. The cycles were repeated three times. Before and after each freeze/thaw cycle, three samples were analyzed.

2.8.2. Stability at room temperature

The room temperature stability of ED-71 in human serum was evaluated using QC samples at 50 and 2400 pg/mL. The samples were stored at room temperature for 24 h. Before and after storage, each of the three samples was analyzed.

2.8.3. Stability in the autosampler

The stability of ED-71 extracted from human serum in the autosampler was examined using QC samples at 50 and 2400 pg/mL. After the pretreatment procedure, the samples were stored in the autosampler at 5 °C. Each of the three samples was analyzed at 0, 24 and 48 h.

2.9. Cross-validation

The bioavailability study was undertaken by the Jikei University School of Medicine, ED-71 (1 μg, tablet or capsule) was single orally administrated to healthy volunteers. Blood samples for ED-71 analysis were collected by the range from 0.5 to 96 h after administration. Serum was separated and then stored at below −20 °C until analysis. Previously, the stability of ED-71 in human serum at −20 °C had been confirmed for six months by the HPLC-RRA method. Informed consent was obtained from healthy volunteers at the Jikei University School of Medicine. The serum samples from the bioavailability study in human were determined by LC/MS/MS and HPLC-RRA. The developed HPLC-RRA in our laboratory had been fully validated, LLOQ is 25 pg in 1 mL human serum aliquots (not published). RRA analysis using bovine thymus $1\alpha,25(OH)_2D_3$ receptor was performed after solid-phase extraction on Bond Elut C8 cartridge and following purification by reversed-phase HPLC.

The cross-validation experiment was performed by evaluating the differences of two determinations, employing the previously reported method [31]. Statistical comparisons were performed using linear regression analysis, a paired *t*-test, and random error in Microsoft Excel 2000 (Microsoft, Redmond, WA). The fixed systematic error and proportional systematic error were determined from the 95% confidence interval and *t*-statistic about the intercept and slope. The *t*-critical value was taken from the *t* table with $(n - 2)$ degrees of freedom at the appropriate significance level ($p = 0.05$). Random error was estimated from %difference normalized to the reference concentration obtained by the HPLC-RRA method and the standard deviation of %difference was calculated. The combined assay precisions for two assays were calculated from the individual inter-assay precision data ac-

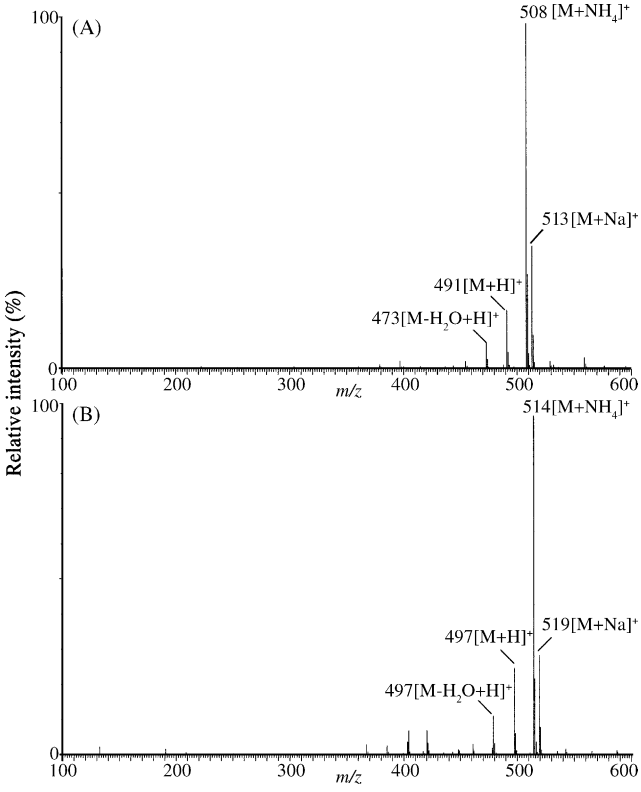

Fig. 2. Electrospray positive ion mass spectra of (A) ED-71 and (B) D6-ED-71 obtained by infusion of 10 μL/min using 10 mmol/L ammonium acetate–methanol (10:90, v/v) as a mobile phase.

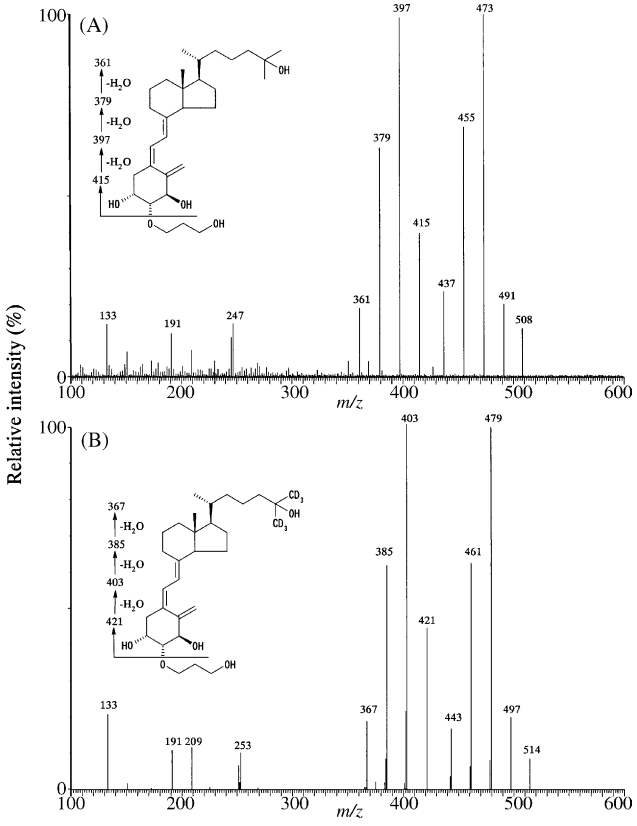

Fig. 3. Product ion mass spectra of (A) ED-71 and (B) D6-ED-71 obtained by infusion of 10 μL/min using 10 mmol/L ammonium acetate–methanol (10:90, v/v) as a mobile phase. Precursor ion, *m/z* 508.4 and 514.4, respectively; collision energy, 10 eV.

cording to the reference method [31]. The combined %R.S.D. value at a low QC level (50 pg/mL) near the ED-71 concentrations observed in the bioavailablity study was compared with the random error.

3. Results and discussion

3.1. LC/ESI–MS/MS analysis

For highly sensitive and selective determination of trace compounds in biological fluids, tandem mass spectrometry is effective. Previously, we reported that OCT was characterized by an intense adduct ion $[M + NH_4]^+$ with a high abundance of product ion formed by cleavage of 22-ether group in LC/ESI–MS/MS and achieved the LLOQ of 20 pg/mL [25]. Therefore, an initial effort was made to examine efficient ionization condition and SRM setting of ED-71 in the ESI mode.

The ESI mass spectra of ED-71 and D6-ED-71 (internal standard) were obtained in 10 mmo/L ammonium acetate–methanol (10:90, v/v) by infusion of 10 μL/min. Typical spectra of ED-71 and D6-ED-71 are shown in Fig. 2. ED-71 and D6-ED-71 exhibited an ammonium adduct ion $[M + NH_4]^+$ as a most intense ion at *m/z* 508.4 and 514.4, respectively. The effects of capillary and cone voltage on the intensity of an ammonium adduct ion were examined; optimal ionization conditions were 2.8 kV and 15 V for the capillary and cone voltage, respectively. Furthermore, the effects of pH and concentration of ammonium acetate in a mobile phase for the effective detection of ammonium adduct ion were investigated. The decreasing of concentration of ammonium acetate brought about an enhancement of intensity of an ammonium adduct ion, whereas pH had no effect on the intensity of an ammonium adduct ion (data not shown). As a result, the concentration of ammonium acetate was set at 2 mmol/L, which was the minimum concentration to obtain higher sensitivity and the efficient chromatographic peak shape. For D6-ED-71, the ratio of the unlabeled component to fully labeled component was determined in the selected ion monitoring (SIM) mode to be less than 1/2000, which was sufficiently available isotopic purity as an internal standard.

The product ion mass spectra of ED-71 and D6-ED-71 using ammonium adduct ion $[M + NH_4]^+$ as a precursor ion for finding the effective SRM condition were determined (Fig. 3). ED-71 and D6-ED-71 were easily fragmented in the low collision energy, and the fragment ions formed by elimination of the 2β-hydroxypropoxy group and water molecule were observed. When the SRM transitions of the *m/z* 508.4 > 397.3 and 514.4 > 403.3 for ED-71 and D6-ED-71 using the product ions formed by the elimination of the 2β-

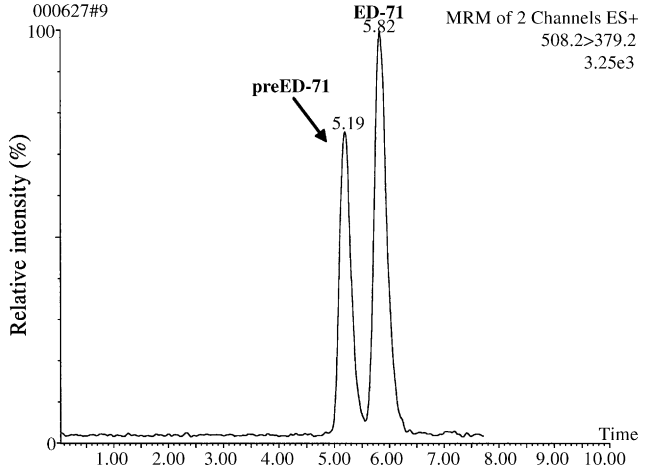

Fig. 4. Selected reaction monitoring (SRM) ion chromatogram of an authentic mixture of ED-71 and preED-71. SRM transition, *m/z* 508.4 > 397.3.

hydroxypropoxy group and one water molecule with 10 eV of a collision energy were chosen, the most sensitive and specific determination was attained, the limit of detection was 20 fmol (ED-71, 10 pg)/injection at a signal-to-noise ratio of 10.

As shown in Fig. 4, the chromatographic separation of ED-71 and preED-71 having the same molecular weight as ED-71 was attained using 2 mmol/L ammonium acetate–methanol (15:85, v/v) as a mobile phase on a Symmetry C18 column.

3.2. Pretreatment of serum sample

Because ED-71 is a neutral compound, the pretreatment method by two different separation modes is effective for the purification of biological specimens. To eliminate coexisting substances such as protein and inorganic salts in biological fluids, plasma sample was subjected to solid-phase extraction with Bond Elut C18 and Bond Elut NH2, as described in Section 2. As shown in Table 1, the extraction recoveries of ED-71 and D6-ED-71 were greater than 80% at each QC level. The extraction recovery of preED-71 was 84.2% at 2400 pg/mL, which was the same result as that of ED-71. In contrast, the previtamin D form of OCT is unstable compared with preED-71, completely decomposed by the treatment with hydrochloric acid during pre-

Table 1
Extraction recovery of ED-71 and D6-ED-71 from human serum

Nominal concentration of ED-71 (pg/mL)	Recovery (%)			
	ED-71		D6-ED-71[a]	
	Mean	S.D.	Mean	S.D.
50	85.0	8.5	80.1	10.1
400	90.2	3.0	90.9	1.8
2400	92.1	5.1	93.2	4.5

$n = 5$.
[a] The concentration of D6-ED-71 is 1600 pg/mL.

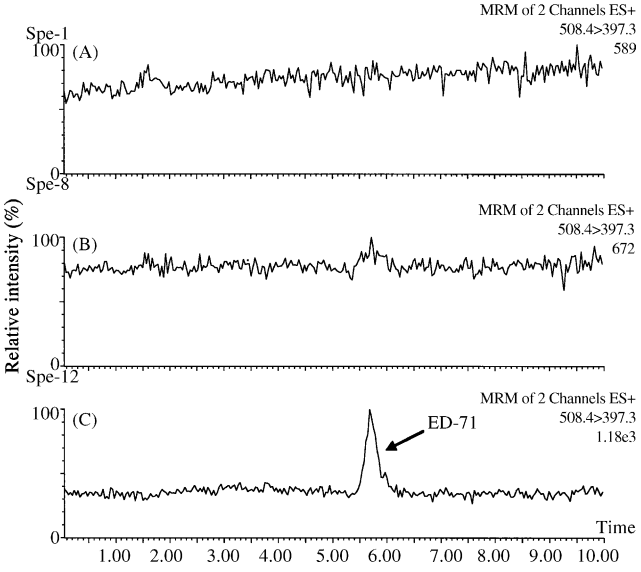

Fig. 5. Specificity of ED-71: selected reaction monitoring ion chromatograms of (A) blank human serum; (B) human serum spiked with D6-ED-71 (ca. 1600 pg/mL) and (C) human serum spiked with ED-71 (25 pg/mL) and D6-ED-71 (ca. 1600 pg/mL). SRM transition: *m/z* 508.4 > 397.3 for ED-71.

treatment procedure in the reference method [25]. Therefore, the chromatographic separation of ED-71 and preED-71 is required.

Typical SRM chromatograms obtained from blank serum, calibration blank (0 pg/mL), and the lowest calibration standard (25 pg/mL) are illustrated in Figs. 5 and 6. In these chromatograms, there was no interfering peak derived from endogenous substances and unlabeled component of D6-ED-71 at the retention times of ED-71 and D6-ED-71.

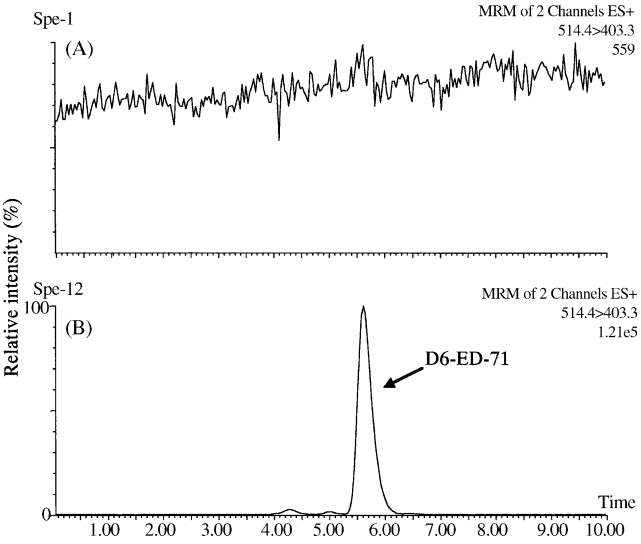

Fig. 6. Specificity of D6-ED-71: selected reaction monitoring ion chromatograms of (A) blank human serum and (B) human serum spiked with D6-ED-71 (ca. 1600 pg/mL). SRM transition: *m/z* 514.4 > 403.3 for D6-ED-71.

Table 2
Linearity of calibration curve for the determination of ED-71 in human serum

Nominal concentration (pg/mL)	Accuracy of back calculated concentration (%)				
	Day 1	Day 2	Day 3	Day 4	Day 5
25	110.8	106.0	105.6	116.8	107.2
50	101.2	85.8	96.8	89.2	101.0
100	98.3	95.3	96.1	92.5	95.8
200	95.5	103.0	98.5	99.5	97.5
400	101.3	113.5	105.8	104.5	104.5
800	91.0	103.6	95.8	98.3	92.0
1600	98.9	90.7	101.7	98.1	100.3
3200	102.9	102.2	99.7	101.1	101.5
Coefficient of correlation	0.9984	0.9964	0.9997	0.9997	0.9992

3.3. Calibration curve

Calibration curves were constructed for five days by the weighted ($1/x$) linear regression using the ratio of the peak area of ED-71 to that of D6-ED-71, plotted against the concentrations. The results are shown in Table 2. The linearity was good from 25 to 3200 pg/mL [correlation coefficient (r) > 0.9964], and the accuracies of back calculated concentrations were within 85.8–116.8%.

3.4. Precision and accuracy of assay

The precision and accuracy of assay are summarized in Table 3. The intra-assay precision and accuracy were evaluated by analyzing five replicates of QC samples at the concentrations described in Section 2. The inter-assay precision and accuracy were determined by measuring the same samples on three different days. The intra- ($n = 5$) and inter-assay ($n = 5$, three days) coefficient of variations (CVs) were less than 9.7 and 3.4% at all levels, respectively. The intra- and inter-assay accuracies were within 90.8–107.0% of the nominal concentration. From the results, the LLOQ was assigned as 25 pg/mL.

Table 3
Intra- and inter-assay precision and accuracy for the determination of ED-71 in human serum

Nominal concentration (pg/mL)	Found concentration (pg/mL, mean ± S.D.)	CV (%)	Accuracy (%)
Intra-assay[a]			
25	25.2 ± 2.0	7.9	100.8
50	47.6 ± 4.6	9.7	95.2
400	428 ± 10	2.3	107.0
2400	2439 ± 66	2.7	101.6
Inter-assay[b]			
25	26.5 ± 0.9	3.4	106.0
50	45.4 ± 0.9	2.0	90.8
400	421 ± 4	1.0	105.3
2400	2355 ± 48	2.0	98.1

[a] $n = 5$.
[b] $n = 5$, three days.

Table 4
Freeze/thaw stability of ED-71 in human serum[a]

Nominal concentration (pg/mL)	Percentage of expected			
	Freeze/thaw cycle			
	0	1	2	3
50	97.8	90.2	94.4	102.0
2400	97.1	95.7	97.9	104.8

[a] $n = 3$.

3.5. Stability

The freeze/thaw stability of ED-71 in human serum was examined, which indicated that ED-71 was stable up to three freeze/thaw cycles (Table 4). ED-71 was stable in human serum at room temperature for at least 48 h (Table 5). ED-71 extracted from human serum was also found to be stable in the autosampler at 5 °C for 48 h (Table 5).

3.6. Cross-validation

The present method was applied to the determination of serum samples from healthy subjects who received a single oral administration (1 μg). A typical SRM chromatogram obtained from a serum sample in the bioavailability study is illustrated in Fig. 7. In this chromatogram, there was no peak of preED-71. It was confirmed that mostly

Table 5
(A) Room temperature stability in human plasma and (B) stability of ED-71 extracted from human serum in the autosampler[a]

Nominal concentration (pg/mL)	Percentage of expected		
	Time (h)		
	0	24	48
(A)			
50	89.4	102.0	98.2
2400	99.2	101.7	99.2
(B)			
50	104.2	102.0	108.8
2400	94.4	98.1	100.3

[a] $n = 3$.

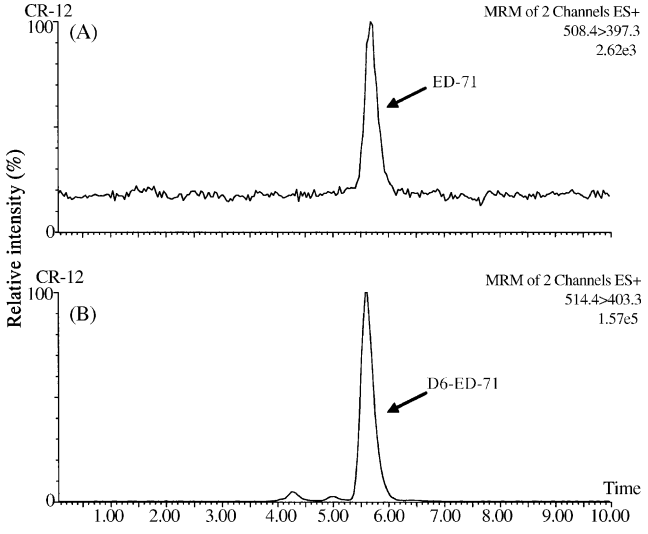

Fig. 7. Selected reaction monitoring ion chromatogram of human serum 5 h after a single oral administration of 1 μg ED-71 tablet. SRM transitions: *m*/*z* 508.4 > 397.3 for ED-71; *m*/*z* 514.4 > 403.3 for D6-ED-71.

biologically active ED-71 was exposed to human blood stream since preED-71 was stable during the pretreatment procedure.

As depicted in Fig. 8, comparable results were obtained using the LC/MS/MS method and HPLC-RRA, and indicated a good correlation ($r = 0.8845$). The summary of statistics during a cross-validation experiment is shown in Table 6. Unweighted linear regression analysis showed an intercept of 12.9 pg/mL with a confidence interval from −0.873 to 26.7 pg/mL, which contains zero. The observed *t*-statistic was less than the *t*-critical with $(n - 2)$ degrees of freedom. There is, therefore, no significant fixed bias. Similarly, the results

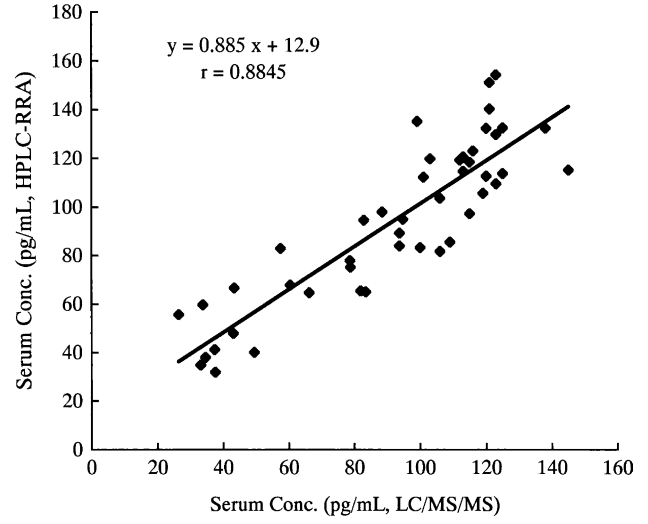

Fig. 8. Cross-validation: comparison of the serum ED-71 concentrations determined by both LC/MS/MS and HPLC-RRA. Serum samples were obtained from the healthy volunteers received a single oral administration (1 μg) in the bioavailability study.

Table 6
Summary of statistics obtained in the cross-validation of LC/MS/MS and HPLC-RRA

Test	Statistic
1. Random error (%difference)	
Mean	14.4
S.D.	11.7
2. Regression analysis (intercept)[a]	
Coefficient	12.9
Standard error	6.8
Confidence interval	−0.873–26.7
Observed *t*[b]	1.89
3. Regression analysis (slope)[a]	
Coefficient	0.885
Standard error	0.070
Confidence interval	0.743–1.03
Observed *t*[b]	−1.64

[a] Unweighted least squares regression.
[b] *t*-Critical: 2.02 ($p = 0.05$), $n = 46$, degrees of freedom: 44.

Table 7
Combined assay precisions for two assays

	LC/MS/MS		HPLC-RRA[a]
Mean (pg/mL)	45.4		52.6
S.D. (pg/mL)	0.9		12.5
CV (%)	2.0		23.8
$S.D._{comb}$ (pg/mL)[b]		12.5	
$\%R.S.D._{comb}$[c]		25.1	

[a] In-house data.
[b] $S.D._{comb} = \sqrt{(S.D._{LC/MS/MS})^2 + (S.D._{HPLC\text{-}RRA})^2}$.
[c] $\%R.S.D._{comb} = [S.D._{comb}/\text{concentration}] \times 100$.

of the statistical tests for the calculated slope (0.885) showed no significant proportional error, using *t*-statistic (−1.64) and a confidence interval (0.743–1.03). The random error (standard deviation of a %difference) of 11.7% was within the combined assay precision ($\%R.S.D._{comb}$) of 25.1% (Table 7) indicate that the cross-validation can be considered successful. From these results, the LC/MS/MS method described here can be used as a rapid and reliable assay alternative to complicated HPLC-RRA.

4. Conclusion

A highly sensitive and specific method has been developed for the determination of unchanged ED-71 in human serum and fully validated with good accuracy and reproducibility. The method achieved the LLOQ of 25 pg/mL and the separation from preED-71 isomer. The data obtained in this study demonstrate that LC/MS/MS can provide a powerful alternative to HPLC-RRA for the determination of Vitamin D analog. Further clinical studies are also now being implemented with the proposed method. It is hoped that the availability of the present method for the determination of ED-71 in human serum will contribute to provide ED-71 for the patients of osteoporosis immediately.

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References

[1] K. Miyamoto, E. Murayama, K. Ochi, H. Watanabe, N. Kubodera, Chem. Pharm. Bull. 41 (1993) 1111.
[2] Y. Ono, H. Watanabe, A. Shiraishi, S. Takeda, Y. Higuchi, K. Sato, N. Tsugawa, T. Okano, T. Kobayashi, N. Kubodera, Chem. Pharm. Bull. 45 (1997) 1626.
[3] Y. Ono, A. Kawase, H. Watanabe, A. Shiraishi, S. Takeda, Y. Higuchi, K. Sato, T. Yamauchi, T. Mikami, M. Kato, N. Tsugawa, T. Okano, N. Kubodera, Bioorg. Med. Chem. 6 (1998) 2517.
[4] H. Reichel, H.P. Koeffler, A.W. Norman, N. Engl. J. Med. 320 (1989) 980.
[5] E. Abe, C. Miyaura, H. Sakagami, M. Takeda, K. Konno, T. Yamazaki, S. Yoshiki, T. Suda, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 4990.
[6] J.M. Lemire, J. Cell. Biochem. 49 (1992) 26.
[7] Y. Seino, H. Tanaka, K. Yamaoka, H. Yabuuchi, Bone Miner. 2 (1987) 479.
[8] H. Orimo, M. Shiraki, Y. Hayashi, T. Hoshino, T. Onaya, S. Miyazaki, H. Kurosawa, T. Nakamura, N. Ogawa, Calcif. Tissue Int. 54 (1994) 370.
[9] T. Okano, N. Tsugawa, S. Masuda, A. Takeuchi, T. Kobayashi, Y. Takita, Y. Nishii, Biochem. Biophys. Res. Commun. 163 (1989) 1444.
[10] K. Sato, Y. Nishii, F.N. Woodiel, L.G. Raisz, Bone 14 (1993) 47.
[11] H. Tsurukami, T. Nakamura, K. Suzuki, K. Sato, Y. Higuchi, Y. Nishii, Calcif. Tissue Int. 54 (1994) 142.
[12] Y. Tanaka, T. Nakamura, S. Nishida, K. Suzuki, S. Takeda, K. Sato, Y. Nishii, J. Bone Miner. Res. 11 (1996) 325.
[13] K. Yamane, T. Okano, H. Kishimoto, H. Hagino, Bone 24 (1999) 187.
[14] Y. Uchiyama, Y. Higuchi, S. Takeda, T. Masaki, A. Shiraishi, K. Sato, N. Kubodera, K. Ikeda, E. Ogata, Bone 30 (2002) 582.
[15] M.W. France, B. Lalor, Ir. J. Med. Sci. 150 (1981) 310.
[16] C. Lind, J. Chen, I. Byrjalsen, Clin. Chem. 43 (1997) 943.
[17] S. Scharla, H. Schmidt-Gayk, H. Reichel, E. Mayer, Clin. Chim. Acta 142 (1984) 325.
[18] A.P. De Leenheer, R.M. Bauwens, Clin. Chem. 31 (1985) 142.
[19] N. Kobayashi, K. Shimada, H. Taki, Y. Sakamoto, S. Kamachi, Biol. Pharm. Bull. 18 (1995) 382.
[20] J.S. Chandler, J.W. Pike, L.A. Hagan, M.R. Haussler, Methods Enzymol. 67 (1980) 522.
[21] T.A. Reinhardt, B.W. Hollis, Methods Enzymol. 123 (1986) 176.
[22] T. Okano, N. Tsugawa, T. Kobayashi, J. Chromatogr. 493 (1989) 63.
[23] S. Masuda, T. Okano, M. Kamao, Y. Kanedai, T. Kobayashi, J. Pharm. Biomed. Anal. 15 (1997) 1497.
[24] M. Shimizu, X. Wang, S. Yamada, J. Chromatogr. B 690 (1997) 15.
[25] M. Ishigai, Y. Asoh, K. Kumaki, J. Chromatogr. B 706 (1998) 261.
[26] A.M. Kissmeyer, K. Sonne, E. Binderup, J. Chromatogr. B 740 (2000) 117.
[27] T. Higashi, D. Awada, K. Shimada, Biomed. Chromatogr. 15 (2001) 133.
[28] T. Higashi, D. Awada, K. Shimada, Biol. Pharm. Bull. 24 (2001) 738.
[29] T. Higashi, D. Awada, K. Shimada, J. Chromatogr. B 772 (2002) 229.
[30] R. Bouillon, L.A. Sarandeses, K. Allewaert, J. Zhao, J.L. Mascarenas, A. Mourino, S. Vrielynck, P. de Clercq, M. Vandewalle, J. Bone Miner. Res. 8 (1993) 1009.
[31] M.T. Gilbert, I. Barinov-colligon, J.R. Miksic, J. Pharm. Biomed. Anal. 13 (1995) 385.