Food Chemistry **45** (1992) 215–225
Analytical Methods Section

A method for the simultaneous determination of vitamins D_2,D_3 and their metabolites in plasma and its application to plasma samples obtained from normal subjects and patients

Sonoko Masuda, Toshio Okano & Tadashi Kobayashi

Department of Hygienic Sciences, Kobe Women's College of Pharmacy, Kobe 658, Japan

(Received 2 November 1990; revised version received 24 April 1991; accepted 2 May 1991)

A method has been established for the simultaneous determination of vitamins D_2,D_3 and their metabolites in plasma. Vitamin D_2/D_3, 25-hydroxyvitamin D_2/D_3 (25-OH-D_2/D_3), 24*R*,25-dihydroxyvitamin D_2/D_3 [24,25$(OH)_2D_2/D_3$] and 1α,25-dihydroxyvitamin D_2/D_3 [1,25$(OH)_2D_2/D_3$] in 1·5 ml of a plasma sample were simultaneously assayed. The method includes extraction of lipid, followed by three steps of high-performance liquid chromatography (HPLC) for clean-up and separation. Vitamin D_2/D_3 and 25-OH-D_2/D_3 were quantified by a UV detector, while 24,25$(OH)_2D_2/D_3$ and 1,25$(OH)_2D_2/D_3$ were assayed by competitive protein binding assay (CPBA) and radioreceptor assay (RRA) methods, respectively. Existing interfering substances in a sample could be effectively eliminated by HPLC and reliable results with small coefficients of variation were obtained. The method was applied to plasma samples obtained from normal subjects and patients with epilepsy, liver cirrhosis, leukaemia, diabetes, sensorineural deafness and hypophosphataemic osteomalacia; satisfactory results were obtained.

INTRODUCTION

It is well known that vitamins D_2 and D_3 with different side chain structures show practically the same physiological activity in mammals including human beings. ('Vitamin D' is used as a general name for vitamins D_2 and D_3 and the same nomenclature system is applied to the metabolites.) The vitamins are similarly metabolized to 25-OH-D in the liver and subsequently to 1,25$(OH)_2D$ or 24,25$(OH)_2D$ in the kidney according to lower or higher levels of calcium in plasma than normal, respectively (DeLuca *et al.*, 1976; Lawson & Davie, 1979). 7-Dehydrocholesterol in the epidermis of mammal skin is photochemically and thermally converted into vitamin D_3 by sun exposure and the endogenously formed vitamin is transported to the kidney via the liver to be metabolized to 1,25$(OH)_2D_3$ or 24,25$(OH)_2D_3$ via 25-OH-D_3 in the same manner as orally taken vitamin D_3 (Holick *et al.*, 1977; Okano *et al.*, 1977). Since no vitamin D_3 is found in most foods except some fish, e.g. eel, skipjack, tuna and Japanese pilchard (Kobayashi *et al.*, 1986), the vitamin and its metabolites found in plasma of normal subjects are mainly derived from the endogenous material formed in the skin. In contrast, since vitamin D_2 is not endogenously formed (like vitamin D_3) and is widely used in commercial drugs and enriched foods in Japan, all of the D_2 compounds in plasma originate from exogenous sources. Therefore, separative determination of the D_2 and D_3 compounds in human plasma allows distinction between endogenous and exogenous sources. Moreover, when vitamin D_2 is used for nutritional and/or clinical purposes, the assayed values of the D_2 compounds in plasma give direct information on the fate of exogenous vitamin D_2. The authors previously reported a method for the determination of 25-OH-D_2 and 25-OH-D_3 in plasma using high-performance liquid chromatography (HPLC) (Okano *et al.*, 1981) and applied it to the plasma of mothers and neonates (Kuroda *et al.*, 1981), and healthy subjects (Kobayashi *et al.*, 1983). However, the results are limited to the determination of 25-OH-D_2/D_3. In order to evaluate the nutritional and clinical problems completely, the

Food Chemistry 0308-8146/92/$05.00

plasma levels of vitamin D_2/D_3, $24,25(OH)_2D_2/D_3$ and $1,25(OH)_2D_2/D_3$ in addition to 25-OH-D_2/D_3 should be assayed. From these considerations, the authors have established a method for the simultaneous determination of vitamins D_2,D_3 and their metabolites in plasma, and applied it to plasma samples obtained from normal subjects and patients with epilepsy, liver cirrhosis, leukaemia, diabetes, sensorineural deafness and hypophosphataemic osteomalacia.

EXPERIMENTAL

Compounds and reagents

Among the standard compounds for the assay, commercially available crystalline vitamin D_2, vitamin D_3, 25-OH-D_3, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ (Dupher Co., Weesp, The Netherlands) were used. Purified compounds of 25-OH-D_2 and $1,25(OH)_2D_2$ were obtained by in-vivo (Okano *et al.*, 1984) and in-vitro (Horiuchi *et al.*, 1984) experiments, respectively, while $24,25(OH)_2D_2$ was chemically synthesized by the present authors (Katsumi *et al.*, 1987). These compounds were dissolved in absolute ethanol with a stream of argon gas to make appropriate concentrations and stored at −20°C until used. Commercial grades of [1α,2α(*n*)-^{3}H]-vitamin D_3 (specific activity, 740 GBq mmol^{-1}), [23,24(*n*)-^{3}H]-25-OH-D_3 (specific activity, 3·3 TBq mmol^{-1}), [23,24(*n*)-^{3}H]-24*R*,25$(OH)_2D_3$ (specific activity, 2·3 TBq mmol^{-1}) and [26,27-methyl-^{3}H]-1α,25$(OH)_2D_3$ (specific activity, 6·7 TBq mmol^{-1}) were purchased from Amersham Co. (Buckinghamshire, UK). 2,5-Diphenyloxazole, 1,4-bis-2-(4-methyl-5-phenyl-oxazoyl)-benzene, Triton X and toluene for liquid scintillator were purchased from Nakarai Chem. Co. (Kyoto, Japan). Organic solvents of analytical grades were distilled before use. Other guaranteed reagents were used.

Plasma samples

Plasma samples were obtained from normal subjects with or without oral administration of vitamin D_2, and patients with epilepsy, liver cirrhosis, leukaemia, diabetes, sensorineural deafness and hypophosphataemic osteomalacia were used. The samples were stored at −20°C until used. The studies are approved by each of the medical ethics committees of the hospitals.

Procedure for the simultaneous determination of vitamin D_2/D_3, 25-OH-D_2/D_3, $24,25(OH)_2D_2/D_3$ and $1,25(OH)_2D_2/D_3$ in plasma

The proposed procedure for the simultaneous determination of vitamins D_2/D_3, 25-OH-D_2/D_3, $24,25(OH)_2D_2/D_3$ and $1,25(OH)_2D_2/D_3$ in plasma is summarized in Fig. 1. Shimadzu LC-6A high-performance liquid chromatographs (Kyoto, Japan) with Shimadzu SPD-6A (265 nm; absorbance unit of full scale (AUFS) 0·001) detectors for HPLC analysis and a minifraction collector with a drop counter (Model 201, Gilson Co., Wisconsin, USA) were used.

Extraction of lipid

A plasma sample (1·5 ml) is placed in a glass centrifuge tube (10 ml) with a screw cap and then the radioactive standard solutions (2500 dpm in 25 μl of ethanol) of [1α,2α(*n*)-^{3}H]-vitamin D_3, [23,24(*n*)-^{3}H]-25-OH-D_3, [23,24(*n*)-^{3}H]-$24,25(OH)_2D_3$ and [26,27-methyl-^{3}H]-1,25$(OH)_2D_3$ ethanolic solutions are added for measuring recovery. After standing for 1 h at room temperature, tetrahydrofuran (THF) (0·75 ml) is added with stirring and then ethyl acetate (4·5 ml). Extraction of lipid is

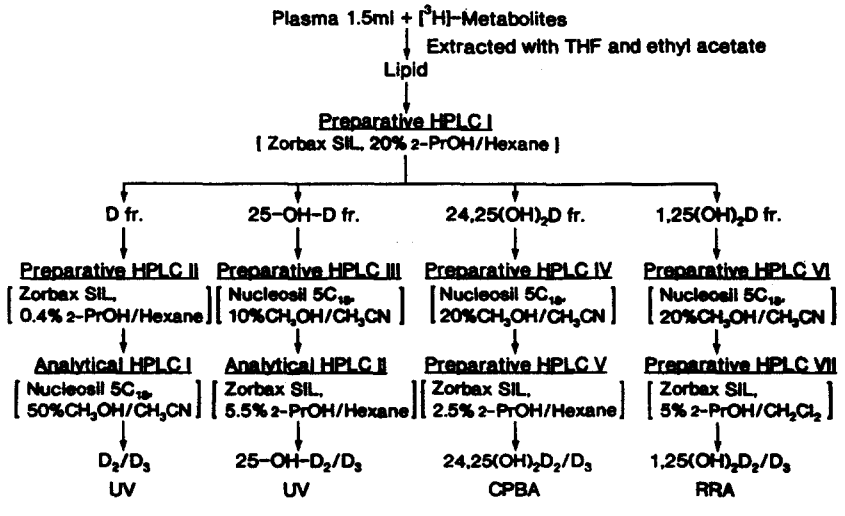

Fig. 1. Procedure for simultaneous determination of vitamins D_2,D_3 and their metabolites in plasma.

performed by vortex mixing for 2 min, standing for 10 min, and again vortex mixing for a further 2 min. After centrifugation at 3000 rpm for 10 min, the separated ethyl acetate layer is taken in another centrifuge tube (10 ml) and the remaining water layer is extracted again with ethyl acetate (3·0 ml). The combined ethyl acetate extract is dehydrated with anhydrous sodium sulphate and centrifuged at 3000 rpm for 5 min. The separated ethyl acetate layer is evaporated under reduced pressure.

Separation of vitamin D, 25-OH-D, 24,25$(OH)_2$D and 1,25$(OH)_2$D fractions

The residue thus obtained is dissolved in 20% (v/v) 2-propanol in *n*-hexane (2·0 ml) and then filtered through a membrane filter (Kurabo Co., Kurashiki, Japan) to eliminate any remaining sodium sulphate. The filtrate is evaporated under reduced pressure and the resulting residue is dissolved in 20% (v/v) 2-propanol in *n*-hexane (200 μl). The solution is applied to the following preparative HPLC I to collect the fractions of vitamin D, 25-OH-D, 24,25$(OH)_2$D and 1,25$(OH)_2$D.

Preparative HPLC I
Column: Zorbax SIL (4·6 i.d. × 250 mm, DuPont Co., Wilmington, DE, USA)
Mobile phase: 20% (v/v) 2-propanol in *n*-hexane
Flow rate: 1·0 ml min^{-1} (40 kg cm^{-2})
Collection: vitamin D fraction, 270–320 drops (retention time, 4·7–5·6 min); 25-OH-D fraction, 320–370 drops (retention time, 5·6–6·5 min); 24,25$(OH)_2$D fraction, 370–470 drops (retention time 6·5–8·3 min); 1,25$(OH)_2$D fraction, 490–620 drops, (retention time, 8·6–10·9 min)

Assay of vitamins D_2 and D_3

The collected vitamin D fraction is evaporated under reduced pressure and the resulting residue is dissolved in 0·4% (v/v) 2-propanol in *n*-hexane (200 μl) for application to the following preparative HPLC II for further clean-up.

Preparative HPLC II
Column: Zorbax SIL (4·6 i.d. × 250 mm)
Mobile phase: 0·4% (v/v) 2-propanol in *n*-hexane
Flow rate: 1·8 ml min^{-1} (60 kg cm^{-2})
Collection: vitamin D fraction, 2300–2630 drops (retention time, 20·0–22·0 min)

The collected vitamin D fraction is evaporated under reduced pressure and the resulting residue is dissolved in 50% (v/v) methanol in acetonitrile (200 μl) for application to the following analytical HPLC I for assaying vitamins D_2 and D_3.

Analytical HPLC I
Column: Nucleosil $5C_{18}$ (7·5 i.d. × 300 mm, Chemco Scientific Co., Osaka, Japan)
Mobile phase: 50% (v/v) methanol in acetonitrile
Flow rate: 2·0 ml min^{-1} (45 kg cm^{-2})
Retention time: vitamin D_2, 21·0 min; vitamin D_3, 22·7 min

The peak heights corresponding to vitamins D_2 and D_3 in the profile of analytical HPLC I are estimated and the vitamin D_3 fraction is collected. After adding the liquid scintillator (3 ml) to the collected solution, the radioactivity is measured by a liquid scintillation counter (Aloka LSC-700, Aloka Co., Tokyo, Japan) for measuring the recovery of vitamin D_3. The counting efficiency is approximately 45%. The recovery value is also applied to vitamin D_2. Exactly 100 μl of vitamin D_2 or D_3 standard solution (the concentrations are made as 100 ng ml^{-1} in 50% (v/v) methanol in acetonitrile) is applied to the analytical HPLC I to estimate the peak heights corresponding to vitamin D_2 and D_3. The concentration of vitamin D_2 or D_3 (ng ml^{-1}) is calculated using the following formula:

$$\text{Concentration of vitamin } D_2 \text{ or } D_3 \text{ in plasma (ng ml}^{-1}\text{)} = S \times \frac{Psa}{Pst} \times \frac{1}{R} \times \frac{1}{V} \times 100$$

where S is the quantity of vitamin D_2 or D_3 in the respective standard solutions (ng), *Psa* is the peak height of vitamin D_2 or vitamin D_3 in the HPLC chromatogram obtained from a sample, *Pst* is the peak height in the HPLC chromatogram obtained from the respective standard solution, R is recovery (%), and V is the volume of a sample (ml) taken for assay.

Assay of 25-OH-D_2 and 25-OH-D_3

The 25-OH-D fraction obtained from the preparative HPLC I is evaporated under reduced pressure and the resulting residue is dissolved in 10% (v/v) methanol in acetonitrile (200 μl) for application to the preparative HPLC III for further clean-up.

Preparative HPLC III
Column: Nucleosil $5C_{18}$ (7·5 i.d. × 300 mm)
Mobile phase: 10% (v/v) methanol in acetonitrile
Flow rate: 1·4 ml min^{-1} (30 kg cm^{-2})
Collection: 25-OH-D fraction, 1250–1600 drops (retention time, 16·0–20·5 min)

The 25-OH-D fraction obtained from the preparation HPLC III is evaporated under reduced pressure and the resulting residue is dissolved in 2·5% (v/v) of 2-propanol in *n*-hexane (200 μl) for application to the analytical HPLC II for assaying 25-OH-D_2 and 25-OH-D_3.

Analytical HPLC II
Column: Zorbax SIL (4·6 i.d. × 250 mm)
Mobile phase: 2·5% (v/v) 2-propanol in *n*-hexane
Flow rate: 1·3 ml min^{-1} (40 kg cm^{-2})
Retention time: 25-OH-D_2, 16·8 min; 25-OH-D_3, 20·8 min

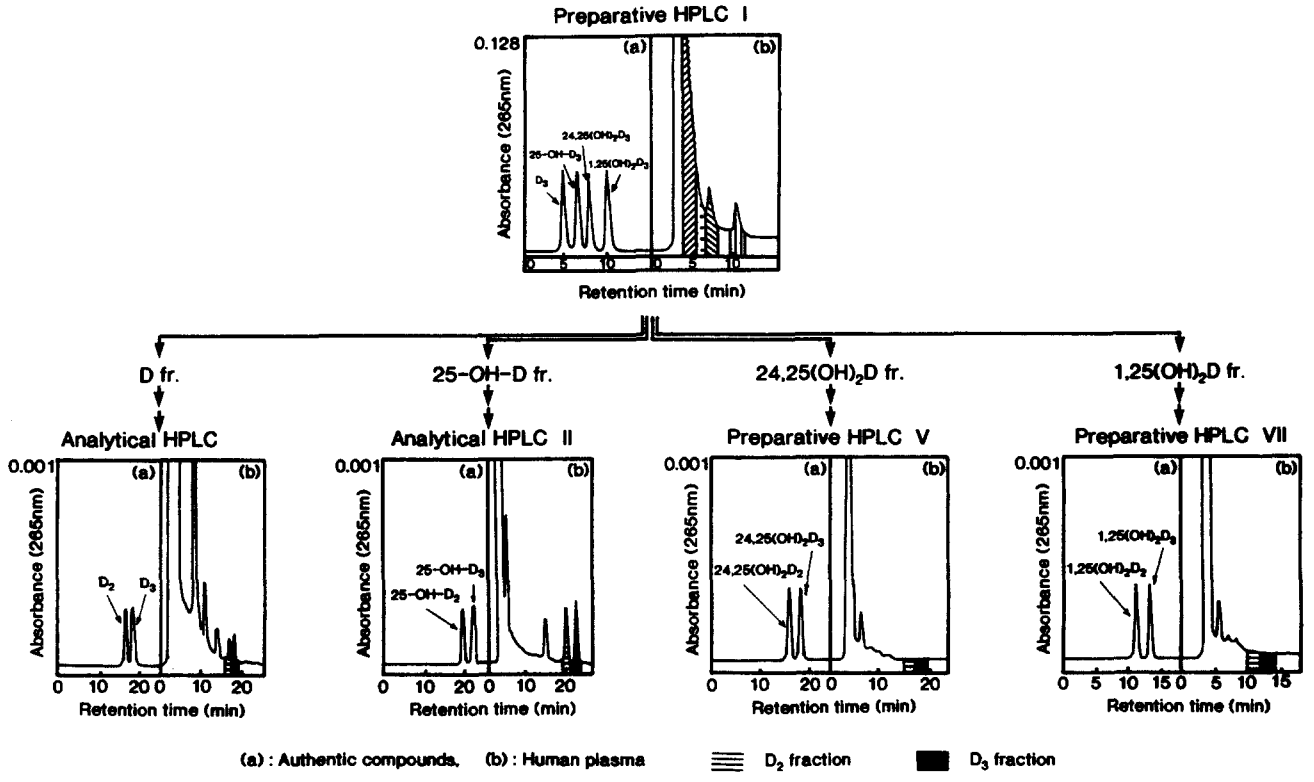

Fig. 2. Chromatograms of preparative HPLC and analytical HPLC of vitamins D_2,D_3 and their metabolites in plasma.

The peak heights corresponding to 25-OH-D_2 and 25-OH-D_3 in the chromatogram obtained from analytical HPLC II are estimated and the 25-OH-D_3 fraction is collected. After adding the liquid scintillator (3 ml) to the collected solution, the radioactivity is measured by a liquid scintillation counter for measuring the recovery of 25-OH-D_3. The counting efficiency is approximately 45%. The value of 25-OH-D_3 is also applied to 25-OH-D_2. Exactly 100 μl of 25-OH-D_2 or 25-OH-D_3 standard solution (the concentrations are made as 100 ng ml^{-1} in 2·5% (v/v) 2-propanol in *n*-hexane) is applied to the analytical HPLC II to estimate the peak heights corresponding to 25-OH-D_2 and 25-OH-D_3. The concentration of 25-OH-D_2 or 25-OH-D_3 (ng ml^{-1}) is calculated using the following formula:

$$\text{Concentration of 25-OH-}D_2\text{ or 25-OH-}D_3\text{ in plasma (ng ml}^{-1}\text{)} = S \times \frac{Psa}{Pst} \times \frac{1}{R} \times \frac{1}{V} \times 100$$

where S is the quantity of 25-OH-D_2 or 25-OH-D_3 in the respective standard solution (ng), *Psa* is the peak height of 25-OH-D_2 or 25-OH-D_3 in the HPLC chromatogram obtained from a sample, *Pst* is the peak height on the HPLC chromatogram obtained from the respective standard solution, R is recovery (%), and V is volume of a sample (ml) taken for assay.

Assay of 24,25(OH)$_2$D$_2$ and 24,25(OH)$_2$D$_3$

The collected 24,25(OH)$_2$D fraction from the preparative HPLC I is evaporated under reduced pressure and the resulting residue is applied to the procedure of competitive protein binding assay (CPBA) described in a previous paper (Matsuoka *et al.*, 1989) to assay 24,25(OH)$_2$D$_2$ and 24,25(OH)$_2$D$_3$.

Assay of 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$

The collected 1,25(OH)$_2$D fraction from the preparative HPLC I is evaporated under reduced pressure and the

Table 1. Analytical data obtained by the proposed method

	n	Vitamin D_3	25-OH-D_3	24,25(OH)$_2$D$_3$	1,25(OH)$_2$D$_3$
Detection limit		0·3 ng	0·5 ng	12·5pg	2 pg
Recovery (%)	4	56·6	60·6	52·7	56·4
Intra-assay					
x (ml^{-1})	4	1·7 ng	18·1 ng	2·2 ng	52·8 pg
CV (%)		9·6	9·0	4·6	5·4
Inter-assay					
x (ml^{-1})	4	1·8 ng	19·7 ng	2·3 ng	50·3 pg
CV (%)		3·3	5·3	6·6	10·9

resulting residue is applied to the procedure of radioreceptor assay (RRA) described in a previous paper (Matsuoka *et al.*, 1989) to assay $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$.

RESULTS

Chromatograms of the preparative and analytical HPLC

As shown in Fig. 2, the respective D_2 and D_3 standard compounds were completely separated in the chromatograms of the final HPLC. Successful separations between vitamins D_2 and D_3 and between 25-OH-D_2 and 25-OH-D_3 were also observed in the respective chromatograms of the sample as shown in Fig. 2. No significant peaks corresponding to $24,25(OH)_2D_2$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were observed in the respective chromatograms, but no apparent peaks due to interfering substances could be observed in the fractions.

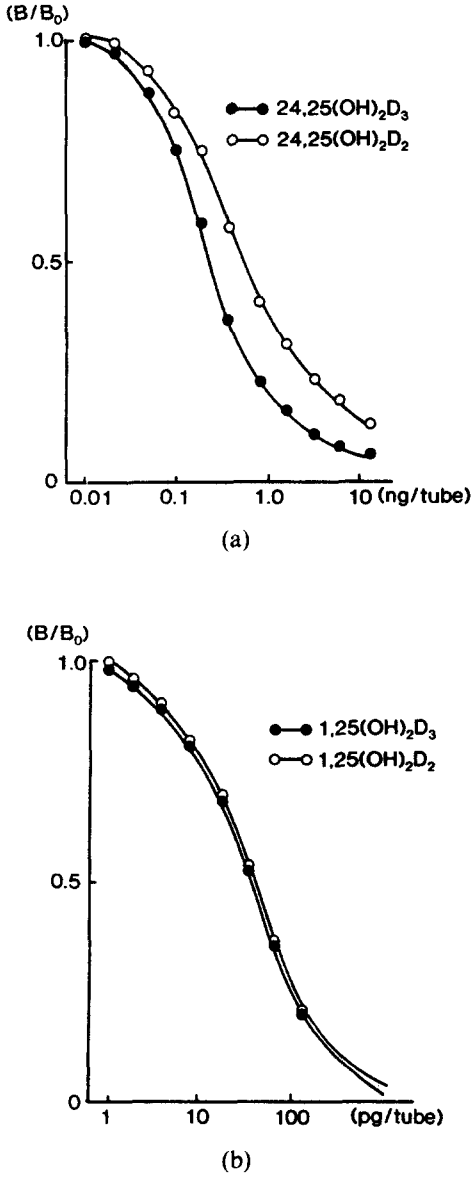

Fig. 3. (a) Calibration curves of $24,25(OH)_2D_2$ and $24,25(OH)_2D_3$; vitamin-D-deficient rat plasma was used as a binding protein for CPBA. (b) Calibration curves of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$; chick embryonal intestinal receptor was used as a binding protein for RRA.

Calibration curves of $24,25(OH)_2D_2$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$

Figures 3(a) and (b) show the calibration curves of $24,25(OH)_2D_2$ and $24,25(OH)_2D_3$ for CPBA and those of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ for RRA, respectively. Satisfactory sensitivities were obtained for both assays. Since both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ gave practically the same calibration curve, as shown in Fig. 3(b), the latter compound, easily obtained from commercial sources, can be used as a standard compound for RRA of both compounds. On the other hand, since the displacement potency of $24,25(OH)_2D_2$ to vitamin D protein (DBP) was 1·7 times weaker than that of $24,25(OH)_2D_3$, as shown in Fig. 3(a), the latter could not be substituted for the former's assay and $24,25(OH)_2D_2$ itself should be used as a standard compound for the assay.

Analytical data on the proposed method

The proposed method was applied to pooled plasma samples of healthy subjects to check the sensitivity and reproducibility. As shown in Table 1, the detection limits of vitamin D_3, 25-OH-D_3, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were 0·3 and 0·5 ng and 12·5 and 2·0 pg per tube, respectively. All of the overall recoveries were higher than 50%. The coefficients of variation (CV) values of the intra- and inter-assay of 25-OH-D_3, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were smaller than 15% (a satisfactory result), although those of vitamin D_3 were comparatively high.

Comparison between the assayed values of 25-OH-D in plasma by the proposed and CPBA methods

The authors' proposed method for determination of 25-OH-D_2 and 25-OH-D_3 is based on direct UV detection of the corresponding peaks observed in HPLC. In order to check the reliability of the assayed values, those of 25-OH-D_2 and 25-OH-D_3 in eight plasma samples obtained by the authors' proposed method were compared with those obtained by a CPBA method which was performed by applying the fractions of 25-OH-D_2 and 25-OH-D_3 from the analytical HPLC II in the authors' proposed method to a method according to Belsey *et al.* (1974).

As shown in Table 2, the respective assayed values were very close to each other, and there was a highly significant correlation ($P < 0·01$) between the two

Table 2. Comparison between the assayed values of 25-OH-D in plasma by HPLC and CPBA methods

Sample	25-OH-D_2 (ng ml^{-1})		25-OH-D_3 (ng ml^{-1})	
	HPLC	CPBA	HPLC	CPBA
1	ND	ND	21·8	26·5
2	ND	ND	20·1	23·7
3	ND	ND	26·7	25·5
4	ND	ND	27·2	31·3
5	ND	ND	20·5	24·9
6	ND	ND	33·8	29·1
7	4·6	6·6	22·7	28·2
8	345·9	332·1	5·1	4·8

Correlation (HPLC versus CPBA): $r = 1{\cdot}00$
$n = 10$ (25-OH-D_2 + 25-OH-D_3)
$P < 0{\cdot}01$

ND, Not detected.
Samples 1–6 were normal subjects. Samples 7 and 8 were obtained from patients with hypophosphataemic osteomalacia, who received orally massive doses of vitamin D_2.

groups. The results show that the authors' proposed HPLC method for determination of 25-OH-D_2 and 25-OH-D_3 using UV detection gives reliable data without application of CPBA.

Comparison between the assayed values of 1,25$(OH)_2$D in plasma by the proposed method and the previously used method

The authors previously assayed 1,25$(OH)_2$D in plasma by an RRA method (Takeuchi *et al.*, 1988). The method includes extraction of lipid, preliminary purification on a silica Sep-Pak cartridge, clean-up by HPLC using a Zorbax SIL column with 10% 2-propanol in *n*-hexane as a mobile phase and application to the RRA (Eisman *et al.*, 1976). However, the authors found a few samples, including those from normal adults and children, and various kinds of patients gave unexpectedly high values which might be due to insufficient clean-up. The examples are shown in Table 3. Five kinds of plasma samples obtained from normal subjects and patients with diabetes and epilepsy were studied by the previous method and the presently proposed method and they were compared with the respective assayed values. As shown in Table 3, the assayed values by the previous method were 3–6 times higher than the respective values assayed by the proposed method. When the 1,25$(OH)_2D_3$ fraction obtained from the HPLC of the previous method were successively applied to the preparative HPLC VI and VII and RRA described in the proposed method, all of the assayed values decreased to reach closely the respective values assayed by the proposed method. Therefore, the authors have concluded that the abnormal high values observed in the samples by the previous method were due to insufficient clean-up and use of additional one or two steps of HPLC is essential for complete clean-up.

Table 3. Comparison between the assayed values of 1,25-$(OH)_2D_3$ in plasma by the proposed method and previously used method

Case	Previous method[a] (pg ml^{-1})	Proposed method (pg ml^{-1})
Normal adult	287·8	58·8
Normal child	238·6	85·6
Patient with diabetes	271·3	41·0
Patient with epilepsy	214·0	50·2
Patient with epilepsy	229·8	72·2
Normal range	35–65	

[a] The previous method involves extraction with ethyl acetate and THF, preliminary purification on a silica Sep-Pak cartridge, followed by final purification on a normal-phase HPLC using 10% 2-propanol in *n*-hexane. Quantitation of 1,25$(OH)_2D_3$ is achieved using radioreceptor assay.

Concentrations of vitamin D and its metabolites in plasma of normal subjects

The proposed method was applied to the plasma samples obtained from 29 normal male subjects aged in their twenties in December 1988. The assayed values of vitamin D_3, 25-OH-D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ and their distributions are shown in Fig. 4. Vitamin D_2 and its metabolites were not detected in any of the samples. As shown in Fig. 4, the histograms of 25-OH-D_3 and 1,25$(OH)_2D_3$ show regular distributions, while those of vitamin D_3 and 24,25$(OH)_2D_3$ were irregular.

Table 4 shows the concentrations of vitamins D_2,D_3 and their metabolites in the plasma samples obtained from seven normal male volunteers aged in their twenties who have orally taken 400 international units (IU) per day for 10 months (Dec. 1988–Oct. 1989) via multivitamin preparations.

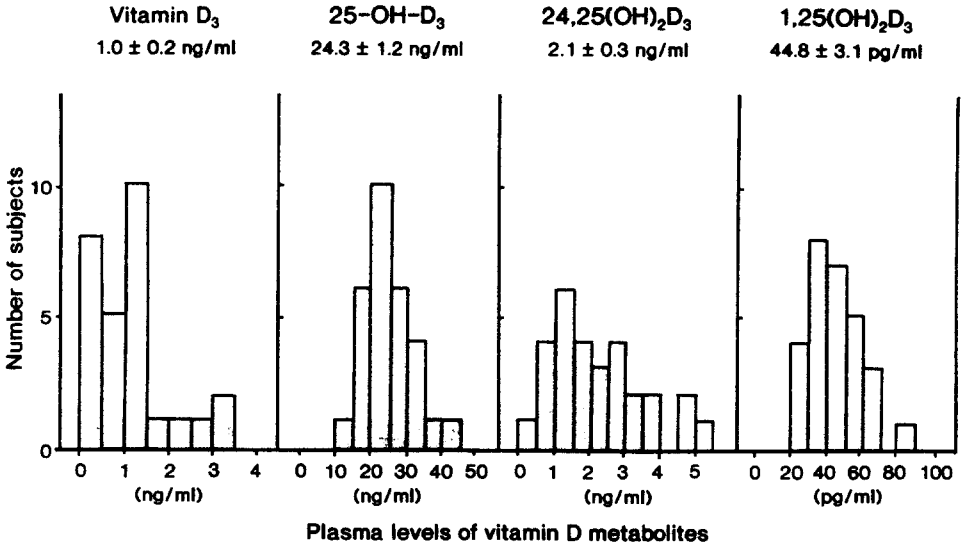

Fig. 4. Histograms of levels of vitamin D metabolites in plasma of normal subjects. Concentrations of vitamin D metabolites in normal subjects are expressed as mean ± SE.

Concentration of 25-OH-D_3 and 1,25$(OH)_2D_3$ in plasma of various diseases

Figure 5 shows the assayed values of 25-OH-D_3 and 1,25$(OH)_2D_3$ in the plasma samples obtained from patients with epilepsy, liver cirrhosis, leukaemia, diabetes and sensorineural deafness. All samples were taken before therapy with vitamin D_2 or D_3 and/or 1α-hydroxyvitamin D_3 (1α-OH-D_3). The D_2 compounds were not detected in any of the samples. The plasma levels of 25-OH-D_3 in the patients with decompensated liver cirrhosis (10·5 ± 1·5 ng ml^{-1}, mean ± SE) and leukaemia (9·3 ± 1·0 ng ml^{-1}) were significantly lower than those of normal adults (24·7 ± 1·0 ng ml^{-1}). The plasma levels of 1,25$(OH)_2D_3$ in patients with epilepsy (64·8 ± 2·3 pg ml^{-1}) were close to those of the normal children (59·9 ± 3·3 pg ml^{-1}). The levels of the patients with diabetes mellitus type II (63·8 ± 5·5 pg ml^{-1}) were slightly higher than the normal adults (46·6 ± 2·8 pg ml^{-1}) but not significantly so, while those in patients with decompensated liver cirrhosis (20·9 ± 2·5 pg ml^{-1}), leukaemia (29·4 ± 4·7 pg ml^{-1}) and sensorineural deafness (22·4 ± 7·5 pg ml^{-1}) were significantly lower than in normal adults.

Table 4. Changes in the plasma levels of vitamin D and its metabolites of normal subjects after daily oral administration of vitamin D_2 for 10 months

	Before	After 10 months
Vitamin D_2 (ng ml^{-1})	ND	1·7 ± 0·2
Vitamin D_3 (ng ml^{-1})	1·2 ± 0·2	2·8 ± 0·3
25-OH-D_2 (ng ml^{-1})	ND	14·8 ± 1·9
25-OH-D_3 (ng ml^{-1})	23·0 ± 1·5	24·6 ± 2·4
24,25$(OH)_2D_2$ (ng ml^{-1})	ND	ND
24,25$(OH)_2D_3$ (ng ml^{-1})	1·5 ± 0·3	2·2 ± 0·3
1,25$(OH)_2D_2$ (pg ml^{-1})	ND	29·8 ± 5·9
1,25$(OH)_2D_3$ (pg ml^{-1})	40·2 ± 6·0	48·2 ± 7·8

Data were expressed as mean ± SE of seven samples; ND, Not detected.
Each normal subject was orally administered 400 IU per day of vitamin D_2 for 10 months.

Changes in the concentrations of vitamin D_2 and its metabolites in the plasma obtained from a patient with hypophosphataemic osteomalacia orally receiving a massive dose of vitamin D_2

Figure 6 shows changes in the concentrations of vitamins D_2,D_3 and their metabolites in plasma of a patient with hypophosphataemic osteomalacia who orally received a massive dose of vitamin D_2. After oral administration of vitamin D_2 (2000 IU per day) for two weeks, the patient orally received a single dose of 500 000 IU of vitamin D_2 followed by 1 000 000 IU per day of vitamin D_2 for a week. Furthermore, the patient orally received 1 g per day of sodium phosphate for two weeks and subsequently 12–20 μg per day of 1,25$(OH)_2D_3$ for three weeks. Blood was taken at least once a week and the plasma samples were studied using the proposed method. At the initial stage, the concentrations of vitamin D_3 (not detected), 25-OH-D_3 (9·2 ng ml^{-1}), 24,25$(OH)_2D_3$ (0·6 ng ml^{-1}) and 1,25$(OH)_2D_3$ (24·4 pg ml^{-1}) were lower than the respective normal ranges. The plasma levels of vitamin D_2, 25-OH-D_2 and 24,25$(OH)_2$-D_2 rapidly increased by the administration of massive dose of vitamin D_2 and gradually decreased after stopping it. In contrast, although the plasma levels of 1,25$(OH)_2D_2$ significantly increased ($P < 0·01$) from the initial level (not detected) by the administration of a massive dose of vitamin D_2, the increase was not so large and the total levels of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ were kept in the normal range. By the administration of 1,25$(OH)_2D_3$, the plasma levels of 1,25$(OH)_2D_2$ gradually decreased according to the in-

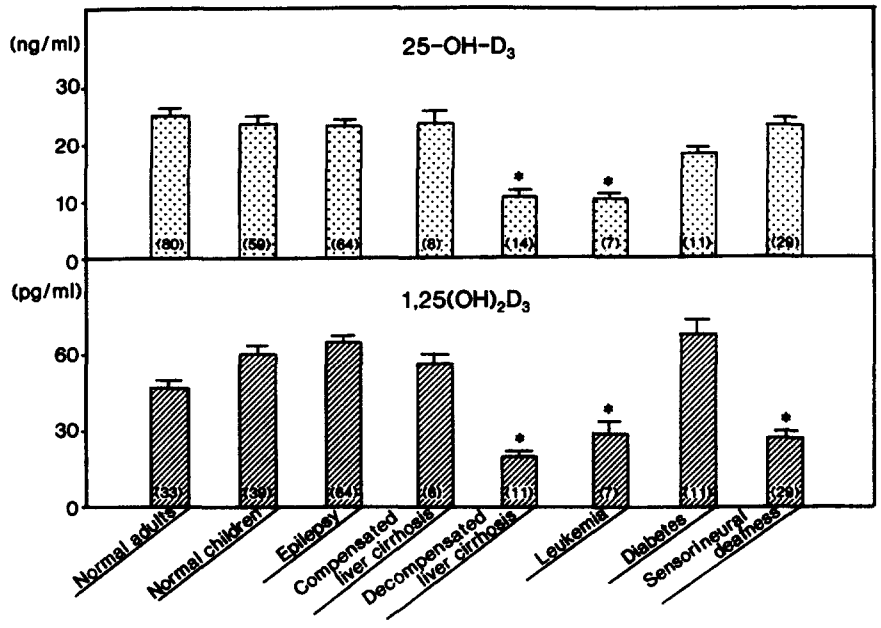

Fig. 5. Concentrations of 25-OH-D_3 and $1,25(OH)_2D_3$ in plasma of patients with various diseases. The numbers of subjects in parentheses; *, significantly different from normal adults, $P < 0{\cdot}01$.

crease of $1,25(OH)_2D_3$ levels and the total levels of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were strictly controlled within 50–80 pg ml^{-1}. The plasma levels of vitamin D_3 compounds were little changed by the administration of vitamin D_2.

DISCUSSION

Recently, significant progress has been made in the field of assay of vitamin D and its metabolites in biological fluids and many methods have been reported (Belsey *et al.*, 1974; Eisman *et al.*, 1976; Bikle, 1983; Horst, 1985; Jones *et al.*, 1985; Porteous *et al.*, 1987; Takeuchi *et al.*, 1988). Simultaneous determination of vitamin D and its metabolites has been also reported in a few papers (Lambert *et al.*, 1981; Parviainen *et al.*, 1981; Bouillon *et al.*, 1983; Hollis & Pittard, 1984). However, most of these assayed vitamin D and its metabolites without separating the respective D_2 and D_3 compounds. Since all of the vitamin D_2 compounds are derived from exogenous sources, separative assay of

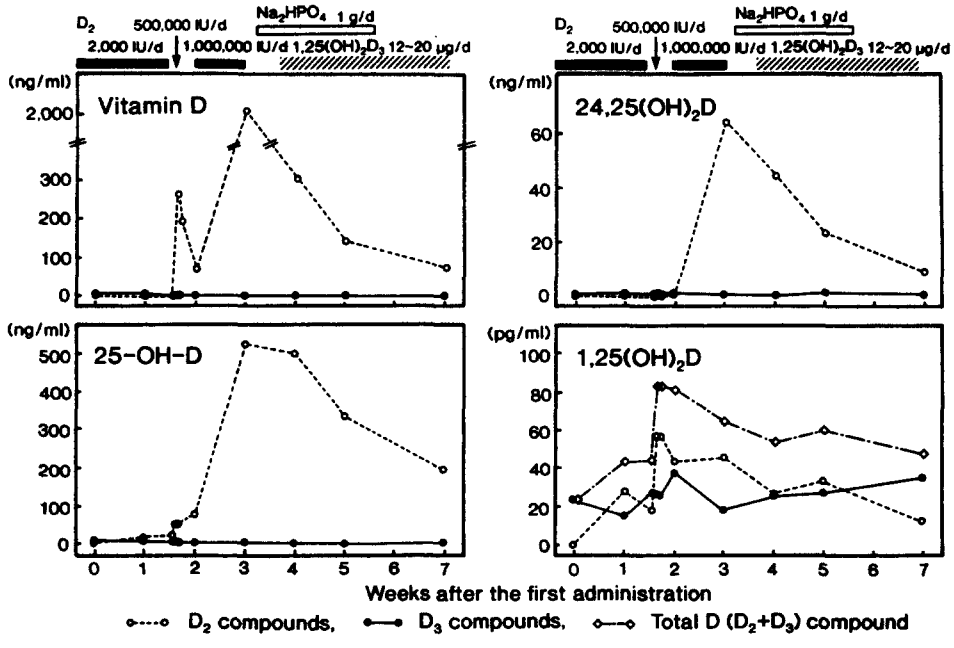

Fig. 6. Changes in the plasma concentration of vitamin D and its metabolites after oral administration of vitamin D_2 or $1,25(OH)_2D_3$ in a patient with hypophosphataemic osteomalacia.

the D_2 and D_3 compounds in human plasma allows distinction between the endogenous and exogenous sources. Gas chromatography–mass spectrometry (GC–MS) was used for simultaneous determination of the D_2 and D_3 compounds of 25-OH-D, $24,25(OH)_2D$ and $25,26(OH)_2D$ in plasma samples and successful results were reported (Coldwell *et al.*, 1989).

However, since the sensitivity of GC–MS is insufficient for the determination of pg amounts in samples, its application to the determination of $1,25(OH)_2D$ in plasma is difficult.

The authors previously reported a method for the simultaneous determination of $24,25(OH)_2D_2$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ in plasma (Matsuoka *et al.*, 1989) and in bone marrow (Masuda *et al.*, 1989). In this paper, the authors have added the determination of vitamins D_2,D_3, 25-OH-D_2 and 25-OH-D_3 and completed a method for the simultaneous determination of the D_2 and D_3 compounds of vitamin D, 25-OH-D, $24,25(OH)_2D$ and $1,25(OH)_2D$ in plasma.

As shown in Fig. 2, good separations between the D_2 and D_3 standard compounds were obtained by the proposed method. Successful separations between vitamins D_2 and D_3 and between 25-OH-D_2 and 25-OH-D_3 in a plasma sample were also obtained without disturbance by interfering substances. In order to confirm the reliability of the authors' assayed values of 25-OH-D_2 and 25-OH-D_3, eight plasma samples were chosen in which levels of the metabolites were assayed by the presently proposed method and the CPBA method (Belsey *et al.*, 1974). As shown in Table 2, the assayed values were close to each other and a highly significant correlation was observed between the two groups. Therefore, the authors' proposed method for the assay of 25-OH-D_2 and 25-OH-D_3, based on direct UV measurement, is confirmed to be a highly reliable one.

As shown in Fig. 2, no significant peaks corresponding to $24,25(OH)_2D_2$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were observed in the chromatograms of a sample, because plasma levels of the metabolites were too low to be detected by a UV detector. Therefore, the fractions corresponding to the metabolites previously confirmed by the standard compounds were collected and applied to the CPBA or RRA method. As shown in Fig. 2, no apparent peak due to interfering substances was observed in the corresponding fractions of the final HPLC. This phenomenon is very important for carrying out the assay successfully. If apparent interfering peaks are observed in the fractions, it means there is insufficient clean-up and abnormally high values of $24,25(OH)_2D$ and $1,25(OH)_2D$ are usually obtained.

Table 3 shows the comparison between the values of $1,25(OH)_2D_3$ in five kinds of plasma samples assayed by the previously used method (Takeuchi *et al.*, 1988) and the proposed method. The assayed values by the previous method were approximately 3–6 times higher than the respective values obtained by the proposed method. Since the steps of clean-up procedures in the previous method are less than those in the proposed method, the high values might be derived from insufficient clean-up. In fact, the high values obtained by the previous method could be decreased to values close to the proposed method's values by further clean-up of the $1,25(OH)_2D_3$ fractions. Abnormally high values were not always obtained by the previous method and these were rather rare cases. More than 90% of plasma samples assayed by the previous method gave reasonable values, while the remaining samples gave high values. The authors first found such samples giving abnormal high values in patients with diabetes and epilepsy, and therefore it was thought that these phenomena might be special in the patients. Therefore, it was thought that the problem could be solved by application of additional one or two steps of HPLC to such samples. However, overestimation was observed even in the plasma obtained from normal adults and children. The appearance of such abnormality was irregular and could not be predicted. Therefore, the authors conclude that the three steps of HPLC used in the proposed method are essential for the accurate determination of $1,25(OH)_2D$ is plasma, because the appearance of samples giving such abnormal high values cannot be predicted.

Table 4 shows the concentrations of vitamins D_2,D_3 and their metabolites in the plasma samples obtained from normal subjects who had orally taken 400 IU per day for 10 months via a multivitamin preparation. Vitamin D_2 and its metabolites could not be detected in any of the samples at the initial stage and this suggests that nobody took exogenous vitamin D_2 via pharmaceutical preparations or foods at the time. When the volunteers received orally 400 IU per day of vitamin D_2 for 10 months, the plasma levels of vitamins D_2, 25-OH-D_2 and $1,25(OH)_2D_2$ significantly ($P < 0{\cdot}05$) increased, but those of $24,25(OH)_2D_2$ were still below the limit of detection (Table 4). Since $24,25(OH)_2D$ is formed from 25-OH-D when plasma calcium concentrations are higher than normal (DeLuca, 1976), the fact that no significant amount of $24,25(OH)_2D_2$ was observed in the plasma suggests that intake of 400 IU per day of vitamin D_2 has little risk of hypervitaminosis for vitamin D.

Although the histograms of 25-OH-D_3 and $1,25(OH)_2D_3$ show regular distributions, those of vitamin D_3 and $24,25(OH)_2D_3$ were irregular (Fig. 4). It is well known that endogenously photosynthesized vitamin D_3 and exogenously taken vitamin D_3 and D_2 are rapidly incorporated in adipose tissue or rapidly metabolized to 25-OH-D_3 or 25-OH-D_2, and therefore plasma levels of vitamin D itself are labile and usually much lower than those of 25-OH-D (Lawson *et al.*, 1986; Liel *et al.*, 1988). The authors' results, that the distribution of plasma vitamin D_3 levels did not show a regular histo-

gram, might be due to the properties of vitamin D *in vivo*. In contrast, plasma levels of 25-OH-D are not labile and directly reflect incorporated amounts of vitamin D, the levels usually show regular distribution and the nutritional status of vitamin D_3. The authors' results, that the plasma levels of 25-OH-D_3 showed a regular histogram, agreed with the theory. Since metabolism of 25-OH-D to 1,25$(OH)_2$D is strictly feedback controlled by plasma calcium and phosphorus levels and other factors (DeLuca, 1976), abnormal plasma levels of 1,25$(OH)_2$D suggest that some disorders in the feedback control system have occurred. Therefore, plasma levels of normal subjects are usually within the normal range and the distribution usually shows a regular histogram. The authors' results on the plasma levels of 1,25$(OH)_2D_3$ agreed with the theory. It is unclear why 24,25$(OH)_2$D did not show a regular histogram. It is well known that 25-OH-D is metabolized into 24,25$(OH)_2$D in the kidney when plasma calcium levels are normal or higher than normal. If 24-hydroxylation of 25-OH-D in the kidney is controlled only by plasma calcium concentrations, the plasma levels of 24,25$(OH)_2D_3$ should show a regular histogram. However, the results did not show a regular histogram and they suggest that the conversion may be not regulated by plasma calcium concentration levels but by other unknown factors.

The proposed method was applied to the plasma samples obtained from patients with epilepsy, liver cirrhosis, leukaemia, diabetes and sensorineural deafness (Fig. 5). The patients with decompensated liver cirrhosis and leukaemia shown significantly lower 25-OH-D_3 levels than normal adults, and the results strongly suggest that the patients might have little chance of exposure to sunshine because of their serious illness. The lower plasma levels of 1,25$(OH)_2D_3$ in patients with decompensated liver cirrhosis, leukaemia and senso-rineural deafness suggest that disorder of vitamin D metabolism might be derived from their illness.

The proposed method was also applied to the plasma samples obtained from a patient with hypophosphataemic osteomalacia receiving massive doses of vitamin D_2. As shown in Fig. 6, the plasma levels of vitamin D_2 itself immediately increased by single dose of 500 000 IU vitamin D_2, but these rapidly decreased within three days and increased again by sequential doses of 1 000 000 IU of vitamin D_2. The changes suggest that incorporation of vitamin D_2 in adipose tissue and/or metabolism to 25-OH-D_2 in liver might occur rapidly. The plasma levels of 25-OH-D_2 and 24,25$(OH)_2D_2$ rapidly increased and gradually decreased after stopping the administration. The results suggest that the in-vivo formation of the two compounds might be not regulated by physiological factors but correlated with intake of vitamin D_2. In contrast, the formation of 1,25$(OH)_2D_2$ was not so great even after a massive dose of vitamin D_2 and the results suggest that the formation of 1,25$(OH)_2$D from 25-OH-D_2 in the kidney was strictly controlled by a feedback regulation system and that the regulation system acted equally on 25-OH-D_2 and 25-OH-D_3 without distinguishing the two compounds.

As mentioned above, the presently proposed method has been confirmed to be useful as a routine method for the simultaneous determination of the D_2 and D_3 compounds of vitamin D—25-OH-D, 24,25$(OH)_2$D and 1,25$(OH)_2$D—in biological fluids, especially in human plasma. The authors applied the method not only to normal subjects but also to patients with epilepsy, liver cirrhosis, leukaemia, diabetes, sensorineural deafness and hypophosphataemic osteomalacia; successful results were obtained. Simultaneous determination of the D_2 and D_3 compounds in 20 plasma samples can be performed within seven days by the proposed method.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Sumiaki Okamoto, Nagasaki University School of Medicine, for kind arrangement of plasma samples of hypophosphataemic osteomalacia. Thanks are due to Prof. Makoto Mino, Osaka Medical College, Prof. Hirotoshi Morii, Osaka City University Medical School, Dr Hakumei Oh, School of Medicine, Chiba University, Dr Toshihiko Suematsu, Osaka Prefectural Hospital, and Dr Katsuhisa Ikeda, Tohoku University School of Medicine, for kind supply of plasma samples. The authors are indebted to Setsu Matsuoka and Yoko Tobita of Kobe Women's College of Pharmacy for their technical assistance.

REFERENCES

Belsey, R. E., DeLuca, H. F. & Potts Jr, J. T. (1974). A rapid assay for 25-OH-vitamin D_3 without preparative chromatography. *J. Clin. Endocrinol. Metab.*, **38**, 1049–51.

Bikle, D. D. (1983). *Assay of Calcium Regulating Hormones.* Springer Verlag, New York.

Bouillon, R. (1983). Radiochemical assays for vitamin D metabolites: Technical possibilities and clinical applications. *J. Steroid Biochem.*, **19**, 921–7.

Coldwell, R. D., Trafford, D. J. H., Varley, M. J., Kirk, D. N. & Makin, H. L. J. (1989). Measurement of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_2 and 25,26-dihydroxyvitamin D_2 in a single plasma sample by mass fragmentography. *Clin. Chem. Acta*, **180**, 157–68.

DeLuca, H. F. (1976). Recent advances in our understanding of the vitamin D endocrine system. *J. Lab. Clin. Med.*, **87**, 7–26.

Eisman, J. A., Hamstra, A. J., Kream, B. A. & DeLuca, H. F. (1976). A sensitive precise and convenient method for determination of 1,25-dihydroxyvitamin D in human plasma. *Arch. Biochem. Biophys.*, **176**, 235–43.

Holick, M. F., Frommer, J. E., McNeill, N. M., Richard, J. W. & Potts Jr, J. T. (1977). Photometabolism of 7-dehydro-

cholesterol to previtamin D_3 in skin. *Biochem. Biophys. Res. Commun.*, **76**, 107–14.

Hollis, B. W. & Pittard, W. B. (1984). Evaluation of the total fetomaternal vitamin D relationships at term: Evidence for racial differences. *J. Clin. Endocrinol. Metab.*, **59**, 652–7.

Horiuchi, N. *et al.* (1984). A rapid sensitive *in vitro* assay of 25-hydroxyvitamin D_3-1α-hydroxylase and 24-hydroxylase using rat kidney homogenates. *Biochem. Biophys. Res. Commun.*, **121**, 174–80.

Horst, R. L. (1985). Recent advances in the quantitation of vitamin D and vitamin D metabolites. In *Vitamin D: Basic and Clinical Aspects*, ed. R. Kumar. Martinus Nijhoff, Boston, pp. 423–78.

Jones, G., Seamark, D. A., Trafford, D. J. H. & Makin, H. L. J. (1985). Vitamin D: Cholecalciferol, ergocalciferol, and hydroxylated metabolites. In *Modern Chromatographic Analysis of Vitamins*, ed. A. P. DeLeenheer, W. E. Lambert & M. G. M. DeRuyrter. Marcel Dekker, New York, pp. 73–128.

Katsumi, K. *et al.* (1987). Syntheses of 24,25-dihydroxyvitamin D_3, 24,25-dihydroxy-22-dehydrovitamin D_3, 25-hydroxy-24-oxo-22-dehydrovitamin D_3 and 22,24,25-trihydroxyvitamin D_3. *Chem. Pharm. Bull.*, **35**, 970–9.

Kobayashi, T. *et al.* (1983). Variation of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 levels in human plasma obtained from 758 Japanese healthy subjects. *J. Nutr. Sci. Vitaminol.*, **29**, 271–81.

Kobayashi, T., Okano, T. & Takeuchi, A. (1986). The determination of vitamin D in foods and feeds using high-performance liquid chromatography. *J. Micronutr. Anal.*, **2**, 1–24.

Kuroda, E. *et al.* (1981). Plasma levels of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 levels in maternal, cord and neonatal blood. *J. Nutr. Sci. Vitaminol.* **27**, 55–65.

Lambert, P. W., DeOreo, P. B., Hollis, B. W., Fu, I. Y. Ginsberg, D. J. & Roos, B. A. (1981). Concurrent measurement of plasma levels of vitamin D_3 and five of its metabolites in normal humans, chronic renal failure patients and anephric subjects. *J. Lab. Clin. Med.*, **98**, 536–84.

Lawson, D. E. M. & Davie, M. (1979). Aspects of the metabolism and function of vitamin D. *Vitam. Horm.*, **37**, 1–67.

Lawson, D. E. M., Sedrani, S. H. & Douglas, J. (1986). Interrelationship in rats of tissue pools of cholecalciferol and 25-hydroxycholecalciferol formed in u.v. light. *Biochem. J.*, **233**, 535–40.

Liel, Y., Shary, U. J., Hollis, B. W. & Bell, N. H. (1988). Low circulating vitamin D in obesity. *Calcif. Tissue Int.*, **43**, 199–201.

Masuda, S. *et al.* (1989). Bone marrow and serum concentrations of 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D, and 1α,25-dihydroxyvitamin D in patients with leukemia and normal subjects. *J. Nutr. Sci. Vitaminol.*, **35**, 211–23.

Matsuoka, M. *et al.* (1989). Changes in the concentrations of vitamin D and its metabolites in the plasma of healthy subject orally given physiological doses of vitamin D_2 by multi-vitamin or vitamin D preparations. *J. Nutr. Sci. Vitaminol.*, **35**, 253–66.

Okano, T., Yasumura, M., Mizuno, K. & Kobayashi, T. (1977). Photochemical conversion of 7-dehydrocholesterol into vitamin D_3 in rat skins. *J. Nutr. Sci. Vitaminol.*, **23**, 165–8.

Okano, T. *et al.* (1981). A method for simultaneous determination of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in human plasma by using two steps of high-performance liquid chromatography. *J. Nutr. Sci. Vitaminol.*, **27**, 43–54.

Okano, T., Masuda, S., Kusunose, S., Komatsu, M. & Kobayashi, T. (1984). A rapid method for purification of 25-hydroxyvitamin D_2 from rabbit plasma by recycle high-performance liquid chromatography. *J. Chromatogr.*, **294**, 460–5.

Parviainen, M. T., Savolainen, K. E., Korhonen, P. H., Alhava, E. M. & Visakorpi, J. K. (1981). An improved method for routine determination of vitamin D and its hydroxylated metabolites in serum from children and adults. *Clin. Chim. Acta*, **114**, 233–47.

Porteous, C. E., Coldwell, R. D., Trafford, D. J. H. & Makin, H. L. J. (1987). Recent developments in the measurement of vitamin D and its metabolites in human body fluids. *J. Steroid Biochem.*, **28**, 785–801.

Takeuchi, A., Okano, T., Tsugawa, N., Katayama, M., Mimura, Y. & Kobayashi, T. (1988). Determination of vitamin D and its metabolites in human and cow's milk. *J. Micronutr. Anal.*, **4**, 193–208.