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Fluorimetric assay of 1α,25-dihydroxyvitamin D_3 in human plasma

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Abstract

The first fluorimetric method for assaying plasma 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is described. Lipid extracts from human plasma were successively purified by a Bond Elut NH_2 column and a normal-phase HPLC column, and the 1,25-$(OH)_2D_3$ fractions obtained were fluorescence-labelled with 4-[2-(6,7-dimethoxy-4-methtyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD). The fluorescent adducts of 1,25-$(OH)_2D_3$ were analyzed by HPLC on a reversed-phase column, after extensive elimination of the degradation products of excess reagents, by a Bond Elut PSA column followed by a normal-phase HPLC column. The intra- and inter-assay coefficients of variation were within 10% in both assays. The analytical recoveries of standard 1,25-$(OH)_2D_3$ added to plasma were quantitative. The present fluorimetric assay was compared with the established radioreceptor assay (RRA) used in measuring plasma 1,25-$(OH)_2D_3$ concentration. The regression analysis of the data afforded a relationship $y=1.049x-2.657$ and a correlation coefficient (r) of 0.900.

Keywords: 1α,25-Dihydroxyvitamin D_3; Vitamins

1. Introduction

The hormonally active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (**1a**) elicits its functions in regulating calcium and phosphorus homeostasis, and others such as induction of cell differentiation, suppression of cell proliferation, and immunomodulation [1–6] by a genomic mechanism, via binding to a specific nuclear vitamin D receptor (VDR) and activating gene transcription [7–10].

The concentration of 1,25-$(OH)_2D_3$ in plasma is the most useful parameter in evaluating clinical disorders involving calcium and bone metabolism [11,12]. The production of 1,25-$(OH)_2D_3$ is tightly regulated by parathyroid hormone and plasma calcium levels [13–15] and its plasma concentration is kept quite low (normal range: 20–65 pg/ml of plasma).

A radioreceptor assay (RRA) employing the receptor protein specific for 1,25-$(OH)_2D_3$ from chick intestine [16,17], bovine thymus [18,19] or bovine mammary gland [20,21] is currently used most generally for the assay of active vitamin D metabolite. However, this assay sometimes gives unexpected values owing to cross-reaction with other vitamin D metabolites and/or nonspecific interfering materials, and thus lacks specificity. A physicochemical method such as gas chromatography–mass spectrometry (GC–MS) [22,23] is specific and conclusive, but it is not appropriate for routine clinical uses

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because of its low sensitivity and complicated handling. HPLC–MS with new, highly sensitive ionization devices such as thermospray [24], electrospray [25,26] and fast atom bombardment [27] have been introduced recently for the determination of vitamin D metabolites, but they are still under development.

Our efforts have been directed toward the establishment of a highly sensitive and accurate assay method applicable generally to all vitamin D compounds. We have developed an excellent fluorescent labelling reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) [28,29]. The reagent was designed to target the *s-cis* diene part common to vitamin D compounds (**1**), the labelling reaction yielding epimeric 6,19-cycloadducts (**2**) as shown in Fig. 1. DMEQ-TAD has been successfully applied to the assay of plasma 25-hydroxyvitamin D_3 (25-OHD_3) (**1c**), 24*R*,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] (**1d**) and 25S,26-dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] (**1e**) [30–33] which are present at much higher levels (1–40 ng/ml of plasma) than the active vitamin D metabolite. This method has now been successfully used in determining 1,25-$(OH)_2D_3$ in plasma. This paper reports a first fluorimetric assay of the active vitamin D_3 in detail.

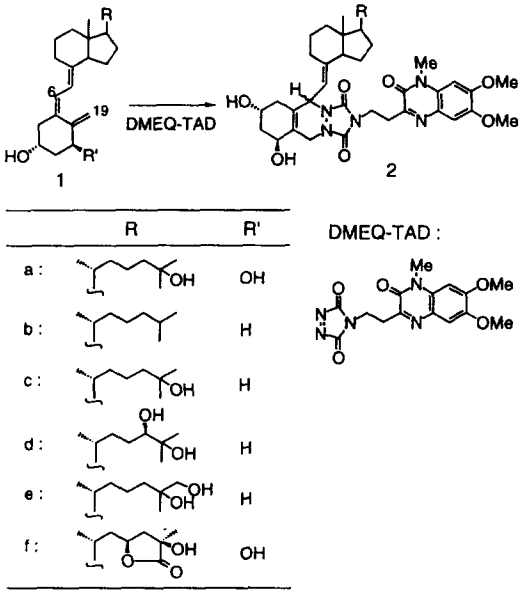

Fig. 1. Fluorescence labelling of vitamin D metabolites with DMEQ-TAD.

2. Experimental

2.1. Chemicals and reagents

25-OHD_3 and 1,25-$(OH)_2D_3$ were purchased from Solvay-Duphar (Amsterdam, Netherlands). 24,25-$(OH)_2D_3$ was a gift from Kureha Chemical Industry (Tokyo, Japan). 1α,25*R*-Dihydroxyvitamin D_3 26,23*S*-lactone [1,25-$(OH)_2D_3$ 26,23-lactone] and a fluorescent dienophile (DMEQ-TAD) were synthesized in our laboratory as reported previously [28,29,34] (DMEQ-TAD is now commercially available from Wako Pure Chemical Industries, Tokyo, Japan). 1α,25-Dihydroxy[26,27-methyl-^{3}H] vitamin D_3 {[^{3}H]-1,25-$(OH)_2D_3$} (specific activity 6.55 TBq/mmol) was obtained from Amersham (Buckinghamshire, UK) and purified by HPLC on a reversed-phase column just before use. Bond Elut cartridge columns (NH_2- and PSA-type, sorbent mass 100 mg) were purchased from Varian (Harbor City, CA, USA). Yamasa RRA assay kits [1,25-$(OH)_2D_3$ receptor from bovine thymus and dextran coated charcoal] were purchased from Yamasa Shoyu (Chiba, Japan). All organic solvents used were of HPLC grade from Cica-Merck (Tokyo, Japan) and water (H_2O) was purified with a Milli-Q water-purification system from Waters (Milford, MA, USA). Dry dichloromethane (CH_2Cl_2) was prepared as described previously [29]. Radioactivity was determined with a Packard Model 460CD liquid scintillation counter using the liquid scintillation cocktail Econofluor and Aquasol from Du-Pont/NEN Research Products (Boston, MA, USA).

Concentrations of standard solutions were determined by ultraviolet (UV) spectroscopy using molar absorptivity $\epsilon = 18200$ [λ_{max} 265 nm in 95% ethanol (EtOH)] for all the vitamin D compounds.

2.2. Liquid chromatographic system

We used a high-performance liquid chromatograph equipped with a Model 801-SC solvent programmer, a Model 880-PU pump, a Model FP-920 fluores-

cence detector (flow cell volume, 16 μl), a Rheodyne Model 7125 injector and a Model 860-CO column oven from JASCO (Tokyo, Japan). The excitation (Ex) and emission (Em) wavelengths were set at 370 and 440 nm, respectively. Separation and quantification were performed on a normal-phase LiChrospher Si 60 column (250×4 mm I.D., particle size 5 μm) (Cica-Merck) and a reversed-phase YMC-pack ODS-AM AM-302 column (150×4.6 mm I.D., particle size 5 μm) (YMC, Tokyo, Japan). The chromatograms were recorded and data-processed with a Model 807-IT integrator (JASCO).

2.3. Plasma samples

Plasma samples were collected from healthy adult subjects (our research group members aged 22–46 years) during February, March, April and May. None of the individuals were taking vitamin D_3 or vitamin D_2 drugs. Plasma pools were kept frozen at −80°C until use.

2.4. Extraction and purification of 1,25-$(OH)_2D_3$

The extraction and purification procedure are outlined in the flow diagram shown in Fig. 2. A mixture of plasma samples (2.5–5 ml) and [^{3}H]-1,25-$(OH)_2D_3$ (ca. 5000 dpm in 20 μl of 95% EtOH) was allowed to stand for 30 min in the dark at room temperature and was extracted with CH_2Cl_2–methanol (MeOH) (1:2) as described in our earlier paper [30].

A Bond Elut NH_2 cartridge was rinsed with MeOH–ethyl acetate (AcOEt) (5:95) (5 ml) and

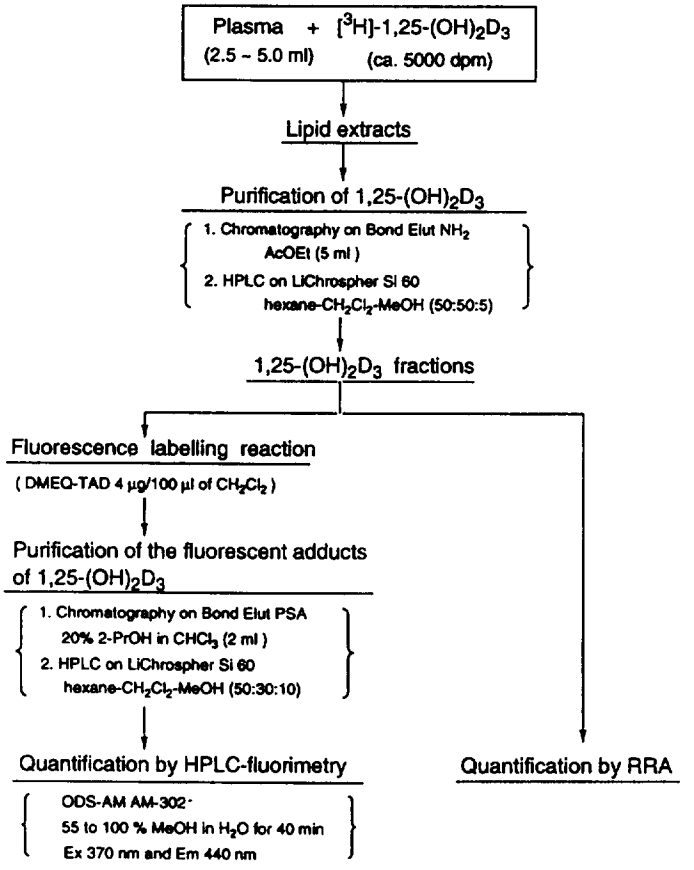

Fig. 2. Flow diagram for the fluorimetric assay of 1,25-$(OH)_2D_3$.

equilibrated in AcOEt–hexane (7:93) (5 ml). The organic extracts dissolved in AcOEt–hexane (7:93) (300 μl) were applied to the top of the column. The column was successively eluted with AcOEt–hexane (7:93) (5 ml), AcOEt–hexane (35:65) (5 ml), AcOEt–hexane (60:40) (5 ml), AcOEt (5 ml) and AcOEt–MeOH (95:5) (5 ml). The AcOEt fraction, containing 1,25-$(OH)_2D_3$, was collected and evaporated in vacuo. The residue dissolved in mobile phase (250 μl) was applied to normal-phase HPLC on LiChrospher Si 60 [mobile phase, hexane–CH_2Cl_2–MeOH (50:50:5); flow-rate, 1 ml/min; temperature, 25–30°C; detection, UV 265 nm]. The fractions eluted between 11 and 14 min were pooled and subjected to fluorescence-labelling and RRA assay. A portion of this fraction was subjected to liquid scintillation counting.

2.5. Fluorescence labelling reaction of 1,25-$(OH)_2D_3$ with DMEQ-TAD

Purified 1,25-$(OH)_2D_3$ fractions as described above were placed in a centrifuge tube (10 ml) and dried in vacuo for 1 h. DMEQ-TAD (ca. 2 μg) dissolved in dry CH_2Cl_2 (50 μl) was added to the dried sample and the mixture was stirred at ambient temperature for 30 min under argon atmosphere. An additional reagent (ca. 2 μg/50 μl of CH_2Cl_2) was added and the entire mixture was further stirred for 2 h at the same conditions. The reaction was quenched by adding MeOH (100 μl) and the solvent was evaporated to dryness.

2.6. Purification of the fluorescence labelled adducts of 1,25-$(OH)_2D_3$

The labelled products were purified by a cartridge column followed by a normal-phase HPLC column. A Bond Elut PSA was conditioned successively with 2-propanol (2-PrOH) (2 ml) and chloroform ($CHCl_3$) (10 ml). The reaction products dissolved in $CHCl_3$ (250 μl) were loaded and the column was eluted with $CHCl_3$ (5 ml) and then 2-PrOH–$CHCl_3$ (2:8) (2 ml). The latter fraction was evaporated, dissolved in the mobile phase (250 μl) and subjected to HPLC [column, LiChrospher Si 60; mobile phase, hexane–CH_2Cl_2–MeOH (50:30:10); flow-rate, 1.5 ml/min; temperature, 25–30°C; fluorescence detection, Ex 370 nm and Em 440 nm]. The fraction eluted between 8 and 10 min was collected and evaporated in vacuo.

2.7. HPLC determination of the DMEQ-TAD adducts of 1,25-$(OH)_2D_3$

The above fractions dissolved in the mobile phase (80 μl) were injected into a reversed-phase HPLC column (YMC-pack ODS-AM AM-302), and eluted with a linear gradient HPLC solvent system (55–100% MeOH in H_2O for 40 min) at a flow-rate of 1 ml/min. The effluent was monitored at Ex 370 nm and Em 440 nm by a fluorimetric detector, which is connected to an integrator. Each peak of the epimeric DMEQ-TAD adducts of 1,25-$(OH)_2D_3$ was calibrated individually on the basis of a two-point calibration graph covering the range of 10–50 pg using radioactive standards (ca. 300–1500 dpm). Each peak was collected and was counted for radioactivity to determine the recovery of the standard adducts from this HPLC column (the average recoveries, 95.8±0.78%, $n=6$). The two peaks (Fig. 3) were integrated for quantification using the calibration graph and corrected for the percent recovery obtained above. The two peaks of interest eluted from the HPLC column were collected together and the radioactivity was counted to calculate the overall recovery.

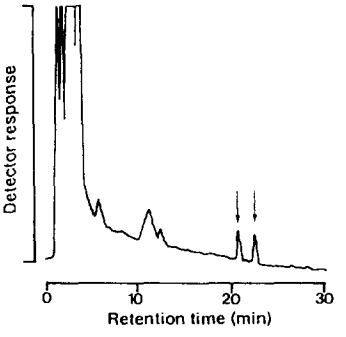

Fig. 3. HPLC profile of the fluorescent adducts of plasma 1,25-$(OH)_2D_3$ from one of the studies. Column, YMC-pack ODS-AM AM-302; mobile phase, gradient elution with 55–100% MeOH in H_2O for 40 min; flow-rate, 1 ml/min; temperature, 35°C; detection, Ex 370 nm and Em 440 nm. The arrows indicate the elution positions of isomeric authentic DMEQ-TAD adducts of 1,25-$(OH)_2D_3$.

2.8. RRA of plasma 1,25-$(OH)_2D_3$

The plasma samples were purified as described above and subjected to the RRA. The RRA followed essentially the method reported by Reinhardt et al. [19] as described in the Yamasa-kit manual, employing bovine thymus receptor.

3. Results

3.1. Extraction and purification of vitamin D metabolites

Lipid extracts from plasma samples were obtained as previously reported [30], and were fractionated by a Bond Elut NH_2 cartridge column. The NH_2 column improved the efficiency of the fractionation of lipid soluble plasma vitamin D metabolites. Recoveries of standard vitamin D metabolites, 25-OHD_3 (**1c**), 24,25-$(OH)_2D_3$ (**1d**), 1,25-$(OH)_2D_3$ (**1a**), and 1,25-$(OH)_2D_3$ 26,23-lactone (**1f**), loaded on a Bond Elut NH_2 column were investigated and the results were shown in Table 1. The metabolites were eluted stepwise with 5-ml portions of varying concentration of AcOEt–hexane (7:93 to 100:0), and MeOH–AcOEt (5:95). 1,25-$(OH)_2D_3$ was eluted mostly (93%) in AcOEt fraction (D) and a small amount (less than 10%) in 5% MeOH in AcOEt effluent (E). Other metabolites, 25-OHD_3, 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ 26,23-lactone, were also cleanly separated in fractions B [AcOEt–hexane (35:65), 90%], C [AcOEt–hexane (60:40), 80%], and E [MeOH–AcOEt (5:95), 90%], respectively. To examine the recovery of 1,25-$(OH)_2D_3$ from plasma, [^{3}H]-1,25-$(OH)_2D_3$ was added to plasma prior to extraction and the lipid extracts were separated similarly on a Bond Elut NH_2 column. The total radioactivity recovered in AcOEt and MeOH–AcOEt (5:95) effluents was 92% (Table 1, the value in parenthesis). The 1,25-$(OH)_2D_3$ fraction eluted with AcOEt was further purified by a normal-phase HPLC column before fluorescence labelling.

3.2. Fluorescence labelling of 1,25-$(OH)_2D_3$, purification of the labelled products, and determination of plasma 1,25-$(OH)_2D_3$

The 1,25-$(OH)_2D_3$ fractions from normal-phase HPLC were labelled with a large excess of DMEQ-TAD in CH_2Cl_2 at room temperature for 2.5 h. To remove interfering compounds due to the large excess reagent the labelled 1,25-$(OH)_2D_3$ had to be purified by two chromatographic runs prior to quantification. First the products were passed through a Bond Elut PSA cartridge and then purified by HPLC on a normal-phase HPLC column. In a model experiment using authentic DMEQ-TAD adducts of 1,25-$(OH)_2D_3$, the recovery of the adducts was 90.4±10.2% (mean±S.D., $n=8$) after the two purification steps described above. The purified sample was analyzed on a reversed-phase HPLC column. Under the conditions of labelling reaction, 1,25-$(OH)_2D_3$ produces two epimeric 6,19-cycloadducts

Table 1
Recoveries of major vitamin D metabolites from Bond Elut NH_2 cartridge

Compounds	Recovery (mean±S.D., $n=8$) (%)				
	A	B	C	D	E
25-OHD_3 (**1c**)	ND[a]	90±6	ND	ND	ND
24,25-$(OH)_2D_3$ (**1d**)	ND	ND	80±4	<10	ND
1,25-$(OH)_2D_3$ (**1a**)	ND	ND	ND	93± 2	<10
	(ND)	(ND)	(ND)	(81±11)	(11± 2)
1,25-$(OH)_2D_3$ 26,23-lactone (**1f**)	ND	ND	ND	ND	90±12

A mixture of 25-OHD_3, 24,25-$(OH)_2D_3$, 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ 26,23-lactone (each 50 ng) dissolved in AcOEt–hexane (7:93) (100 μl) was directly applied to a Bond Elut NH_2 column and eluted successively with (A) AcOEt–hexane (7:93) (5 ml), (B) AcOEt–hexane (35:65) (5 ml), (C) AcOEt–hexane (60:40) (5 ml), (D) AcOEt (5 ml), and (E) MeOH–AcOEt (5:95) (5 ml). The solvent was evaporated, each fraction dissolved in the mobile phase was subjected to HPLC [LiChrospher Si 60, hexane–CH_2Cl_2–MeOH (50:50:5), 1.5 ml/min, room temperature, UV 265 nm] to determine the recovery of the standard vitamin D metabolites. Values in parenthesis indicate the recoveries of [^{3}H]-1,25-$(OH)_2D_3$ added to plasma after fractionation and quantification as described above.

[a] ND=not detected. Recovery was obtained from the results of eight independent assays.

(Fig. 1) in a 1:1 ratio and the labelled products are detected as twin peaks by HPLC analysis. Therefore, the two peaks were integrated for quantification. An example of the final HPLC chromatogram of the fluorescent adducts of plasma 1,25-$(OH)_2D_3$ is displayed in Fig. 3, indicating well separated twin peaks of two epimers with no interfering peaks in the area of interest. The overall recovery of the radioactive internal standard through the whole procedure was 16–18% (Table 2) and the average yields of the fluorescence labelling of the plasma 1,25-$(OH)_2D_3$ fraction was calculated to be 20–25%.

3.3. Intra- and inter-assay variation

The precision and reproducibility of the assay using 2.5 ml plasma were assessed by analyzing 1,25-$(OH)_2D_3$ in a pool of the same plasma and the results are summarized in Table 2. The results of the inter-assay variation were obtained from the seven independent assays run on the same sample pool over a 1-month period. The concentrations of 1,25-$(OH)_2D_3$ were 44.3±4.5 pg/ml in the intra-assay and 41.0±3.5 pg/ml in the inter-assay, and coefficients of variation (C.V.) were 10.2% and 8.5%, respectively. The limit of quantification using the standard fluorescent derivative of 1,25-$(OH)_2D_3$ was around 10 pg (this quantity corresponds to ca. 5 pg of unlabelled vitamin D). The limit of detection of 1,25-$(OH)_2D_3$ in plasma, assuming 16.5% overall recovery from 2.5 ml plasma sample, is estimated to be 12 pg/ml.

3.4. Recovery of added standard 1,25-$(OH)_2D_3$

In validating the present fluorimetric method, the analytical recovery of 1,25-$(OH)_2D_3$ spiked to plasma was determined. A volume of 100, 200 and 300 pg of standard 1,25-$(OH)_2D_3$ together with the radioactive tracer for the determination of the overall recovery of 1,25-$(OH)_2D_3$ were added to 5 ml volumes of plasma pools and the increase of assayed peaks was measured. Table 3 depicts the calculated recoveries and an average of 97.4% of the added 1,25-$(OH)_2D_3$ was recovered.

Table 3
Analytical recoveries of standard 1,25-$(OH)_2D_3$ added to plasma (5 ml)

1,25-$(OH)_2D_3$ (pg/5 ml)		Recovery[c] (%)
Added	Corrected[a]	
0	389.9[b]	–
100	477.9	88.0
100	476.3	86.4
200	589.2	99.7
200	596.2	103.2
300	707.5	105.9
300	694.1	101.4
Average recovery		97.4±8.2

Plasma samples supplemented by known amounts of standard 1,25-$(OH)_2D_3$ (0, 100, 200 and 300 pg/5 ml plasma) together with the radioactive tracer were processed as described in Section 2 and were assayed.

[a] The values are corrected for the recovery of the added radioactive tracer.

[b] Concentration of endogenous 1,25-$(OH)_2D_3$ in 5 ml plasma. The result expresses the means of two experiments.

[c] The recovery of 1,25-$(OH)_2D_3$ added to plasma samples was calculated by the equation: Recovery (%)=$[(F-F_0)/A]\times 100$, where A=quantity of 1,25-$(OH)_2D_3$ added plasma, F=concentration of 1,25-$(OH)_2D_3$ in supplemented plasma sample, F_0=concentration of 1,25-$(OH)_2D_3$ in unsupplemented plasma sample.

3.5. Assay specificity

To test the effect of plasma volumes on the assay linearity, three volumes of plasma (2.5, 5 and 7.5 ml) were assayed in triplicate and the equation of the regression line was found to be: $y=50.159x+5.594$,

Table 2
Intra- and inter-assay variation

Assay	n	Recovery (%)	1,25-$(OH)_2D_3$ (pg/ml)	C.V. (%)
Intra-assay	7	16.4±1.6	44.3±4.5	10.2
Inter-assay	7	17.4±3.0	41.0±3.5	8.5

Results are expressed as mean±S.D. For the inter-assay, the 1,25-$(OH)_2D_3$ concentration in a single plasma was measured in duplicate on seven different occasions (total 14 samples) over a period of one month. Recovery shows the overall recovery of the radioactive internal standard throughout the whole procedure.

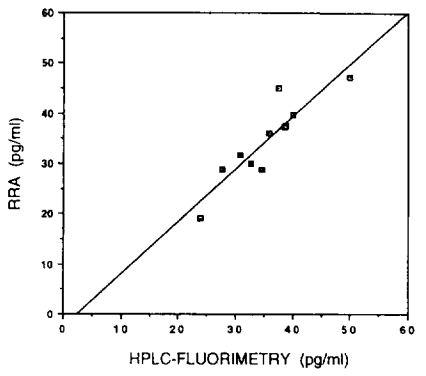

Fig. 4. Correlation between the HPLC–fluorimetric method and the RRA method. Regression analysis of the data gave the correlation coefficient $r=0.900$ and the equation of the regression line is $y=1.049x-2.657$ ($n=11$), slope $=1.049\pm0.383$, intercept $=-2.657\pm13.841$ ($P=0.05$).

where y is the concentration of 1,25-$(OH)_2D_3$ (pg/ml) and x is the volume of plasma (ml). The correlation coefficient (r) was 0.997, and the S.D. of the intercept and the slope were 19.362 ($P=0.05$) and 3.813 ($P=0.05$), respectively.

3.6. Comparison with the independent assay method

The fluorimetric assay was compared with the RRA in 11 individuals. The results were plotted as shown in Fig. 4. Regression analysis of the data afforded the relationship $y=1.049x-2.657$ and the correlation coefficient of 0.900.

4. Discussion

We have demonstrated the fluorimetric method for assaying 1,25-$(OH)_2D_3$ in human plasma. The fluorimetric assay of 1,25-$(OH)_2D_3$ was made possible by the availability of the diene selective and highly reactive fluorescent labelling reagent, DMEQ-TAD. We designed the reagent to tag fluorescence on vitamin D with high specificity [28,29]. This reagent has been successfully used in the fluorimetric assay of major vitamin D metabolites in plasma, such as 25-OHD_3, 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ [30]. The most advantageous feature of the reagent is that it forms a pair of fluorescent adducts with vitamin D [29–31] and affords characteristic twin peaks on HPLC (Fig. 3). Living organisms often use dimer-formation techniques in molecular recognition processes to make those steps accurate. The formation of twin peaks in the fluorescence labelling provides enormous accuracy to this fluorimetric assay. Nonetheless, the fluorimetric determination of plasma 1,25-$(OH)_2D_3$ levels had considerable difficulties, because its plasma concentration is lower by an order or two than those of the above mentioned vitamin D metabolites and the efficiency of fluorescence-labelling is lower in the 1α-hydroxylated metabolite (20–25%) than in non 1α-hydroxylated metabolites mentioned above (70–80%) under the assay conditions. These difficulties were overcome by using large excess of DMEQ-TAD for labelling and especially by extensive purification (Bond Elut cartridge column and normal-phase HPLC) of the DMEQ-TAD-labelled product prior to reversed-phase HPLC for quantification.

The fluorimetric method for assaying 1,25-$(OH)_2D_3$ was fully evaluated through the following analytical dada. There was reasonable agreement between the intra- and inter-assay and these results show precision and reproducibility of the present method. The analytical recoveries were quantitative proving the method to be accurate. Assay linearity was obtained in 2.5- to 7.5-ml plasma volumes, indicating that the increase of interfering substances in plasma did not affect the quantitative results of 1,25-$(OH)_2D_3$, and 2.5 ml of plasma is sufficient to determine the concentration of 1,25-$(OH)_2D_3$ accurately. This new method was compared with the RRA in combination with HPLC purification. Good correlation was obtained between these two independent assay methods, as the correlation coefficient ($r=0.900$) indicates. Thus we proved by physicochemical means that the RRA in combination with HPLC purification accurately measures the plasma concentration of 1,25-$(OH)_2D_3$. However, no correlation was found when this method was compared with a non-HPLC RRA (commercial base assay, data not shown).

A number of clinically useful active vitamin D analogs have been developed and many of those analogs are effective at much lower doses than 1,25-$(OH)_2D_3$ [35–38]. The RRA is not always applicable to those analogs, since they are less potent than

1,25-$(OH)_2D_3$ in binding to VDR. Therefore, a generally applicable assay method for vitamin D compounds, other than the existing methods, has been awaited for long years. The present method has the possibility to satisfy this demand. The advantages of the fluorimetric method over the RRA method are precision and specificity, because (1) the compound under study can be quantified as visible, well separated peaks, (2) absolute concentration can be determined, and (3) the identity of the peaks is doubly confirmed by the twin peaks as mentioned above. However, this fluorimetric method is tedious and time-consuming compared to the RRA method. We believe the method can be simplified by omitting HPLC purification prior to fluorescence-labelling and possibly by using a column-switching technique. Since the fluorigenic reagent (DMEQ-TAD) is a stable crystalline compound, an automated technique may be applicable to this assay.

In the assay of clinical samples, it is very important to determine accurately the physiological changes in the concentration of 1,25-$(OH)_2D_3$ together with the levels of 25-OHD_3 and 24,25-$(OH)_2D_3$. We did not measure the concentration of plasma 1,25-$(OH)_2D_2$, since no appreciable amounts of 25-OHD_2 were observed in the plasma pool used in the present studies and 1,25-$(OH)_2D_2$ is not usually present in blood in measurable concentrations of residents in Japan, except for patients receiving vitamin D_2 medication. We have already established a fluorimetric method for assaying 25-OHD_3 and 24,25-$(OH)_2D_3$. Thus multiple assay of major vitamin D metabolites by a single fluorimetric method using 2.5 ml of plasma is now possible.

In conclusion, a HPLC–fluorimetric method for the hormonally active vitamin D metabolite 25-$(OH)_2D_3$ in human plasma was developed. This assay method is expected to be useful for the exact analysis of 1,25-$(OH)_2D_3$ in clinical diagnosis and therapy of vitamin D dependent diseases.

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