Determination of Vitamin D_3 and 25-Hydroxyvitamin D_3 in Sera by Column-Switching High Performance Liquid Chromatography with Fluorescence Detection

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A column-switching high performance liquid chromatographic method with fluorescence detection for the determination of vitamin D_3 and 25-hydroxyvitamin D_3 in human and rat sera is described. The vitamins in a lipid extract from serum, obtained by solid-phase extraction technique using a Bond-Elut C_{18} cartridge, are converted with 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide into the corresponding fluorescent derivatives. The derivatives are separated from endogenous interfering substances by column-switching chromatography. The chromatographic system consisted of a precolumn chromatography for clean-up of the derivatives and an analytical column chromatography for the complete separation of the derivatives. The derivatives are detected fluorometrically at excitation and emission wavelengths of 360 and 440 nm, respectively. The detection limits (S/N=3) for vitamin D_3 and 25-hydroxyvitamin D_3 are 15 and 8 fmol, respectively, in a 10 µl injection volume. The sensitivity permits simultaneous determination of the vitamins in 1 ml of normal human and rat sera.

Keywords Vitamin D_3, 25-hydroxyvitamin D_3, column-switching high performance liquid chromatography, fluorescence detection, 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide, human serum, rat serum

Vitamin D_3 is hydroxylated in the liver to 25-hydroxyvitamin D_3 (25-OHD_3), which is further hydroxylated in the kidney either to 1α,25-dihydroxyvitamin D_3 or to 24,25-dihydroxyvitamin D_3. The determination of these compounds in human plasma/serum is very important for the assessment of "vitamin D_3 status" in healthy and diseased persons. Among these compounds, vitamin D_3 and 25-OHD_3 occur in serum at relatively high concentrations. Thus, their determination is still widely used for the assessment of vitamin D_3 status and provides significant results in many clinical situations.

Various methods for the determination of vitamin D_3 and/or 25-OHD_3 in human plasma/serum have been reported.[1-22] The methods include lipid extraction, purification by chromatography and quantification by physicochemical methods such as UV-absorption[1-14] and mass fragmentography[15], competitive protein binding assays[8-14,17-22] or radioimmunoassay.[14] Among these methods, the UV-high performance liquid chromatographic (HPLC) methods have been widely used for the simultaneous determination of vitamin D_3 and 25-OHD_3 in plasma/serum. Such methods, however, include an extremely complicated pretreatment of plasma/serum, and require a very long time for the determination.

We have developed 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide (DMEQ-CON_3) as a highly sensitive and reactive fluorescence derivatization reagent for alcohols.[23] The reagent has then been applied to the determination of cholesterol and cholestanol in human serum[24,25], and 7-dehydrocholesterol (previtamin D_3) in rat[26] and human skin surfaces.[27] Recently, we found that vitamin D_3 and 25-OHD_3 react with DMEQ-CON_3 to give the corresponding highly fluorescent carbamic acid esters. This research aims to establish a sensitive column-switching HPLC method utilizing DMEQ-CON_3 for the simultaneous determination of vitamin D_3 and 25-OHD_3 in human and rat sera.

Experimental

Chemicals and solutions

Unless stated otherwise, all chemicals and solvents were of reagent grade. Deionized and distilled water was used. Vitamin D_3 and its hydroxyl derivatives were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMEQ-CON_3 was prepared as described previously.[23] DMEQ-CON_3 solution (2.0 mM) was prepared in benzene. The solution could be used for

two days when stored at 4°C in the dark.

Bond-Elut C_{18} cartridges were obtained from Analytichem International (Harbor City, CA, USA). The cartridges were washed successively with hexane (2 ml), isopropanol (3 ml), methanol (3 ml) and water (5 ml) before use.[6]

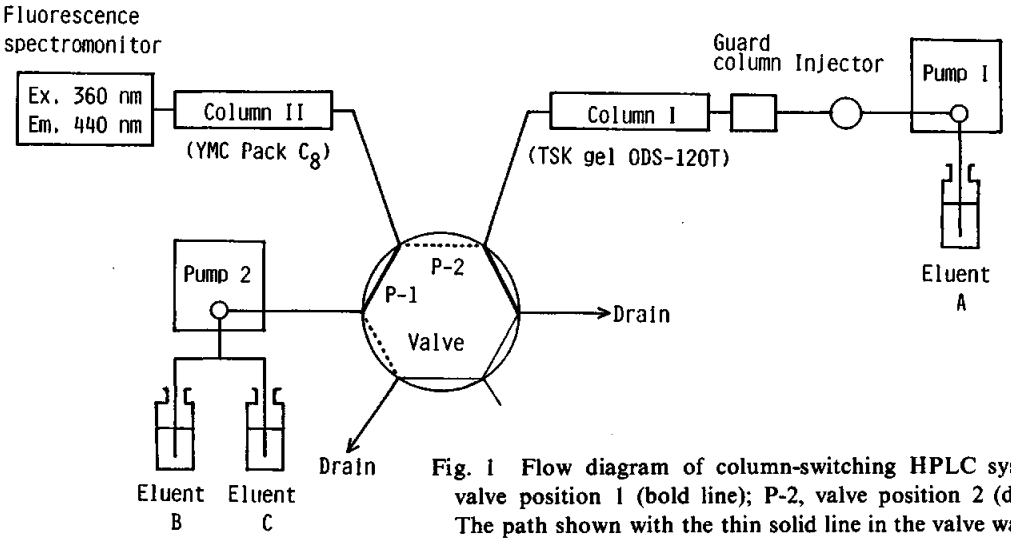

Fig. 1 Flow diagram of column-switching HPLC system. P-1, valve position 1 (bold line); P-2, valve position 2 (dotted line). The path shown with the thin solid line in the valve was not used.

Table 1 Program for controlling the column-switching system

Time/min	Valve position	Eluent (A, and B or C)	Effect
0	P-1	A	The reagent blank components were eluted from column I.
		B	Column II was equilibrated with eluent B.
10.5	P-2	A	The fluorescent derivative of 25-OHD_3 retained on column I was transferred to column II.
		B	Wasted.
12.0	P-1	B	The fluorescent derivative of 25-OHD_3 (peak 3) was separated on column II.
		A	Interfering substances (peaks at retention times of *ca.* 12–23 min in Fig. 2(B)) were removed from column I.
23.0	P-1	C	Column II was equilibrated with eluent C.
		A	Interfering substances (peaks at retention times of *ca.* 23–26.7 min in Fig. 2(B)) were removed from column I.
26.7	P-2	A	The fluorescent derivative of vitamin D_3 (peak 6) was transferred from column I to column II.
		C	Wasted.
29.2	P-1	C	Vitamin D_3 (peak 6) was separated on column II.
		A	Interfering substances (peaks at retention times of *ca.* 29–40 min in Fig. 2(B)) were eluted from column I.
60.0			

All glassware used for the extraction of vitamin D_3 and 25-OHD_3 from sera was rinsed with chloroform and methanol before use.[28]

Apparatus and HPLC conditions

A flow-diagram of the column-switching HPLC system and a time program of the system controller are shown in Fig. 1 and Table 1, respectively. The system consisted of a preliminary HPLC separation of the DMEQ derivatives of vitamin D_3 and 25-OHD_3 from endogenous substances that came from human and rat sera and an analytical column HPLC for a complete separation of the derivatives.

The column-switching HPLC was performed with a Waters 510 high-pressure pump (pump 1) equipped with a U6K Universal injector valve (10 µl loop), a Hitachi L-6200 intelligent pump (pump 2), a Hitachi F1100 fluorescence spectromonitor equipped with a 12 µl flow-cell and a Hitachi six-way automatic valve. A TSK gel ODS-120T column (column I; 150×4 mm i.d.; 5 µm; Tosoh, Tokyo, Japan), fitted with a guard column (TSK gel ODS-120T, 10×4 mm i.d.; 5 µm), and a YMC Pack C_8 column (column II; 150×4 mm i.d.; 5 µm; Yamamura Chemical Labs., Kyoto, Japan) were used as preliminary separation columns and an analytical column, respectively. The columns were maintained at 50±0.2°C with a Shimadzu CTO-6A column oven (Kyoto, Japan). Aqueous 93%(v/v) methanol (eluent A) and mixtures of acetonitrile–0.2 M sodium chloride [80:20 (eluent B) and 85:15 (eluent C), v/v] were used as mobile phases. The flow rates of the three eluents were all 1.0 ml min^{-1}. The fluorescence intensity was monitored at 440 nm (emission) and 360 nm (excitation).

Preparation of human and rat serum samples

Human sera were obtained in December from healthy volunteers (9 males and 3 females; 22–41 years old) in our laboratories. Vitamin D_3 deficient rats were prepared by the following procedure. Male Sprague-Dawley rats weighing 40–50 g (3 weeks old) were fed by the rachitogenic diet of Steenbock and Herting[9] for 6 weeks. After a 6-week depletion period, the rat sera were obtained by decapitation.

Lipids including vitamin D_3 and 25-OHD_3 were extracted from serum according to the method of Hollis and Frank.[6] A human or rat serum sample (1.0 ml) was mixed with saturated ammonium hydrogen sulfate solution (1.0 ml), ethanol (2.0 ml), 0.4 M dipotassium hydrogen phosphate (pH 10.5) (1.0 ml) and acetonitrile (1.0 ml). After vortex-mixing for *ca.* 1 min, the mixture was centrifuged at 1000*g* for *ca.* 10 min. The supernatant (4.0 ml) was applied onto a Bond-Elut C_{18} cartridge. The cartridge was washed successively with water (5.0 ml) and aqueous 70%(v/v) methanol (5.0 ml). A fraction containing vitamin D_3 and 25-OHD_3 was eluted with 6.0 ml of acetonitrile at a flow rate of *ca.* 0.4 ml min^{-1}. The eluate was transferred into a screw-capped 10-ml tube and evaporated to dryness under a nitrogen stream.

Derivatization procedure

To the residue (or a 10 µl portion of a standard mixture of vitamin D_3 and 25-OHD_3) in the screw-capped vial, 0.2 ml of the DMEQ-CON_3 solution was added. Then the vial was tightly closed and heated at 80°C for 80 min. The reaction mixture was diluted with 0.2 ml of methanol and the final solution (10 µl) was injected into the HPLC system.

Calibration

The amounts of vitamin D_3 and 25-OHD_3 were calibrated by means of the standard addition method: 10 µl of one of standard mixtures of vitamin D_3 (1.3–130 pmol) and 25-OHD_3 (12.5–250 pmol) was added to 1.0 ml of serum. The mixture was allowed to stand at room temperature for *ca.* 60 min, and then treated in the same way as above. The net peak heights of the individual vitamins were plotted against the concentrations of the vitamins.

Results and Discussion

Derivatization and precolumn HPLC conditions

Figure 2(A) shows a chromatogram of the DMEQ derivatives of vitamin D_3 and 25-OHD_3, obtained by the preliminary HPLC that used only the guard column, column I and eluent A. Vitamin D_3 and 25-OHD_3 reacted with DMEQ-CON_3 to give two and four fluorescent products, respectively. The main products for peaks 3 and 6 may be due to the DMEQ carbamic acid esters of 3-hydroxyl group in 25-OHD_3 and vitamin D_3, respectively. Peak 5 increased in height for longer reaction times and higher reaction temperatures for the derivatization. This indicates that the peak component is the isomer of vitamin D_3, provitamin D_3, which is produced by heating vitamin D_3.[29] Peak 4 can be attributed to the DMEQ esters of 25-hydroxyl group (tertiary alcohol) in 25-OHD_3; tertiary alcohols are less active than secondary alcohols.[23] Peaks 1 and 2 are probably due to the isomers of the components of peaks 3 and 4, respectively. Peaks 3 and 6, which have the most intense fluorescence in the DMEQ esters of vitamin D_3 and 25-OHD_3, respectively, were employed for the quantification.

Maximum and constant peak heights for peaks 3 and 6 were attained at DMEQ-CON_3 concentrations greater than 0.9 mM in the reagent solution; 2.0 mM was selected as a sufficient concentration. The derivatization reaction of vitamin D_3 and 25-OHD_3 proceeded more rapidly when the reaction mixture was heated at higher temperatures (Fig. 3). However, at 90°C, peaks 3 and 6 decreased in height with time. The heights of peaks 3 and 6 reached almost maxima and became constant after heating at 80°C for 70 and 60 min, respectively. Thus, heating at 80°C for 80 min was recommended in the procedure.

The derivatization was affected by the solvent used. Of the solvents examined (benzene, toluene, acetone,

acetonitrile, ethyl acetate, diethyl ether, chloroform, dimethylsulfoxide and *N*,*N*-dimethylformamide), benzene gave the most intense peaks. Although benzene is prohibited in many laboratories because of its toxicity, we found no other suitable solvent for derivatization of the vitamins.

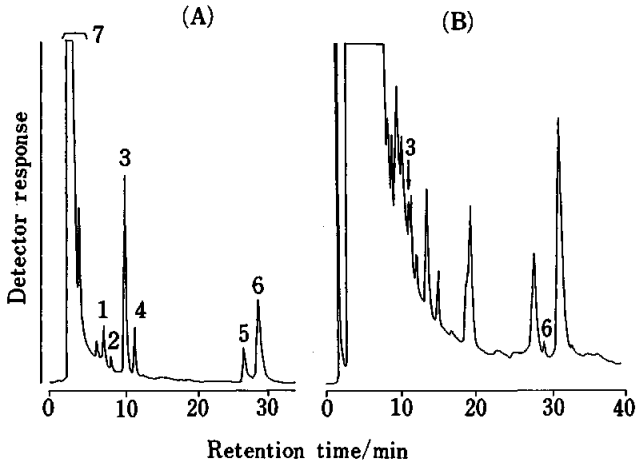

Fig. 2 Chromatograms of the DMEQ derivatives of (A) a standard mixture of vitamin D_3 and 25-OHD_3 and (B) a lipid extract of normal human serum, obtained by using only the guard column, column I and eluent A. A 10 µl portion of a standard mixture of the vitamins (65 nmol ml^{-1} each) was treated according to the derivatization procedure. A 1.0 ml portion of serum was treated according to the serum sample preparation and derivatization procedures. Peaks: 1 and 2, DMEQ esters of 3- and 25-hydroxyl groups in the isomer of 25-OHD_3 (provitamin D_3), respectively; 3 and 4, DMEQ esters of 3- and 25-hydroxyl groups in 25-OHD_3, respectively; 5, DMEQ ester of the isomer of vitamin D_3; 6, DMEQ ester of vitamin D_3; 7, reagent blank components; others, endogenous substances in serum. Detector sensitivities: A, 0.2; B, 2.

The within-day precision was examined using a standard mixture of vitamin D_3 and 25-OHD_3 (20 pmol 10 µl^{-1} each); the relative standard deviations (n=10) for vitamin D_3 and 25-OHD_3 were 2.5 and 4.2%, respectively. The detection limits (S/N=3) for vitamin D_3 and 25-OHD_3 were 15 and 8 fmol, respectively, in a 10 µl injection volume. This method was much more sensitive than that of the HPLC method with UV detection[1-14] and comparable in sensitivity to those of the mass fragmentography[15], the competitive protein binding assays[8-14,17-22] and the radioimmunoassay.[14] The relationships between the peak heights and the amounts of vitamin D_3 and 25-OHD_3 were linear up to at least 50 pmol for both compounds in a 10 µl injection volume.

Many biogenic substances examined gave no fluorescent derivatives under the described conditions, at a concentration of 10 nmol per 10 µl; *i.e.* sugars (D-xylose, D-ribose, 2-deoxy-D-ribose, D-fucose, D-glucose, D-galactose, D-fructose, D-glucosamine, maltose, cellobiose, gentiobiose and lactose), hydroxycarboxylic acids (lactic and malic acids) and seventeen different L-amino acids. Some other vitamin D_3-related hydroxysteroids gave fluorescent products (Table 2). However, under the conditions of the preliminary HPLC separation, DMEQ derivatives of these steroids have retention times different from those of peaks 3 and 6 in Fig. 1. Accordingly, these substances did not interfere with the determination of vitamin D_3 and 25-OHD_3.

Determination of vitamin D_3 and 25-OHD_3 in serum

The solid-phase extraction of lipids including vitamin

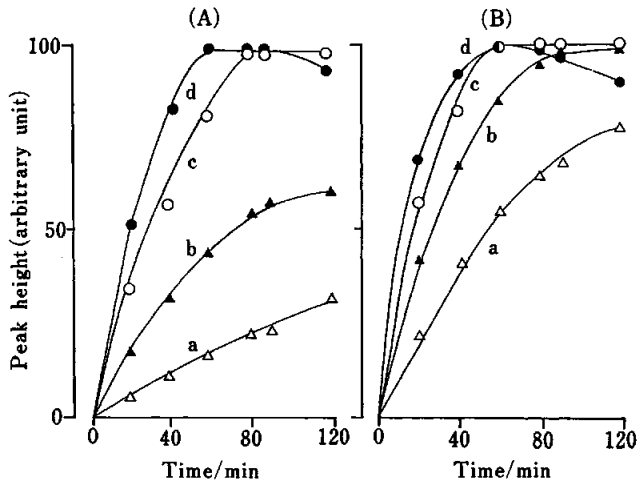

Fig. 3 Effect of reaction time and temperature on the fluorescence derivatization of (A) vitamin D_3 and (B) 25-OHD_3. Portions (10 µl) of a standard mixture of vitamin D_3 and 25-OHD_3 (65 nmol ml^{-1} each) were treated as in the derivatization procedure at various temperatures for various periods. Temperatures: a, 60°C; b, 70°C; c, 80°C; d, 90°C.

Table 2 Retention times for the DMEQ derivatives of vitamin D_3 hydroxysteroids

Compound	Retention time/min
Vitamin D_3	27.2
	28.7
25-OHD_3	8.1
	9.0
	11.2
	12.4
Cholestanol	57.8
Cholesterol	42.5
7-Dehydrocholesterol	36.2
Vitamin D_2	25.9
	27.2
1α-Hydroxyvitamin D_3	12.9
	14.5
	17.2
1α,25-Dihydroxyvitamin D_3	5.7
	6.7
	8.6
24,25-Dihydroxyvitamin D_3	5.0
	5.5
	6.3

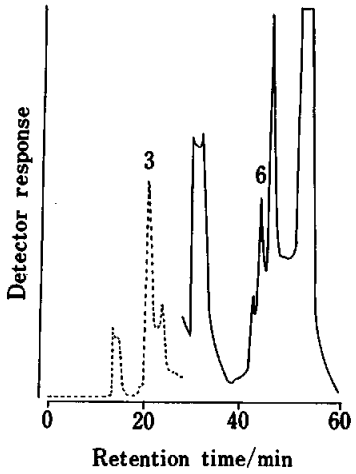

Fig. 4 Chromatogram obtained with a normal human serum. A 1.0 ml portion of serum was treated according to the whole procedure. For peaks 3 and 6, see Fig. 2.

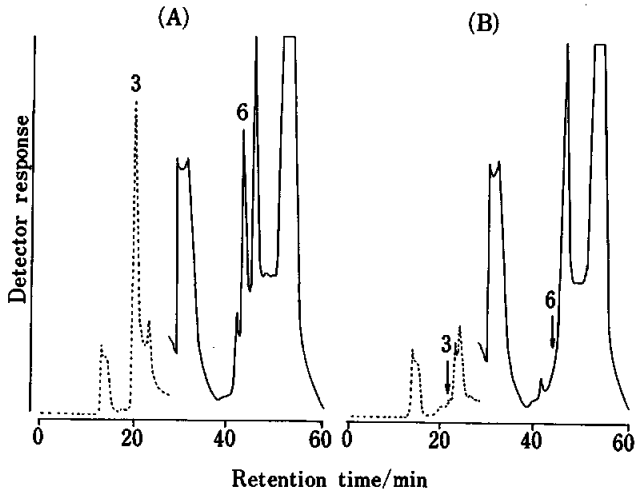

Fig. 5 Chromatograms obtained with sera from (A) normal and (B) vitamin D_3 defficient rats. A 1.0 ml portion of rat serum was treated according to the whole procedure. For peaks 3 and 6, see Fig. 2. Arrows 3 and 6 correspond to the retention times for peaks 3 and 6, respectively. Detector sensitivities: ·····, 1; —, 10.

D_3 and 25-OHD_3 from serum[6] worked well for sample preparation for the derivatization. Without the solid-phase extraction, large peaks appeared on the chromatogram and even small peaks for the vitamins could not be detected.

When the lipid extract of human serum was treated according to the derivatization procedure and the reaction mixture was subjected to an HPLC using only the guard column and column I, large peaks due to endogenous substances other than vitamin D_3 and 25-OHD_3 in human serum appeared on the chromatogram (Fig. 2(B)) and interfered with the determination of the vitamins. Thus, a column-switching HPLC was introduced to diminish the interfering substances.

Figure 4 shows a chromatogram obtained with normal human serum. Peaks 3 and 6 in Fig. 4 were identified as the DMEQ derivatives of vitamin D_3 and 25-OHD_3, respectively, on the basis of their retention times and fluorescence excitation and emission spectra compared with those of the standard compounds, and co-chromatography of the standards and sera with aqueous 50 – 100% methanol as mobile phase. Further evidence was provided when a serum sample from a vitamin-D_3-deficient rat was treated as in the procedure; no peaks were observed at the retention times for peaks 3 and 6 (Fig. 5(B)), though peaks 3 and 6 were observed in the chromatogram of normal rat serum (Fig. 5(A)), the pattern of which was quite similar to that of the chromatogram given by human serum (Fig. 4).

Linear relationships were observed between the peak heights and the amounts of vitamin D_3 and 25-OHD_3 (up to 500 pmol each) added to 1.0 ml of serum.

The within-day precision was determined using a normal human serum containing vitamin D_3 and 25-OHD_3 at mean concentrations of 5.21 and 45.0 pmol ml^{-1}, respectively. The relative standard deviations were 9.4 and 6.5% for vitamin D_3 and 25-OHD_3, respectively (n=10). The precision almost equaled those of the other methods.[1-22] Although we tried to find an internal standard substance suitable both for the column-switching HPLC separation and the fluorescence derivatization to improve the precision, we could not find such a substance. The recoveries of vitamin D_3 (15.4 pmol) and 25-OHD_3 (145 pmol) added to human serum (1.0 ml) were 53.8±4.9 and 76.9±7.2% (mean±standard deviation, n=8), respectively.

Table 3 Concentrations of 25-OHD_3 and D_3 in normal human sera

Age	Sex[a]	25-OHD_3/ ng(pmol) ml^{-1}	D_3/ ng(pmol) ml^{-1}
41	M	21.0 (52.4)	1.26 (3.28)
38	M	19.0 (47.4)	2.10 (5.46)
31	M	6.2 (15.4)	3.49 (9.07)
25	M	8.7 (21.7)	1.18 (3.07)
25	M	17.3 (43.2)	4.55 (11.83)
24	M	17.3 (43.2)	1.14 (2.96)
23	M	10.3 (25.7)	1.14 (2.96)
22	M	12.4 (30.9)	1.34 (3.48)
22	M	13.6 (33.9)	1.30 (3.38)
21	M	28.0 (69.9)	1.75 (4.55)
26	F	15.7 (39.2)	0.37 (0.96)
23	F	10.7 (26.7)	0.90 (2.34)
22	F	10.1 (25.2)	1.20 (3.12)
Mean		14.6 (36.5)	1.67 (4.34)
Standard deviation		5.7 (14.2)	1.09 (2.84)

a. M, male; F, female.

Table 4 Concentrations of 25-OHD_3 and D_3 in rat sera

Sample	25-OHD_3/ ng(pmol) ml^{-1}	D_3/ ng(pmol) ml^{-1}
1	54.1 (135)	10.2 (26.0)
2	43.1 (107)	6.41 (16.6)
3	85.1 (221)	8.90 (23.1)
4	53.5 (113)	7.41 (19.2)
5	59.5 (148)	6.62 (17.1)
6	31.0 (77)	7.43 (19.2)
Mean	54.4 (137)	7.82 (20.2)
Standard deviation	16.6 (44.2)	1.24 (3.08)

The concentrations of vitamin D_3 and 25-OHD_3 in normal human serum are given in Table 3. The concentrations of vitamin D_3 in serum were in good agreement with those obtained by Jones[8] and by Shepard *et al.*[9], although the values were slightly lower than those given by Aksnes[10] and Seamark *et al.*[15] The mean value of 25-OHD_3 obtained by this study was similar to those reported by other workers.[1-22]

The concentrations of the vitamins in normal rat serum determined by the method are shown in Table 4. The levels of the vitamins in rat serum were *ca.* 3 - 5 times higher than those in human serum.

This study provided the first HPLC method with fluorescence detection for the simultaneous determination of vitamin D_3 and 25-OHD_3 in human and rat sera. The method has adequate sensitivity to measure the vitamins in 1.0 ml of normal human and rat sera. The method is relatively more simple and rapid than the other methods are. The method should therefore be useful for physiological and pharmacological investigation of vitamins.

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