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Liquid chromatography–mass spectrometric method combined with derivatization for determination of 1α-hydroxyvitamin D_3 in human plasma

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Abstract

A stable isotope dilution liquid chromatography–tandem mass spectrometric (LC–MS–MS) method for the determination of plasma 1α-hydroxyvitamin D_3 [1α(OH)D_3] has been developed. The method employed derivatization, the reaction with 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione and acetylation, which significantly improved the ionization efficiency of 1α(OH)D_3 with a detection limit of 6.3 fmol per injection. The plasma 1α(OH)D_3 was extracted with acetonitrile, purified with disposable cartridges, derivatized and subjected to LC–MS–MS analysis using atmospheric pressure chemical ionization. The intra- and inter-assay coefficients of variation were below 10.6 and 4.7%, respectively, and the analytical recovery of 1α(OH)D_3 was quantitative. The limit of quantitation was 25 pg/ml for a 1.0-ml plasma aliquot. The application of the developed method to the sample of a volunteer orally given 1α(OH)D_3 was also described.

Keywords: Derivatization, LC–MS; 1α-Hydroxyvitamin D_3

1. Introduction

1α-Hydroxyvitamin D_3 [1α(OH)D_3] is now well-known as a synthetic prodrug of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], a hormonally active form of vitamin D_3, and has been clinically used for the treatment of rickets, hypovitaminosis, hypocalcemia, chronic renal failure and osteoporosis [1]. Although there have been a few papers which describe the change in the plasma/serum concentration of 1,25$(OH)_2D_3$ after the administration of 1α(OH)D_3 (oral dose: several micrograms per body) [2,3], the plasma/serum concentration of 1α(OH)D_3 has been hardly clarified. Recently, the pharmacokinetics of 1α(OH)D_3 in the rat were investigated using a tritium-labeled compound with high specific radioactivity [4]. This method is highly sensitive, but it requires special equipment due to the use of a radioisotope (RI). Therefore, a practical non-RI method for the determination of 1α(OH)D_3 in biological fluids is strongly required, though developing the method is one of the most challenging subjects in the field of vitamin D analysis.

Although vitamin D metabolites and analogs have conventionally been measured by saturation analysis

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using binding proteins, such as competitive protein binding assay and radioreceptor assay based on the vitamin D binding protein and vitamin D receptor, respectively [5], 1α(OH)D_3 does not have competent binding affinity to both proteins. Based on these data, we attempted to prepare a specific antibody to 1α(OH)D_3 for development of its immunoassay using the 1α(OH)D_3-3-hemiglutarate-bovine serum albumin conjugate as an immunogen, but the desired antibody could not be obtained (unpublished results). On the other hand, high-performance liquid chromatography (HPLC) has also been examined for the determination of many vitamin D compounds [5]. However, HPLC with UV detection does not demonstrate the required sensitivity for measurement of 1α(OH)D_3 in biological fluids (several dozens picograms per millilitre). To obtain a higher sensitivity, HPLC methods with electrochemical detection [6] and fluorimetric detection based on a fluorescence (FL)-labeling reaction [7] for some other vitamin D compounds have been reported, but these methods required complicated pretreatment steps including preparative HPLC prior to the determination, so that these methods are not necessarily suitable for the analysis of a great number of samples.

Liquid chromatography–mass spectrometry (LC–MS) employing electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) as an interface is considered to be a rapid and convenient method for the determination of vitamin D compounds in biological fluids. For some synthetic analogs, highly sensitive LC–MS methods have actually been developed [8,9]. However, because 1α(OH)D_3 has few polar functional groups (only two hydroxy groups), its ionization efficiency is very low for various ionization methods, which is the utmost reason that it is hard to develop a practical LC–MS method for 1α(OH)D_3.

The Cookson-type reagent is a 4-substituted 1,2,4-triazoline-3,5-dione (TAD), and TAD is a powerful dienophile which rapidly and quantitatively reacts with the *s-cis*-diene of the vitamin D compound to form a Diels–Alder adduct. Derivatives with Cookson-type reagents have also been examined in various ionization methods to enhance the sensitivity [10–15]. We observed that the ionization efficiencies of vitamin D compounds significantly increased by derivatization with Cookson-type reagents being rich in proton-affinitive atoms, such as oxygen and nitrogen, in APCI–MS operating in the positive-ion mode, and developed sensitive LC–APCI–MS methods for 25-hydroxyvitamin D_2 [25(OH)D_2] and 25-hydroxyvitamin D_3 [25(OH)D_3] [14], and 24*R*,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] [15] in human plasma using this derivatization. We had examined different Cookson-type reagents for APCI–MS and found 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-TAD (DMEQTAD) [16] to be the most effective in increasing the ionization efficiencies of the resulting derivatives [14]. Furthermore, acetylation is also known as a simple technique to enhance the sensitivity of a compound having hydroxy groups in the positive APCI–MS [17].

In the present paper, the usefulness of the derivatization in the positive APCI–MS of 1α(OH)D_3 was examined and then its LC–MS method was developed. The application of the developed method for the determination of plasma 1α(OH)D_3 was also described.

2. Experimental

2.1. Materials and chemicals

1α(OH)D_3, [22,22,23,23-$^2H_4$]-1α(OH)D_3 (internal standard, I.S.) [4], 1,25$(OH)_2D_3$, and 1α,24*R*,25-trihydroxyvitamin D_3 [1,24,25$(OH)_3D_3$] were supplied by Chugai Pharmaceutical Co. (Tokyo, Japan). 25(OH)D_3 and 24,25$(OH)_2D_3$ were obtained from Wako Pure Chemical Co. (Osaka, Japan) and Duphar B.V. Co. (Amsterdam, The Netherlands), respectively. 25*S*,26-Dihydroxyvitamin D_3 [25,26$(OH)_2D_3$] was synthesized in our laboratories by the known method [18]. DMEQTAD [16] was purchased from Wako Pure Chemical Co. OASIS HLB cartridges (60 mg: Waters, Milford, MA, USA) were successively washed with AcOEt (2 ml), MeOH (2 ml) and H_2O (2 ml) prior to use. Bond Elut Si cartridges (500 mg: Varian, Harbor, CA, USA) were successively washed with $CHCl_3$–MeOH (30:1, v/v, 4 ml) and $CHCl_3$ (4 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. Plasma samples

Blank plasma samples were obtained from healthy volunteers (age range 21–32 years) known not to have received vitamin D therapy, and stored at −20 °C prior to use.

1α(OH)D_3 (4 μg, ALFAROL® capsules, Chugai Pharmaceutical Co.) was orally administered as a single dose to a healthy male volunteer (58 years). The volunteer fasted for 4 h prior to the administration and broke his fast 3 h after the administration. Blood was collected at 3, 5 and 7 h following the administration, as well as before the administration and then the plasma was separated and stored at −20 °C prior to use. Informed consent was obtained from the volunteer.

2.3. LC–MS(–MS)

LC–MS(–MS) was performed using a ThermoQuest LCQ (San Jose, CA) liquid chromatograph–ion trap-mass spectrometer connected to a JASCO PU-980 (Tokyo) chromatograph, and the APCI was used in the positive-ion mode. A J'sphere ODS H-80 (4 μm, 150×4.6 mm I.D.; YMC, Kyoto, Japan) column was used at a flow-rate of 1 ml/min at 40 °C. For the MS–MS analysis, helium was used as the collision gas. The source current and the sheath gas flow-rate were 5 μA and 80 units, respectively. The heated capillary temperature, the vaporizer temperature, the capillary voltage, the tube lens offset voltage and mobile phase for each analyte were as follows: 175 °C, 550 °C, 5 V, 20 V and MeOH–H_2O (19:1, v/v) for 1α(OH)D_3; 225 °C, 550 °C, 3 V, 15 V and MeCN–H_2O (3:1, v/v) for 1α(OH)D_3-DMEQ-TAD; 225 °C, 475 °C, 3 V, 15 V and MeCN–H_2O (23:2, v/v) for 1α(OH)D_3-DMEQTAD-1,3-diacetate (Ac).

2.4. Pretreatment of plasma sample

The I.S. solution (ca. 400 pg in 10 μl of EtOH) was added to a plasma sample (1.0 ml) placed in a borosilicate glass tube and the mixture was allowed to equilibrate for 15 min at room temperature, which was then added to MeCN (1.0 ml) placed in another tube. The former tube was rinsed with MeCN (0.25 ml) and the washings were combined with the above sample. The mixture was vortex-mixed for 30 s and subjected to centrifugation at 1500 *g* for 10 min. Water (2 ml) was added to the supernatant and the sample was then passed through an OASIS HLB cartridge. After washing with H_2O (2 ml), 70% MeOH (2 ml) and hexane (1 ml), 1α(OH)D_3 and I.S. were eluted with AcOEt (1 ml), which was evaporated under a N_2 gas stream. The residue was dissolved in $CHCl_3$ (0.2 ml×2) and applied to a Bond Elut Si cartridge. After washing with $CHCl_3$ (3 ml) and $CHCl_3$–MeOH (40:1, v/v, 2.2 ml), 1α(OH)D_3 and I.S. were eluted with $CHCl_3$–MeOH (30:1, v/v, 2 ml). After evaporation, the residue was subjected to derivatization as described below.

2.5. Derivatization

2.5.1. Reaction with DMEQTAD

The pretreated plasma samples or standard samples were dried for 10 min in vacuo and then dissolved in AcOEt (25 μl) containing DMEQTAD (2.5 μg). The mixture was kept at room temperature for 30 min, then an additional reagent (2.5 μg/25 μl of AcOEt) was added and the entire mixture was further kept at room temperature for 1 h to form the DMEQTAD derivatives [7]. EtOH (40 μl) was added to the mixture to decompose any excess reagent, and the solvent was evaporated.

2.5.2. Acetylation (DMEQTAD-Ac derivative)

The DMEQTAD derivatives were dissolved in the mixture of Ac_2O (10 μl) and pyridine (20 μl), and kept at 50 °C for 1 h. After the addition of EtOH (40 μl), the solvent was evaporated and, unless otherwise indicated, the residue was dissolved in the mobile phase (40 μl), 15 μl of which were subjected to LC–MS(–MS).

2.6. Effect of derivatization for detection responses

The effect of the derivatization for the detection responses was evaluated by the limit of detection (LOD; the amount of 1α(OH)D_3 or derivatives per injection giving a signal-to-noise ratio (*S*/*N*) of 3). Two hundred picograms (500 fmol) of 1α(OH)D_3 were derivatized as described above. These deriva-

tives were dissolved in the mobile phase (200 μl) to prepare the solutions of 2.5 fmol/μl and then subjected to LC–MS. By stepwise decreasing the injection volume of the resulting solution, the amount of derivative giving an S/N of 3 was determined. The LOD of the intact 1α(OH)D_3 was determined using the solution of 20 ng/ml in the same way.

2.7. Calibration curves

Stock solutions of 1α(OH)D_3 were prepared as 50 μg/ml solutions in EtOH, the concentrations of which were confirmed by UV spectroscopy using the molar absorptivity, $\varepsilon = 18\,200$, at 265 nm. Subsequent dilutions were carried out with EtOH to prepare 2.5, 5, 10 and 15 ng/ml solutions. The ethanolic solution of I.S. with the concentration of ca. 40 ng/ml was also prepared.

Calibration curves were constructed in two ways. One used the above standard solutions; 1α(OH)D_3 (25, 50, 100 or 150 pg) and I.S. (400 pg) placed in a tube were derivatized and subjected to LC–MS–MS, and the calibration curve was constructed by plotting the peak area ratios [1α(OH)D_3/I.S.] against the amounts of 1α(OH)D_3 per tube (pg). The other used the blank plasma spiked with a known amount of 1α(OH)D_3; 1.0 ml of plasma was spiked with 25, 50, 100 or 150 pg of 1α(OH)D_3 and 400 pg of I.S., which was then pretreated, derivatized and subjected to LC–MS–MS. The calibration curve was constructed by plotting the peak area ratios [1α(OH)D_3/I.S.] against the concentrations of 1α(OH)D_3 (pg/ml).

2.8. Absolute recovery and influence of endogenous substances for derivatization

One millilitre of blank plasma and 1α(OH)D_3 spiked plasma (100 pg/ml, $n = 3$) were pretreated as above and then 100 pg of 1α(OH)D_3 were added to only the blank sample. To both samples were added I.S. (400 pg) and then derivatized. The absolute recovery of 1α(OH)D_3 during pretreatment was calculated from the peak area ratios [1α(OH)D_3/I.S.] of the spiked sample and the blank sample. Next, 1 ml of blank plasma and I.S. spiked plasma (400 pg/ml, $n = 3$) were pretreated and 400 pg of I.S. were added to only the blank sample. To both samples were added 1α(OH)D_3 (200 pg) and then derivatized. The absolute recovery of I.S. was calculated from the peak area ratios [I.S./1α(OH)D_3] of the spiked sample and the blank sample.

The influence of endogenous substances on the derivatization was examined as follows. I.S. (400 pg) was placed in tubes with plasma (1.0 ml) extract prepared as described above. After derivatization, the peak area was compared with that obtained from the sample without the plasma extract.

2.9. Freeze–thaw stability

The plasma samples spiked with 1α(OH)D_3 (100 pg/ml) were frozen at −20 °C for 24 h and then thawed at room temperature. When the samples were completely thawed, they were frozen for 24 h again. The samples were analyzed after the next thawing.

2.10. Measurement of 1,25(OH)$_2$D in plasma

The plasma 1,25(OH)$_2$D of the volunteer administered 1α(OH)D_3 was measured by radioreceptor assay (Yamasa SRL kit, Choshi, Japan).

3. Results and discussion

3.1. LC–MS(–MS) of 1α(OH)D_3 and its derivatives

DMEQTAD was previously developed as an FL-labeling reagent having a highly reactive dienophile and sensitive fluorophore by Shimizu et al. [16]. When the reagent is applied to the assay of the vitamin D metabolites in plasma using FL–HPLC, a complicated pretreatment to remove the excess reagent is necessary [7]. We have applied this reagent to LC–APCI–MS for the simultaneous determination of 25(OH)D_2 and 25(OH)D_3 in human plasma, in which remarkable enhancement of the sensitivity was observed by the derivatization [14]. This was caused by two major factors; by the derivatization, the proton-affinitive atoms were introduced and the molecular mass of the analyte was shifted to a higher mass range, where background noise is relatively low. Furthermore, the method did not require the

Table 1
Limit of detection in 1α(OH)D_3 and its derivatives[a]

Compounds	Monitoring ions (m/z)	t_R (min)	LOD (per injection)
1α(OH)D_3	Sum of 401 $[M+H]^+$ (9)[b], 383 $[401-H_2O]^+$ (100) and 365 $[401-2H_2O]^+$ (22)	5.4	500 fmol (200 pg)
1α(OH)D_3-DMEQTAD	746 $[M+H]^+$ (100)	6.4	18 fmol
1α(OH)D_3-DMEQTAD-Ac	830 $[M+H]^+$ (100)	6.8	10 fmol
1α(OH)D_3-DMEQTAD-Ac (MS–MS)[c]	830 $[M+H]^+$ (100)	6.8	6.3 fmol

[a] The t_R and LOD values of the derivatives are those of the 6*R*-isomer.
[b] The values in parentheses are the relative intensities.
[c] Precursor ion, m/z 830; relative collision energy, 15%.

step to remove the excess reagent, because a compound having a different molecular mass does not interfere in an LC–MS experiment, in contrast to FL–HPLC. In addition, acetylation is commonly used as a simple technique to increase the ionization efficiency of compounds with hydroxy groups in the positive APCI–MS [17].

Based on these data, we first examined the effect of these derivatizations for the detection responses of 1α(OH)D_3 in the positive APCI–MS (Table 1). The adduct of a vitamin D compound with the Cookson-type reagent consisted of 6*R* and 6*S* isomers (Fig. 1), because the reagent attacked at the *s-cis*-diene of the compound from the α- and β-sides, respectively. In the case of 1α(OH)D_3, the 6*R*-isomer was produced in a slightly higher ratio (6*R*/6*S* = 1.4/1, Table 2) [19], therefore, we used the 6*R*-isomer in the following studies. The LC–MS conditions of intact 1α(OH)D_3, the DMEQTAD derivative and the DMEQTAD-Ac derivative were optimized as described in Section 2, in which the mobile phases were selected to give relatively short chromatographic run-time to obtain a higher run capacity. Intact 1α(OH)D_3 was ionized more efficiently in the

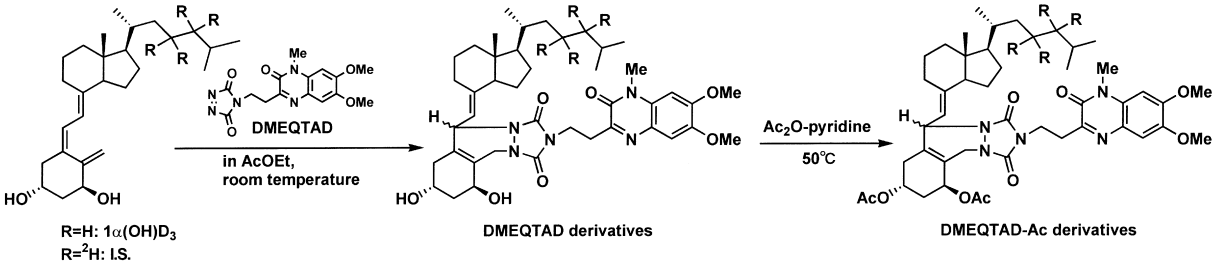

Fig. 1. Derivatization of 1α(OH)D_3 and I.S.

Table 2
Separation of DMEQTAD-Ac derivatives of 1α(OH)D_3 and endogenous vitamin D_3 metabolites

Compounds (DMEQTAD-Ac derivatives)	t_R		Ratio (6*R*/6*S*)	Characteristic ions (m/z)[a]
	(6*R*)	(6*S*)		
1α(OH)D_3	6.8	9.3	1.4/1	830 $[M+H]^+$ (100), 770 $[830-AcOH]^+$ (4)
1,25$(OH)_2D_3$	2.2	2.5	1.3/1	846 $[M+H]^+$ (100), 828 $[846-H_2O]^+$ (5)
1,24,25$(OH)_3D_3$	1.9	2.1	1.7/1	904 $[M+H]^+$ (100), 844 $[904-AcOH]^+$ (5)
25$(OH)D_3$	2.4	2.8	1/4.3	788 $[M+H]^+$ (100), 770 $[788-H_2O]^+$ (9)
24,25$(OH)_2D_3$	2.0	2.2	1/3.6	846 $[M+H]^+$ (100)
25,26$(OH)_2D_3$	2.0	2.2	1/3.6	846 $[M+H]^+$ (100)

[a] Values in parentheses are the relative intensities (%).

mobile phase using MeOH than in that using MeCN. On the contrary, the ionization efficiency of the derivatives were higher in the mobile phase using MeCN. 1α(OH)D_3 gave a protonated molecular ion together with its intense dehydrated ions, whereas the derivatives gave a very intense protonated ion and its dehydrated or AcOH eliminated ion was slight (relative intensity: less than 5%). The ions listed in Table 1 were used as monitoring ions to evaluate the effect of the derivatization for sensitivity.

The derivatization with DMEQTAD significantly increased the ionization efficiency of the 1α(OH)D_3 (LOD: 18 fmol) as observed in the case of 25(OH)D [14]. Additional acetylation provided the higher sensitivity with an LOD of 10 fmol, that was 50 times superior to that obtained without derivatization (Table 1). Considering these results, 1α(OH)D_3 was converted to the DMEQTAD-Ac derivative in the following studies.

The retention times and characteristic ions of the DMEQTAD-Ac derivatives of 1α(OH)D_3 and endogenous vitamin D_3 metabolites are summarized in Table 2. No derivatized metabolite interfered with the analysis of 1α(OH)D_3 under these conditions.

The APCI mass spectra of the derivatized 1α(OH)D_3 is shown in Fig. 2a, in which the protonated molecular ion was observed as the base ion (m/z 830). To obtain greater sensitivity, the MS–MS mode using the protonated molecular ion as the precursor ion was examined, but the ion was so stable that no product ion was formed by the addition of less than 15% of the relative collision energy (Fig. 2b). When the higher collision energy was used, the ion was fragmented, and as an example of which, the product ion mass spectrum of the 1α(OH)D_3 derivative using 25% of the relative collision energy is shown in Fig. 2c. The ions at m/z 770 and 710 were formed by the loss of one or two AcOH molecules from the precursor ion, and the ion at m/z 568 was assigned as the A-ring fragment ion derived from the cleavage of the C-6–7 bond of the vitamin D. However, the intensities of these product ions were considerably weaker in comparison with that of the precursor ion, which indicated that the MS–MS mode using one of these product ions as the monitoring ions was not advantageous in sensitivity. On the other hand, when the MS–MS mode with 15% of the relative energy using the ion at m/z 830 as the precursor ion and the same residual ion as the monitoring ion was used, the noise ions were reduced without decreasing the intensity of the monitoring ion, and as a result, a smaller LOD value was obtained (6.3 fmol: equivalent to ca. 2.5 pg of 1α(OH)D_3). Based on these results, the MS–MS mode with 15% of the relative collision energy was utilized for the quantitative analysis of 1α(OH)D_3.

Although the I.S. was inferred to contain four deuterium atoms from its synthetic method [4], several ions with relatively strong intensities between m/z 831 and 843, which corresponded to the [$^2H_1$]–[$^2H_{13}$]-labeled derivatives, were observed in the mass spectrum of the derivatized I.S. (Fig. 2d). However, the ion at m/z 830 was negligible, which corresponds to the 1α(OH)D_3 derivative having no deuterium. We chose the three stronger ions (m/z 833, 834 and 835) as the precursor and monitoring ions for the I.S. derivatives.

Based on the above results, the following mass chromatographic method was used for the determination of 1α(OH)D_3 in plasma; precursor ions: m/z 827–837, scan range: m/z 825–840, monitoring ions: m/z 830 for 1α(OH)D_3 and m/z 833, 834 and 835 for I.S., respectively.

3.2. Pretreatment of plasma sample and derivatization

The plasma specimen (1.0 ml) was extracted with MeCN and purified with two disposable cartridges, OASIS HLB and Bond Elut Si. The 1α(OH)D_3 fraction was treated with a large excess of DMEQTAD at room temperature and then with Ac_2O–pyridine at 50 °C. Bond Elut Si was useful to remove endogenous substances that prevent DMEQTAD from reacting, so that the derivatization efficiency (Diels–Alder reaction and acetylation) of the plasma sample was almost equal to that of the standard sample (95.2±3.9%, mean±standard deviation (S.D.), $n=4$). The overall absolute recovery rates of 1α(OH)D_3 (100 pg/ml) and I.S. (ca. 400 pg/ml) from the plasma specimen were 62.4±4.7% ($n=3$) and 61.0±2.1% ($n=3$), respectively, and there was no significant difference between both recovery rates.

Typical chromatograms are shown in Fig. 3. The

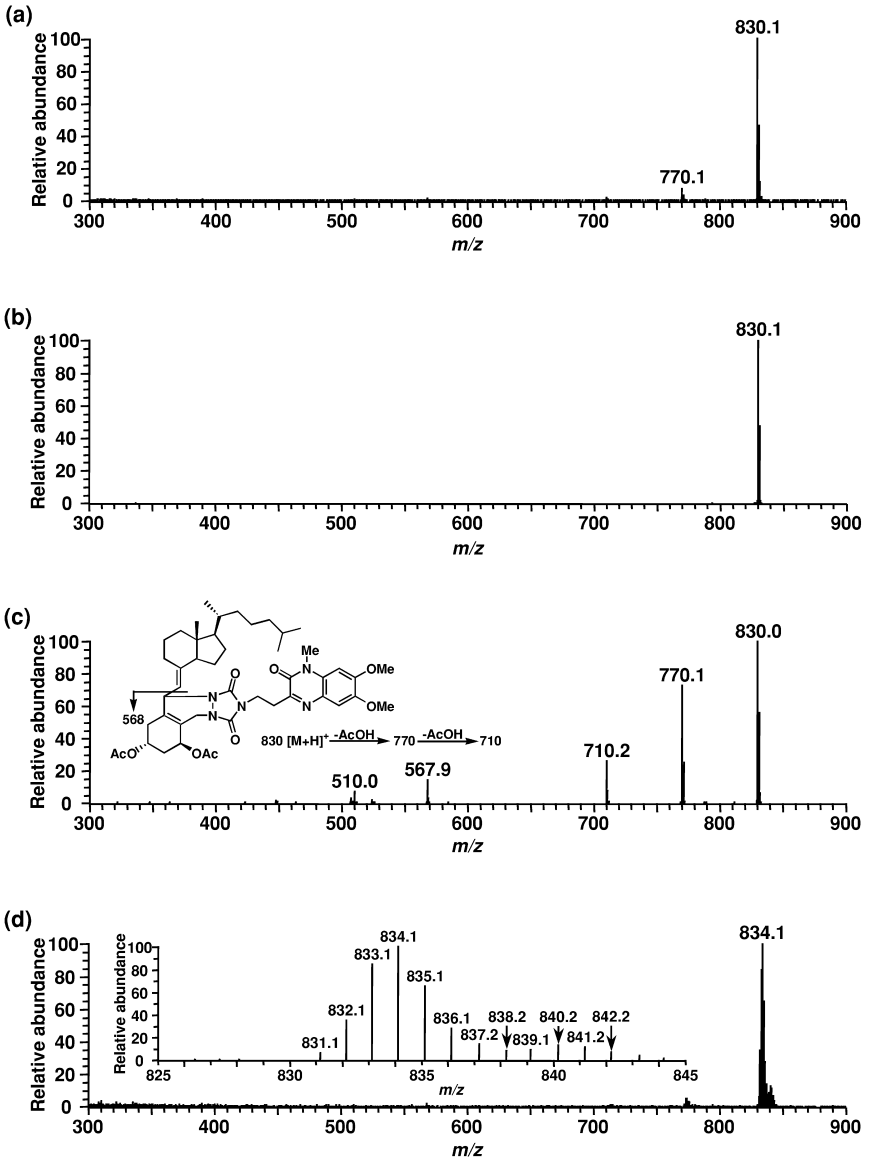

Fig. 2. (a) APCI mass spectrum of 1α(OH)D_3-DMEQTAD-Ac. (b) Product ion mass spectrum of 1α(OH)D_3-DMEQTAD-Ac (precursor ion, *m*/*z* 830; relative collision energy, 15%). (c) Product ion mass spectrum of 1α(OH)D_3-DMEQTAD-Ac (precursor ion, *m*/*z* 830; relative collision energy, 25%). (d) APCI mass spectrum of I.S.-DMEQTAD-Ac.

peaks of 1α(OH)D_3 and the I.S. derivatives were observed at 6.8 and 6.7 min, respectively, so that the LC eluent entered the mass spectrometer from 5.5 to 7.5 min after injection through a diversion valve. Fig. 3a shows the chromatograms obtained in the blank plasma without 1α(OH)D_3 and I.S. These chromatograms revealed that there was no interfering peak derived from the endogenous plasma components and the reagent at the elution positions of the derivatized 1α(OH)D_3 (*m*/*z* 830) and I.S. (sum of *m*/*z* 833, 834 and 835). The chromatograms obtained in the plasma samples spiked with 25 or 100 pg/ml of 1α(OH)D_3 and 400 pg/ml of I.S. are shown in Fig. 3b and c, respectively. In a sample below 25

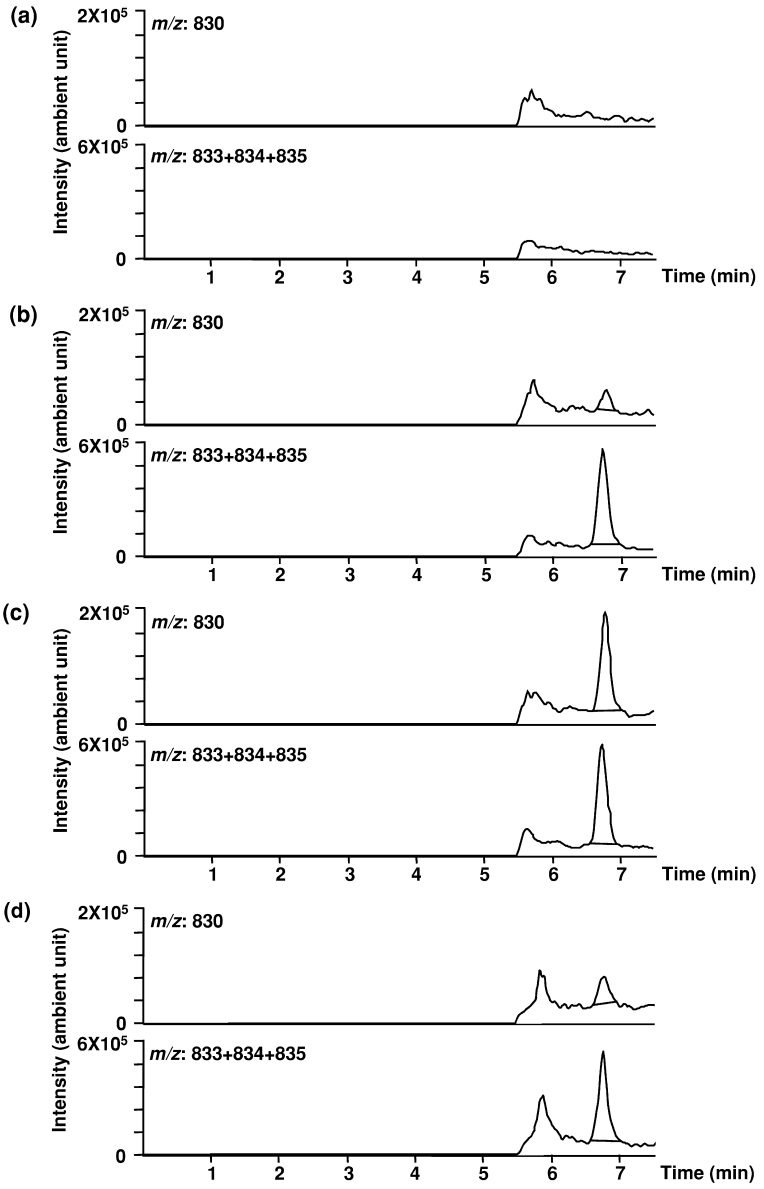

Fig. 3. Typical mass chromatograms of derivatized $1\alpha(OH)D_3$ and I.S. in human plasma. (a) Blank plasma sample. (b) Plasma sample spiked with $1\alpha(OH)D_3$ (25 pg/ml) and I.S. (ca. 400 pg/ml). (c) Plasma sample spiked with $1\alpha(OH)D_3$ (100 pg/ml) and I.S. (ca. 400 pg/ml). (d) Plasma sample from volunteer dosed with 4 μg of $1\alpha(OH)D_3$ (7 h after the administration). Monitoring ion: $1\alpha(OH)D_3$-DMEQTAD-Ac (upper chromatograms) *m/z* 830; I.S.-DMEQTAD-Ac (lower chromatograms) *m/z* 833, 834 and 835 (sum).

pg/ml of $1\alpha(OH)D_3$, the peak shape was not satisfactory and the peak area widely varied, therefore, the limit of quantitation (LOQ) of $1\alpha(OH)D_3$ was determined to be 25 pg/ml when 1 ml of the plasma specimen was used.

3.3. Calibration curves

Although one should calibrate a method with samples of the matrix of the analytical samples (in this case, blank plasma) [20], we examined the

following calibration curves for convenience. Five calibration curves were constructed on five separate days using two materials; one was the standard solutions of 1α(OH)D_3 and the other was the blank plasma spiked with a known amount of 1α(OH)D_3 as recommended [20]. The slope and intercept of five regression lines obtained from the standard solutions were 0.003171±0.000058 (mean±S.D.) and 0.003626±0.003247, respectively. On the contrary, those obtained from the spiked plasma were 0.003204±0.000080 and 0.003735±0.006398, respectively. There was no significant difference between the two regression lines and both methods demonstrated good linearity in the range of 25–150 pg (correlation coefficient (r^2)=0.998). Based on these data and the fact that the standard solutions do not require the pretreatment steps, the calibration curves were constructed using the standard solutions in the following studies.

Although linearity was obtained in the range over 150 pg, the largest amount in the calibration curve was chosen to be 150 pg because clinical samples are expected to contain less than 150 pg/ml.

3.4. Method validation and freeze–thaw stability of 1α(OH)D_3 in human plasma

The plasma specimens to which known amounts of 1α(OH)D_3 had been added were pretreated and analyzed by the present method. The intra-assay coefficient of variation (C.V.) (n=5) of the 25 pg/ml sample was 10.53%, but this is considered to be an acceptable value for clinical sample analysis, and satisfactory inter-assay C.V. values (less than 4.69%, n=5) were also obtained as shown in Table 3. The differences between the value obtained and the nominal one were less than 7%. These data indicate that the present method using the above mentioned calibration curve is accurate and highly reproducible.

The freeze–thaw stability of 1α(OH)D_3 in human plasma was also examined. After two freeze–thaw cycles, the concentrations in two different plasma samples were 102.6 and 96.5% (mean of duplicate assay) of the nominal concentration (100 pg/ml), which demonstrated that 1α(OH)D_3 was stable for two freeze–thaw cycles.

3.5. Determination of 1α(OH)D_3 in the plasma of a healthy subject administered 1α(OH)D_3 capsules

To investigate the possibility of the proposed method for a clinical study, the plasma 1α(OH)D_3 concentration of the subject who had received a single 1α(OH)D_3 administration (4 μg) was measured. Because it has been reported that the maximum plasma/serum concentration of 1,25$(OH)_2$D was reached after about 10 h following the oral administration of 1α(OH)D_3 [2,3], that of 1α(OH)D_3 was presumed to be reached within 10 h after the administration. Therefore, plasma samples were collected before the administration and at 3, 5 and 7 h following the administration. The plasma concentrations of 1α(OH)D_3 as well as 1,25$(OH)_2$D were measured and the following results were obtained (1α(OH)D_3 and 1,25$(OH)_2$D, respectively); before: 0 and 75.1 pg/ml; 3 h: less than 25 (LOQ) and 90.8 pg/ml; 5 h: 32.5 and 109 pg/ml, and 7 h: 35.4 and 119 pg/ml. In this case, the plasma 1α(OH)D_3 was elevated with the elapse of time. The chromatograms obtained in the sample at 7 h after dosing are shown in Fig. 3d.

4. Conclusion

We have demonstrated the LC–MS–MS method for the determination of 1α(OH)D_3 in human plasma. The derivatization procedures, reaction with

Table 3
Assay accuracy and precision

Variation	Nominal conc. (pg/ml, A)	Observed conc. (pg/ml, B)	Accuracy (%, B/A)	Precision (%, C.V.)
Intra-assay	25	23.3±2.5	93.2	10.53
(Pooled plasma, n=5)	100	99.0±4.9	99.0	4.93
Inter-assay	25	24.9±1.2	99.6	4.69
(Five different plasma)	100	101.7±4.1	101.7	4.00

DMEQTAD and acetylation, employed in the method were very useful for enhancing the sensitivity. The assay is specific, accurate and reproducible, and the chromatographic run-time is also short (within 8 min from one injection to the next). Until now, the pharmacokinetics of $1\alpha(OH)D_3$ in humans have been indirectly evaluated by the change in the plasma/serum $1,25(OH)_2D_3$ levels. Using this new LC–MS–MS method, the direct pharmacokinetic study of $1\alpha(OH)D_3$ in humans can be carried out. Moreover, it is expected that the method can be used for monitoring the plasma $1\alpha(OH)D_3$ levels in patients with leukemia who received a high dose of the drug (9–12 μg/body/day) [21] to reduce the risk of inducing hypercalcemia.

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