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Determination of 25-hydroxyvitamin D_3 in human plasma using a non-radioactive tetranorvitamin D analogue as an internal standard

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Abstract

A convenient non-radioactive method for assaying plasma 25-hydroxyvitamin D_3 is described. The method uses 22-hydroxytetranorvitamin D_3 as an internal standard and includes two-step liquid- and solid-phase extractions and quantification by normal-phase HPLC. The intra- and inter-assay coefficients of variation were 2.2% and 2.4%, respectively, and the analytical recovery of 25-hydroxyvitamin D_3 added to plasma was quantitative. Assay linearity was obtained in the range 0.5–4.0 ml of plasma. When compared with the method employing a radioactive 25-hydroxyvitamin D_3 tracer, the correlation coefficient was 0.990 (slope 0.999 and intercept 1.19 ng/ml).

1. Introduction

Vitamin D_3, which is either synthesized from 7-dehydrocholesterol in the skin or taken up from food, is successively metabolized to 25-hydroxyvitamin D_3 (25-OHD_3) in the liver and then to the active hormone 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] in the kidney.

It is well recognized that the concentration of 25-OHD_3 in plasma serves as a useful indication of the vitamin D status, since it reflects correctly the amount of vitamin D_3 produced by sun exposure and/or taken up from the diet [1,2]. Vitamin D_3 in plasma decreases rapidly by metabolism to 25-OHD_3 and incorporation into fat tissues (the half-life is about 1 day) [3]. The concentration of 1α,25-$(OH)_2D_3$ is kept constant under strict control by parathyroid hormone and serum calcium levels, except for specific clinical conditions [4]. Therefore, it is very important to determine accurately the concentration of plasma 25-OHD_3 for the assessment of the clinical and nutritional status of vitamin D.

Many methods have been developed for assaying 25-OHD_3 in plasma [5]. The most common method is competitive protein binding assays (CPBA) [6,7], utilizing vitamin D-binding protein (DBP), but this method lacks specificity and accuracy [8,9]. HPLC methods with a variety of detection method, ultraviolet (UV), fluorimetric and mass spectrometric (MS), have been used frequently when accuracy and high selectivity are required [10–18]. Although gas chromatography–mass spectrometry (GC–MS) offers a specific and definitive method for assay, it cannot be used for routine assay [19,20]. We have

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developed a highly sensitive fluorimetric HPLC method (HPLC–FL) for assaying 25-OHD_3, which has also been applied for the assay of 24,25- and 25,26-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$] [13,14]. With the exception of the GC–MS method, radioisotope-labelled tracers to determine the analytical recoveries are required. In the course of developing a non-radioactive assay method for vitamin D metabolities, we synthesized several vitamin D analogues and found that 22-hydroxy-24,25,26,27-tetranorvitamin D (ISa) is suitable as an internal standard for the HPLC assay of 25-OHD_3. Here we report a convenient and accurate HPLC assay procedure for determining 25-OHD_3 in human plasma without using radioisotopes.

2. Experimental

2.1. Chemicals and reagents

25-OHD_3 was purchased from Solvay-Dupher (Amsterdam, Netherlands). 25-OH-[26,27-^{3}H]D_3 (specific activity 0.69 TBq/mmol) was obtained from Amersham (Amersham, UK) and purified by HPLC just before use. 25-Hydroxyvitamin D_2 (25-OHD_2) was synthesized in our laboratory as described [21]. A Sep-Pak Classic silica cartridge was purchased from Waters (Milford, MA, USA). All organic solvents used were of HPLC grade (Cica-Merck, Tokyo, Japan) and water was purified with a Milli-Q water-purification system (Waters). Radioactivity was determined with a Packard Model 460CD liquid scintillation counter using the liquid scintillation cocktail Econofluor (Du-Pont/NEN Research Products, Boston, MA, USA).

Concentrations of standard solutions were determined using the molar absorptivity $\varepsilon =$ 18 200 (λ_{max} 265 nm) for 25-OHD_3 and the synthetic vitamin D analogues (ISa and ISb) and $\varepsilon = 18\,900$ (λ_{max} 265 nm) for 25-OHD_2.

Mass spectra were measured on a Jeol JMS-AX505HA mass spectrometer and m/z values are given with relative intensities (%) in parentheses. ^{1}H NMR spectra were recorded at 270 MHz on a Jeol GX-270 FT-NMR spectrometer. Chemical shifts are reported as δ values (parts per million) from tetramethylsilane as an internal standard. UV spectra were measured in 95% EtOH on a Hitachi U-3200 spectrophotometer.

2.2. Liquid chromatographic system

The HPLC system consisted of Jasco (Tokyo, Japan) high-performance liquid chromatograph equipped with a Model 880-PU pump, a Model 801-SC solvent programmer, a Uvidec-100-V variable-wavelength UV detector, a Rheodyne Model 7125 injector and a Model 860-CO column oven. Separation and quantification were achieved with a normal-phase Zorbax SIL column (250 mm × 4.6 mm I.D., particle size 5 μm) (DuPont, Wilmington, DE, USA) and a reversed-phase LiChrospher RP-18(e) column (250 mm × 4 mm I.D., particle size 5 μm) (Cica-Merck). The chromatograms were recorded and analysed utilizing a Jasco Model 807-IT integrator.

2.3. Plasma samples

Plasma samples from healthy adult volunteers (men aged 22–45 years) were collected from the Faculty of Medicine Hospital, Tokyo Medical and Dental University. None of the volunteers was taken vitamin D_2 drugs. Plasma samples were stored frozen at −80°C until use.

2.4. Synthesis of internal standards (Fig. 1)

(6S)- and (6R)-SO_2 adducts of 23,24,25,26,27-pentanorvitamin D_3 22-aldehyde (1)

Vitamin D_2 (1 g, 2.52 mmol) was dissolved in liquid sulphur dioxide (SO_2) and the mixture was refluxed for 30 min. The SO_2 was evaporated to afford the crude products, which were purified by silica gel column chromatography (20 g) with hexane–ethyl acetate (AcOEt) (10:90) to give the SO_2 adducts as a ca. 1:1 epimeric mixture at C(6) (1.09 g, 93.7%) [22–24]. This purification step by chromatography can be omitted.

A stirred solution of the SO_2 adducts (1.05 g, 2.27 mmol) in dichloromethane (10 ml) and

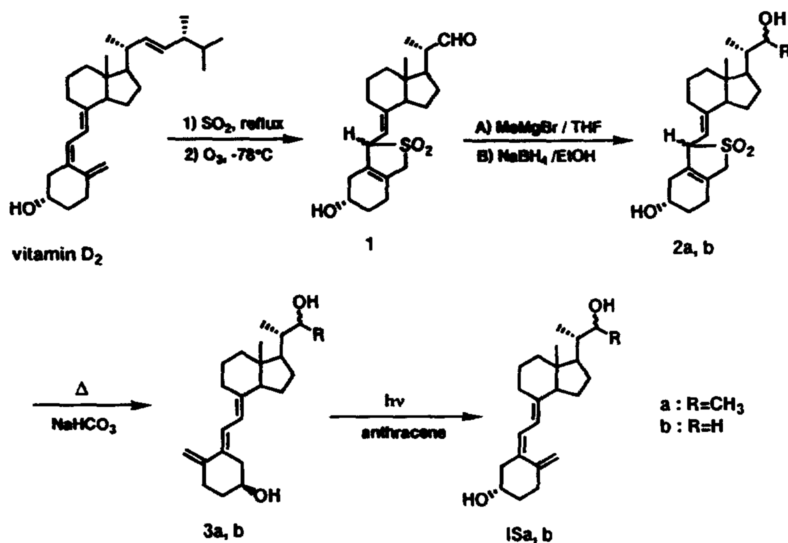

Fig. 1. Synthesis of internal standards.

pyridine (1 ml) at −78°C was treated with ozone at a rate of 8.5 mg/5 min for 1.5 h. The reaction mixture was rinsed successively with 1% HCl, 5% $NaHCO_3$ and brine and then dried over $MgSO_4$. Evaporation of the solvent gave the crude product, which was separated by chromatography on silica gel (20 g) with hexane–AcOEt (50:50) as eluent to yield the aldehyde (**1**, 583.5 mg, 65.3%) and the unreacted starting material (117.8 mg). **1**: ^1H NMR ($CDCl_3$), δ 0.62 and 0.70 (1:1) (3H, s, H-18), 1.14 and 1.15 (1:1) (3H, d, $J = 6.9$ Hz, H-21), 3.68 (2H, br. signal, H-19), 4.09 (1H, m, H-3), 4.57 and 4.66 (1:1) (1H, d, $J = 9.9$ Hz, H-6), 4.76 and 4.79 (1:1) (1H, d, $J = 9.9$ Hz, H-7), 9.58 and 9.60 (1:1) (1H, d, $J = 3.0$ Hz, CHO).

*(6S)- and (6R)-SO_2 adducts of 22-hydroxy-24,25,26,27-tetranorvitamin D_3 (**2a**) and (6S)- and (6R)-SO_2 adducts of 22-hydroxy-23,24,25,26,27-pentanorvitamin D_3 (**2b**)*

To a stirred solution of the aldehyde (**1**, 372 mg, 0.95 mmol) in dry tetrahydrofuran (10 ml) at −20°C was added dropwise a solution of methylmagnesium bromide (3 *M* in diethyl ether, 2.23 mmol) under an argon flow. The mixture was warmed to room temperature and stirred for 45 min, quenched with saturated NH_4Cl and then extracted with AcOEt. The organic phase was washed with 5% $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographed on silica gel (20 g) with hexane–AcOEt (30:70) to afford the alcohol as a mixture of diastereomers (**2a**, 255.2 mg, 51.5%) and the starting material (56 mg). **2a**: MS, m/z (%) 408 (no M^+), 344 (16.1), 326 (74.1), 308 (56.6), 298 (61.7), 253 (34.3), 251 (61.0), 105 (100).

A solution of sodium borohydride (95 mg, 2.52 mmol) in EtOH (2.5 ml) was added dropwise to a stirred solution of the aldehyde (**1**, 500 mg, 1.27 mmol) in dichloromethane (5 ml) at −78°C over a period of 10 min. The mixture was warmed to 0°C and stirred for 30 min, water was added and then the whole mixture was extracted with dichloromethane. The organic layer was washed with brine, dried over $MgSO_4$ and evaporated. The residue was chromatographed on silica gel (20 g) with hexane–AcOEt (30:70) to afford the alcohol as a mixture of C(6) epimers (**2b**, 428 mg, 85.4%). **2b**: ^1H NMR ($CDCl_3$), δ 0.59 and 0.68 (1:1) (3H, s, H-18), 1.07 (3H, d, $J = 6.4$ Hz, H-21), 3.67 (2H, m, H-19), 4.11 (1H, m, H-3).

(5E)-22-Hydroxy-24,25,26,27-tetranorvitamin D_3 (3a)

A solution of **2a** (170.7 mg, 0.42 mmol) in EtOH (15 ml) was heated in the presence of $NaHCO_3$ (704 mg, 8.38 mmol) at 120°C for 2 h in a sealed tube. The mixture was filtered and diluted with AcOEt. The filtrate was washed with 1% HCl and brine, dried over $MgSO_4$ and evaporated. Without purification, the crude product can be used directly for the subsequent photoisomerization reaction. In order to separate C(22) epimers, the reaction mixture was chromatographed on silica gel (10 g) with hexane–AcOEt (50:50) to give the less polar (22*S*)-**3a** and the more polar (22*R*)-**3a'** (total 90.1 mg, 48.1%, **3a**:**3a'** ≈ 4:1). **3a**: ^1H NMR ($CDCl_3$), δ 0.57 (3H, s, H-18), 0.93 (3H, d, *J* = 6.8 Hz, H-21), 1.17 (3H, d, *J* = 6.4 Hz, H-23), 3.89 (1H, m, H-3), 3.97 (1H, dq, *J* = 6.4 and 1.4 Hz, H-22), 4.69 and 4.98 (each 1H, s, H-19), 5.88 and 6.55 (each 1H, d, *J* = 11.6 Hz, H-6, 7). MS, *m/z* (%) 344 (M^+, 63.4), 326 (100), 308 (23.1), 271 (14.2), 253 (40.0), 251 (37.5). **3a'**: ^1H NMR ($CDCl_3$), δ 0.59 (3H, s, H-18), 0.93 (3H, d, *J* = 6.6 Hz, H-21), 1.04 (3H, d, *J* = 6.3 Hz, H-23), 3.89 (1H, m, H-3), 3.93 (1H, m, H-22), 4.69 and 4.98 (each 1H, s, H-19), 5.88 and 6.54 (each 1H, d, *J* = 11.6 Hz, H-6, 7).

(5Z)-22-Hydroxy-24,25,26,27-tetranorvitamin D_3 (ISa)

A cold (0°C) solution of **3a** (9.6 mg, 0.028 mmol) and anthracene (25 mg, 0.14 mmol) in EtOH (95 ml) and benzene (5 ml) was irradiated with a halogen lamp (200 W) (Matsushita Electric, Tokyo, Japan) for 20 min. After evaporation of the solvent, the crude product was purified by HPLC on a reversed-phase column [ODS; H_2O–MeOH (20:80); 1 ml/min; room temperature] to yield the less polar epimer (22*S*)-ISa (7.2 mg, 75.3%). The same procedure and work-up were applied for **3a'** to give the more polar epimer (22*R*)-ISa'. ISa: MS, *m/z* (%) 344 (M^+, 75.7), 326 (93.7), 311 (23.6), 271 (15.6), 253 (41.1), 136 (96.7), 118 (100). ^1H NMR ($CDCl_3$), δ 0.55 (3H, s, H-18), 0.93 (3H, d, *J* = 6.9 Hz, H-21), 1.16 (3H, d, *J* = 6.4 Hz, H-23), 3.95 (2H, m, H-3, 22), 4.82 and 5.05 (each 1H, m, H-19), 6.04 and 6.23 (each 2H, d, *J* = 11.4 Hz, H-6, 7). UV, λ_{max} (EtOH) 265, 212 nm. ISa': ^1H NMR ($CDCl_3$), δ 0.57 (3H, s, H-18), 0.93 (3H, d, *J* = 6.6 Hz, H-21), 1.04 (3H, d, *J* = 6.6 Hz, H-23), 3.93 (2H, m, H-3, 22), 4.82 and 5.05 (each 1H, m, H-19), 6.04 and 6.23 (each 1H, d, *J* = 11.5 Hz, H-6, 7).

(5Z)-22-Hydroxy-23,24,25,26,27-pentanorvitamin D_3 (ISb)

A mixture of the alcohol (**2b**, 100 mg, 0.25 mmol), $NaHCO_3$ (436 mg, 5.07 mmol) and EtOH (5 ml) was heated at 120°C in a sealed tube for 3.5 h. The same work-up as described above gave the (5*E*)-vitamin D (**3b**). Without purification, the crude **3b** dissolved in EtOH–benzene (90:10) (100 ml) was irradiated with a halogen lamp (200 W) in the presence of anthracene (25 mg). The usual work-up and purification as described above afforded the vitamin D derivative ISb (65 mg, 77.6% from **2b**). ISb: MS, *m/z* (%) 330 (M^+, 35.7), 312 (100), 271 (7.2), 253 (32.7), 136 (67.6), 118 (74.8). ^1H NMR ($CDCl_3$), δ 0.57 (3H, s, H-18), 1.06 (3H, d, *J* = 6.4 Hz, H-21), 3.95 (1H, m, H-3), 4.82 and 5.05 (each 1H, m, H-19), 6.04 and 6.23 (each 1H, d, *J* = 11.1 Hz, H-6, 7).

2.5. Extraction and separation of plasma samples

A flow chart of the entire assay protocol is shown in Fig. 3. To a plasma sample (1–2 ml) were added ISa (40 ng in 40 μl of EtOH) and [^3H]-25-OHD_3 (3500 cpm in 20 μl of EtOH) for the determination of the recovery of 25-OHD_3. The plasma samples were allowed to equilibrate for 30 min in the dark and lipids were extracted with dichloromethane–MeOH (1:2) as described previously [13].

A Sep-Pak silica cartridge was washed with AcOEt–hexane (60:40) (10 ml) and equilibrated in AcOEt–hexane (7:93) (10 ml). The lipid extracts dissolved in AcOEt–hexane (7:93) (300 μl) were applied to the cartridge column. The column was eluted with AcOEt–hexane (7:93)

(30 ml) and then with AcOEt–hexane (15:85) (40 ml). The latter fraction, containing mono-hydroxylated vitamins, was evaporated in vacuo.

2.6. HPLC determination of 25-OHD$_3$

The 25-OHD$_3$ fraction from the Sep-Pak column was dissolved in the mobile phase (250 μl) and analysed by normal-phase HPLC on a Zorbax SIL column with hexane–chloroform–MeOH (100:25:2) as the mobile phase at a flow-rate of 1.5 ml/min at room temperature, with UV detection at 265 nm (Fig. 4).

The concentration of 25-OHD$_3$ was determined on the basis of a two-point calibration graph covering the range 10–100 ng of 25-OHD$_3$. Standard solutions prepared by dissolving ISa (40 ng) and 25-OHD$_3$ (10 or 100 ng) were injected into the HPLC system separately. The ratio of the peak area of 25-OHD$_3$ to that of ISa was plotted against the amount of 25-OHD$_3$ (10 and 100 ng).

3. Results

3.1. Synthesis of internal standards

The two internal standards (ISa and ISb) useful for assaying plasma 25-OHD$_3$ were each synthesized from readily available vitamin D$_2$ in three steps. Treatment of vitamin D$_2$ with liquid SO$_2$ and ozonolysis of the resulting C(6) epimeric SO$_2$ adducts gave the aldehyde **1** as a mixture of epimers (1:1). The aldehyde **1** was treated with methylmagnesium bromide to yield the 22-alcohol **2a** as a mixture of diastereomers. The C(22) diastereomer ratio was determined by ^{1}H NMR after desulphonylation. Thermolytic desulphonylation of **2a** in the presence of NaHCO$_3$ afforded the (5*E*)-vitamin D derivatives **3a** and **3a'** in about a 4:1 ratio. The 22*S* configuration was tentatively assigned to the major isomer (**3a**) by comparing analogous stereoselective addition to steroidal 22-aldehydes [25–27]. This was also supported by the Felkin–Anh model [28,29]. The major 22*S*-isomer (**3a**) was photoisomerized to the desired vitamin D (ISa) [30]. The other internal standard (ISb) was synthesized using the same synthetic intermediate (**1**). Reduction of **1** with sodium borohydride gave the corresponding C-22 alcohol (**2b**), which was desulphonylated and photoisomerized to yield ISb. The (22*S*)-alcohol (ISa) was used as an internal standard for the following analyses because its elution volume is closer to that of 25-OHD$_3$ than ISb. However, we observed little difference between the two internal standards (ISa and ISb) in the results of plasma 25-OHD$_3$ assay.

3.2. Chromatographic behaviour of 25-OHD and synthetic internal standards

Recoveries of 25-OHD$_3$, 25-OHD$_2$ and the internal standards (ISa and ISb) from the Sep-Pak silica cartridge column are summarized in Table 1. All substrates of interest were effectively eluted with AcOEt–hexane (15:85), but none was detected using AcOEt–hexane (7:93 and 60:40). The recoveries for the four substrates were similar and satisfactory (>93%). The elution profile of the four substrates in normal-phase HPLC is shown in Fig. 2. A baseline separation of four peaks was achieved with the HPLC system.

Table 1
Recoveries of 25-OHD and internal standards after solid-phase extraction

Compound	Recovery (mean ± S.D.; n = 5) (%)		
	Fr-I	Fr-II	Fr-III
25-OHD$_2$	ND[a]	92.6 ± 3.6	ND
25-OHD$_3$	ND	95.3 ± 2.2	ND
ISa	ND	95.2 ± 2.0	ND
ISb	ND	95.0 ± 2.4	ND

A standard mixture of 25-OHD$_3$, 25-OHD$_2$ and internal standards (ISa and ISb) (each 100 ng) dissolved in AcOEt–hexane (7:93) (300 μl) was applied to a Sep-Pak silica cartridge column and eluted successively with AcOEt–hexane (7:93) (Fr-I), AcOEt–hexane (15:85) (Fr-II) and AcOEt–hexane (60:40) (Fr-III). Each fraction was injected into the HPLC system (analytical conditions as in Fig. 2) to determine the recovery of the standard samples.

[a] ND = not detected.

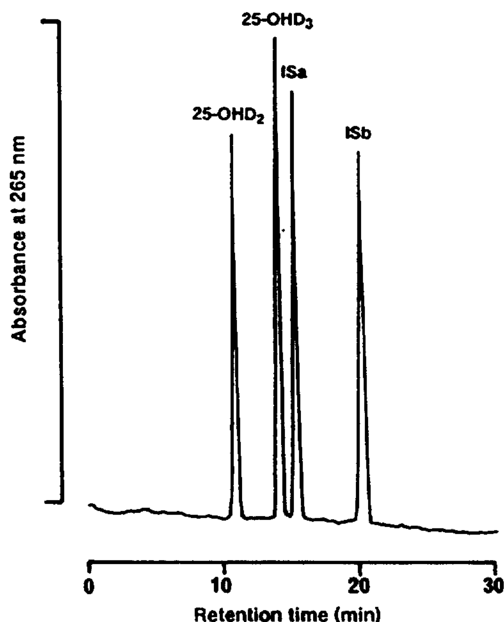

Fig. 2. HPLC profile for a standard mixture of vitamin D metabolites and synthetic internal standards (each ca. 50 ng). HPLC conditions: column, Zorbax SIL; mobile phase, hexane–$CHCl_3$–MeOH (100:25:2); flow-rate, 1.5 ml/min; temperature, room temperature; detection, UV at 265 nm.

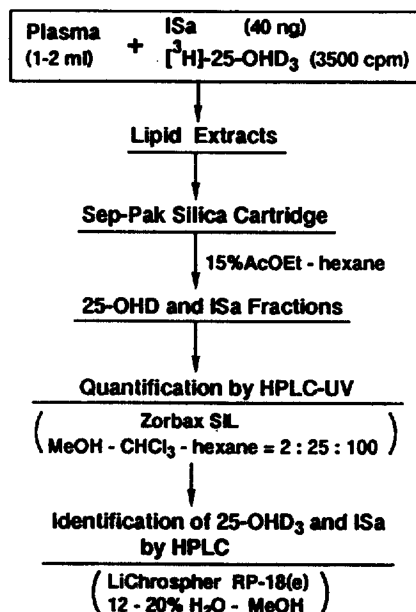

Fig. 3. Flow diagram of the assay procedure for the determination of plasma 25-OHD_3.

3.3. Intra- and inter-assay variation

The precision and reproducibility of the method were evaluated by analysing aliquots taken from the same plasma pool (Fig. 3) and the results are given in Table 2. The peak-area ratios (25-OHD_3 to ISa) were constant in both assays. The intra-assay and inter-assay coefficients of variation (C.V.) were 2.2% and 2.4%, respectively. The overall recovery of the internal standard ISa through the entire procedure was 70% intra-assay and 77% inter-assay.

To confirm the purity of the two peaks quantified on the HPLC trace (Fig. 4), each peak (25-OHD_3 and ISa) was collected and separately rechromatographed on a reversed-phase HPLC column. The results are summarized in Table 3. Neither an interfering nor an impurity peak was detected on the HPLC trace in the time range 0–30 min.

Table 2
Intra- and inter-assay reproducibility

Assay	n	Peak-area ratio (25-OHD_3/ISa)	Recovery of ISa (%)	25-OHD_3 (ng/ml)	C.V. (%)
Intra-assay	8	0443 ± 0.001	69.9 ± 4.1	20.77 ± 0.45	2.2
Inter-assay	7	0.463 ± 0.001	76.9 ± 3.3	21.28 ± 0.50	2.4

Results are expressed as mean ± S.D. Plasma samples for inter-assay measurements were stored at −80°C and analysed in duplicate over a period of 2 weeks. The peak-area ratio was calculated from the peak areas on the chromatogram which was obtained for 25-OHD_3 using ISa (40 ng) as an internal standard in plasma (1 ml).

Table 3
Purities of chromatographic peaks

Compound	t_R (min)	(%) Recovery
25-OHD_3	10.6	103.5 ± 2.4
ISa	10.2	101.3 ± 2.6

Results are expressed as mean ± S.D. ($n = 8$). Each peak (25-OHD_3 and ISa) shown in Fig. 4 was collected separately, known amounts of [^{3}H]-25-OHD_3 were added and then each fraction was rechromatographed to evaluate peak purities. HPLC conditions: column, LiChrospher RP-18(e); mobile phase, H_2O–MeOH (12:88) for the determination of 25-OHD_3 and H_2O–MeOH (20:80) for the determination of ISa; flow-rate, 1 ml/min; temperature, 30°C; detection, UV at 265 nm.

3.4. Analytical recovery

Known amounts of standard 25-OHD_3 (10, 20 and 40 ng/ml plasma) together with internal standard (ISa, 40 ng) were added to plasma samples. Aliquots were analysed and the calculated recoveries are given in Table 4. 25-OHD_3 added to plasma was recovered quantitatively (103.6 ± 3.6%).

Table 4
Analytical recovery of standard 25-OHD_3 added to plasma (1 ml)

25-OHD_3 (ng/ml) Added	Found	Recovery (%)
0	19.4	–
10	30.1	107.3
20	39.3	100.0
40	60.8	103.6

Results are means of duplicate assays.

3.5. Assay specificity

The assay was conducted in duplicate on four volumes of plasma (0.5, 1, 2 and 4 ml). The correlation between the concentration of 25-OHD_3 (y, ng/ml) and the volume of plasma (x, ml) used was excellent: the equation of the line

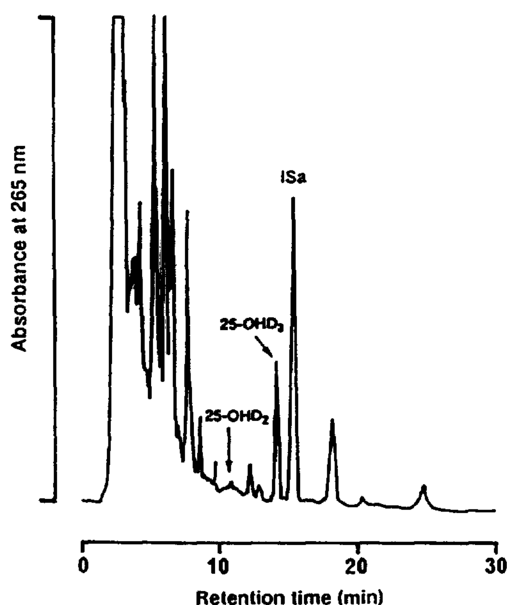

Fig. 4. HPLC profile for the analysis of plasma 25-OHD_3. 25-OHD fractions dissolved in the mobile phase (250 μl) were injected into the HPLC system. HPLC conditions as in Fig. 2. Arrows show the elution position of the standard compounds.

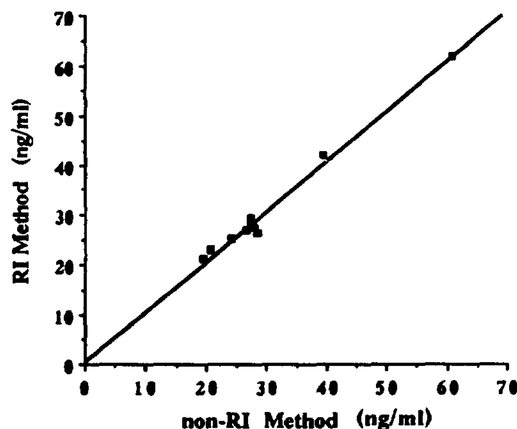

Fig. 5. Correlation between the proposed method (the non-radioactive method) and the method using [^{3}H]-25-OHD_3 as an internal standard (the radioactive method). Regression analysis of the data afforded the correlation coefficient $r = 0.993$ and the equation of regression line is $y = 0.9985x + 1.19$ ($n = 10$), slope = 0.9985 ± 1.0995, intercept = 1.19 ± 3.16 ($p = 0.05$). The samples with the higher concentrations of 25-OHD_3 (>40 ng/ml) indicate the plasma samples to which 25-OHD_3 was added prior to extraction.

was $y = 22.647x - 0.855$, the correlation coefficient (r) was 0.9997 and the intercept was -0.855 ± 0.987 ng ($p = 0.05$).

To evaluate the proposed method, 25-OHD_3 in ten plasma samples obtained from healthy adults was determined by two methods, one employing the tetranorvitamin D analogue (ISa) (the present non-radioactive method) and the other using isotope-labelled 25-OHD_3 (the radioactive method) as internal standards. The results obtained by the two methods are compared in Fig. 5. A regression coefficient of 0.993 and a positive intercept of 1.19 ng/ml were obtained.

4. Discussion

A facile non-radioactive assay method for 25-OHD_3 was developed. For this purpose, we synthesized two vitamin D derivatives (ISa and ISb) which have one hydroxyl group in the side-chain and are appropriate as internal standards for 25-OHD_3 assay. These compounds can be prepared from readily available vitamin D_2 in three-step procedures (Fig. 1) in 10–40% overall yield: (i) SO_2 adduct formation and ozonolysis, (ii) 22-methylation or reduction of the resulting C(22) aldehyde (**1**) and (iii) desulphonylation and photoisomerization.

ISa and ISb showed ideal properties as internal standards under the present assay conditions. The use of a Sep-Pak cartridge offers a simple and effective procedure for the preliminary fractionation of 25-OHD_3. Under these fractionation conditions, ISa and ISb were eluted together with 25-OHD_3 with high efficiency (Table 1). Under the conditions of normal-phase HPLC analysis (Fig. 2), both ISa and ISb were eluted close to 25-OHD_3 with baseline separation. These compounds are not appropriate for analysis on reversed-phase HPLC columns, because they are eluted far from 25-OHD_3 on such columns. It should be noted that under these HPLC conditions 25-OHD_2 is eluted separately from these standards and 25-OHD_3 with baseline resolution. Hence both ISa and ISb are applicable as an internal standard in the HPLC assay of both plasma 25-OHD_3 and 25-OHD_2.

Using the procedure outlined in Fig. 3 and ISa as an internal standard, we assayed plasma 25-OHD_3 and evaluated the results by comparison with existing methods. There was good intra- and inter-assay agreement between the 25-OHD_3 concentrations and the C.V. values were <3% in both assays (Table 2). These results indicate that the method is highly reproducible in routine analysis. The overall recovery of ISa was satisfactory and the analytical recovery of added 25-OHD_3 to plasma was quantitative (Tables 2 and 4). A linear relationship was obtained for the assay of 25-OHD_3 in 0.5–4-ml plasma volumes. This demonstrates that the quantitative result is not dependent on the plasma volume. Thus 0.5 ml of human plasma is sufficient to measure the concentration of 25-OHD_3 accurately. In addition, the 25-OHD_3 concentrations determined by this HPLC method were in excellent agreement with those obtained by the method using [^{3}H]-25-OHD_3 as a tracer (Fig. 5).

An HPLC assay for plasma 25-OHD_2 and 25-OHD_3 using (5*E*)-25-OHD_3 as an internal standard has been reported by Mawer and Hann [31]. However, 5*E*-isomers of vitamin D are known to be unstable relative to the parent vitamin D. Therefore, the recoveries of 25-OHD_3 and its 5*E*-isomer could be different depending on the detailed experimental conditions. Also, it is generally difficult to store (5*E*)-vitamin D for long periods of time even in a freezer. The internal standard must have maximum similarity to the substrate in its physical and chemical properties. Our method is advantageous in that the internal standard has the same vitamin D chromophore so that its stability is nearly the same as that of 25-OHD_3. Once synthesized, it can be stored for an infinite time period in a freezer.

HPLC analysis with UV detection is only applicable to measure circulating plasma vitamin D_3 and 25-OHD_3 at higher concentration levels. We have already developed an HPLC–FL method using 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) as an efficient and

highly sensitive fluorescence-labelling reagent targeting the *s-cis*-diene part of vitamin D structures [13,14,32]. If only plasma 25-OHD_3 is concerned, the method described here is more convenient than the fluorimetric assay. The HPLC–FL method is useful, on the other hand, for assaying metabolites present at low concentrations (from 100 pg to several ng), such as 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$. In preliminary intra-assay experiments, the values obtained by the present method and the HPLC–FL method were similar (23.30 ± 0.41 and 23.24 ± 1.32 ng/ml, respectively; mean ± S.D., $n = 7$). The non-radioactive method is currently being applied to the HPLC–FL assay of dihydroxyvitamin D_3 metabolites.

In conclusion, we have described a reliable, safe and versatile method to determine plasma 25-OHD_3. In the method, which does not use an isotope-labelled standard, the synthetic internal standard ISa is excellent and we believe it will be suitable for routine assays in clinical and research laboratories when ISa becomes commercially available.

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