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Short communication

Determination of 25-hydroxyvitamin D_3 in human plasma by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A method for the determination of 25-hydroxyvitamin D_3, the major metabolite of vitamin D_3 in human plasma, using a non-radioactive internal standard and reversed-phase high-performance liquid chromatography with UV detection (265 nm) has been developed. The method was applied to the determination of the metabolite in plasma from healthy subjects ($n=25$) and from patients with chronic renal failure ($n=12$). 25-Hydroxyvitamin D_3 3-sulfate, a major conjugated metabolite of 25-hydroxyvitamin D_3, was also determined and the correlation between the concentrations of these metabolites was examined. The study showed that almost equal amounts of both compounds were detected in the plasma of healthy subjects, however, in two subjects, the amount of sulfate in the free form was found to be about twice as high as normally detected. In contrast, the free form was predominant in the plasma of patients with chronic renal failure and the sulfate was not detected in four patients.

Keywords: 25-Hydroxyvitamin D_3; 25-Hydroxyvitamin D_3 3-sulfate; Vitamins

1. Introduction

It is widely accepted that vitamin D_3 (D_3) is 25-hydroxylated in the liver as the first step in its conversion to a 1,25-dihydroxylated compound, which is the active metabolite in the intestine and bone. Measurement of the concentration of 25-hydroxyvitamin D_3 [25(OH)D_3] in plasma is widely used as a means of assessing vitamin D status in man and some methods using HPLC have been developed [1]. These methods normally involved the use of a radioactive internal standard (I.S.). Shimizu et al. [2] recently developed a method using a non-radioactive I.S., but they used normal-phase HPLC, which is not always sufficiently versatile for routine analysis.

Despite recent intensive investigation of D_3 metabolism, understanding of the conjugates still remains poor. In previous studies of this series, we confirmed the existence of and a method for the determination of 25(OH)D_3 3-sulfate [25(OH)$D_3$3S] in human plasma without hydrolysis using reversed-phase HPLC with UV (265 nm) detection [3,4]. Although Axelson and Christensen [5,6] clarified that 25(OH)$D_3$3S is the major circulating form of D_3, the biological significance of this compound remains to be determined.

This paper describes the development of a determination method for 25(OH)D_3 using a non-

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radioactive I.S. and reversed-phase HPLC with UV detection (265 nm) and application of the method for the clarification of the correlation between the concentration of 25(OH)D_3 and 25(OH)$D_3$3S in human plasma from healthy subjects and from patients with chronic renal failure.

2. Experimental

2.1. Materials and apparatus

The 25(OH)D_3 was donated by Teikoku Hormone Mfg. (Tokyo, Japan). The 25-hydroxyergosterol (I.S.$_1$) and 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione adduct (I.S.$_2$) of 25-hydroxy-7-dehydrocholesterol were synthesized in this laboratory [7,8]. All chemicals used were of analytical reagent grade. Solvents were purified by distillation prior to use. The Isolute C_{18} (EC) cartridges (International Solvent Technology, Hengoed, UK) were obtained from Uniflex (Tokyo). Silica gel (70-230 mesh, Merck, Darmstadt, Germany) was used for column chromatography.

HPLC was performed using a Shimadzu LC-6A (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SPD-10A UV detector (265 nm) at a flow-rate of 1 ml/min unless otherwise stated.

2.2. Plasma samples

Blood was collected from healthy volunteers (n= 25, November 1995) or from patients with chronic renal failure (n=12, April 1995, kindly donated by First Department of Internal Medicine, School of Medicine, Kanazawa University) in heparinized tubes and centrifuged at 1400 g (15 min, 4°C). The separated plasma was stored at −20°C until analyzed. The fresh and frozen plasma obtained from the Japan Red Cross Service (Tokyo) was used for the preparation of a calibration graph.

To obtain charcoal-treated plasma, activated charcoal (1.2 g) was added to the fresh and frozen plasma (10 ml) and after being shaken for 48 h at room temperature, the mixture was centrifuged at 4500 g (30 min, room temperature). The supernatant was filtered and stored at −20°C until used.

2.3. Determination of 25(OH)D_3 and 25(OH)$D_3$3S

For the determination of 25(OH)D_3, ethanol (1 ml) was added to the plasma (0.5 ml), the mixture was vortex-mixed (1 min) and centrifuged at 1400 g (15 min, room temperature). A solution of I.S.$_1$ (300 ng) in ethanol (75 μl) and 0.2 M KOH (1 ml) were added to the supernatant and the mixture was extracted twice with Et_2O (1 ml). After washing with 25% methanol (2 ml), the organic layer was evaporated in vacuo. The residue was dissolved in hexane–isopropanol (IPA) (98.5:1.5, v/v; 0.3 ml) and passed through the silica gel column (300 mg; 2×0.6 cm I.D.). After washing with hexane–IPA (98.5:1.5, v/v; 10 ml), the eluate with hexane–IPA (84:16, v/v; 2 ml) was evaporated to dryness. The residue was redissolved in methanol (100 μl) and an aliquot of this solution was subjected to HPLC: column, J'sphere ODS-H80 (4 μm) (15×0.46 cm I.D.; YMC, Kyoto, Japan); temperature, 40°C; mobile phase, MeCN–H_2O (7:3, v/v); [retention time (t_R) of 25(OH)D_3, 17.3 min; t_R of I.S.$_1$, 23.5 min].

For the determination of 25(OH)$D_3$3S, the plasma (0.5 ml) was treated with the slightly modified method described in a previous paper [4]; that is, an Isolute C_{18} (EC) cartridge was used instead of a Bond Elut C_{18} cartridge and the difference between these two cartridges did not lead to a difference in the recovery rate of the sulfate.

2.4. Identity of the peak corresponding to 25(OH)D_3

The material providing the peak corresponding to 25(OH)D_3, obtained from HPLC and used for the determination was collected, I.S.$_1$ (300 ng) in ethanol (75 μl) was added and the solvent was evaporated in vacuo. The obtained residue was subjected to the following HPLC conditions: column, YMC-Pack ODS-AM (5 μm) (15×0.46 cm I.D.; YMC); temperature, 40°C; mobile phase, methanol–H_2O (6:1, v/v) [25(OH)D_3, t_R 9.4 min; I.S.$_1$, t_R 11.2 min]; column, Develosil 60-5 (5 μm) (25×0.46 cm I.D.; Nomura, Seto, Japan); temperature, room; mobile phase, hexane–IPA (193:7, v/v) [25(OH)D_3, t_R 10.6 min; I.S.$_1$, t_R 9.3 min], flow-rate, 2 ml/min.

2.5. Absolute recovery of 25(OH)D_3 from blank sample

Solutions of 25(OH)D_3 (5 and 20 ng) and I.S.$_1$ (300 ng) in ethanol were added to 7% bovine serum albumin (BSA) in 0.4 *M* potassium phosphate buffer (pH 7.5, 0.5 ml) and each of the resulting solutions was subjected to the previously described pretreatment procedure. The solution of I.S.$_2$ (50 ng) in ethanol (20 μl) was added to the pretreated sample, which was determined using the previously described HPLC method. The absolute recovery of 25(OH)D_3 and I.S.$_1$ were calculated from the ratio of the height of the corresponding peak to that of I.S.$_2$ (t_R 10.1 min).

2.6. Calibration graph for 25(OH)D_3

Solutions of 25(OH)D_3 (0, 2.5, 5, 10, 15 and 20 ng) in ethanol (20 μl each) were added to the fresh and frozen plasma (0.5 ml), and each of the resulting solutions was assayed using the proposed method. The regression line was constructed using the ratio of the peak height of 25(OH)D_3 to that of I.S.$_1$ against the concentration of added 25(OH)D_3 (ng/ml). The slope of the obtained regression line was used for the calibration graph for the determination of 25(OH)D_3.

2.7. Method validation of determination of 25(OH)D_3

Solutions of 25(OH)D_3 (0, 5 and 10 ng) in ethanol (20 μl each) were added to four different plasma specimens (each 0.5 ml). Each of the resulting solutions was then assayed using the proposed method.

3. Results

3.1. Determination of 25(OH)D_3 in plasma

We developed a method for the determination of 25(OH)D_3 in human plasma using 25-hydroxyergosterol as the I.S. and a reversed-phase column for HPLC (Fig. 1). The chromatographic peaks of 25(OH)D_3 and I.S.$_1$ were well resolved (Fig. 2a) and

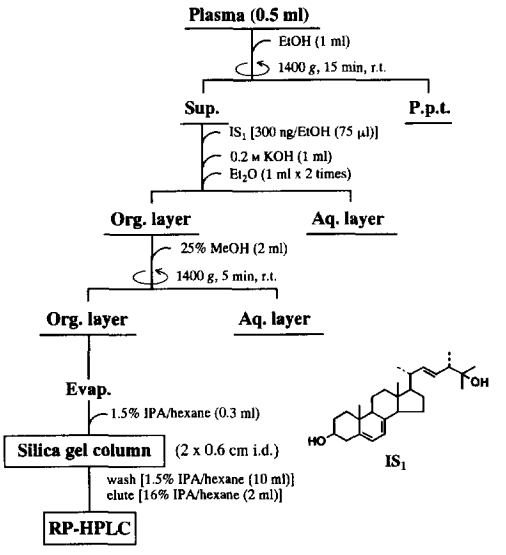

Fig. 1. Procedure for the determination of 25(OH)D_3 in human plasma.

no interference from the endogenous substances in the plasma was detected at the t_R of I.S.$_1$ (Fig. 2b,c). The eluate that corresponds to 25(OH)D_3 was collected, I.S.$_1$ was again added and the mixture was then subjected to HPLC using the other conditions including a normal-phase column. The peak-area ratio of 25(OH)D_3 and I.S.$_1$ was the same under all conditions examined, which confirmed the identity of the peak.

The treatment of the plasma with charcoal did not adsorb endogenous 25(OH)D_3 because of the interaction of D-binding protein in plasma with the compound, these data prompted us to use the 7% BSA solution as a plasma blank [4]. The absolute recoveries of 25(OH)D_3 at two levels and of I.S.$_1$ from 7% BSA in phosphate buffer were examined according to the clean-up procedure. Reasonable recovery rates (>52.0%) with satisfactory precision (R.S.D. <7.1%) were obtained from both compounds throughout the pretreatment procedure (4 steps), as shown in Table 1.

The slope of the regression line constructed using the standard addition method with fresh and frozen plasma showed good linearity (>0.995), reproducibility [$1.13\times10^{-2}\pm8.48\times10^{-4}$ (7.51), mean±S.D. (R.S.D., %), $n=8$] and was used for the calibration

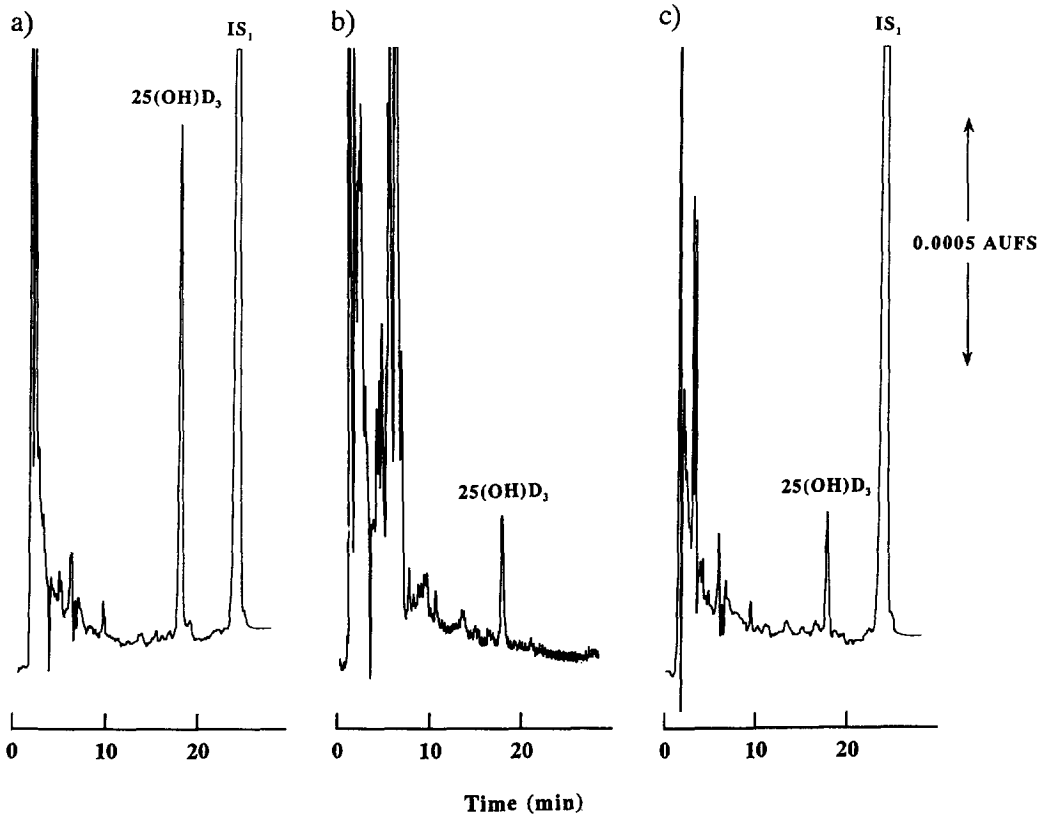

Fig. 2. Chromatograms of 25(OH)D_3. (a) Authentic samples; (b) from human plasma; (c) from human plasma containing I.S. Conditions: column, J'sphere ODS-H80; mobile phase, MeCN–H_2O (7:3, v/v); flow-rate, 1 ml/min; temperature, 40°C; detection, UV (265 nm).

graph for the determination of 25(OH)D_3. The range of the calibration graph and the quantitation limit were 5–40 and 5 ng/ml, respectively.

After adding the standard sample (0, 5 and 10 ng) to four different plasma specimens (each 0.5 ml), the concentrations of 25(OH)D_3 were determined using the proposed method. The intra- and inter-assay parameters that were calculated from these data are summarized in Table 2. Satisfactory recovery rates were obtained with an R.S.D. of less than 9%. These data showed that the proposed method is independent of the plasma used and its accuracy and precision are satisfactory.

Table 1
Absolute recovery of 25(OH)D_3 and I.S.$_1$

	Added[a] (ng)	Recovery[b] (%)	n
25(OH)D_3	5	52.0±0.3 (0.6)	3
	20	59.3±4.2 (7.1)	3
IS$_1$	300	55.2±3.3 (6.0)	5

[a] To 7% BSA in 0.4 *M* potassium phosphate buffer, pH 7.5 (0.5ml).
[b] Mean±S.D. [R.S.D. (%)].

3.2. Correlation between the concentration of 25(OH)D_3 and that of 25(OH)$D_3$3S

The concentrations of 25(OH)D_3 and 25(OH)$D_3$3S in plasma from healthy subjects (n=25) and from patients with chronic renal failure (n=12) have been determined using the proposed method and the previously reported one [4], respectively, and the results are summarized in Fig. 3. Almost equal amounts of both compounds (sulfate, 15.0±3.8 ng/ml; free form, 14.0±4.3 ng/ml) are detected in the plasma of healthy subjects, however, in two of the subjects, the amount of the sulfate form

Table 2
Accuracy and precision of the determination of $25(OH)D_3$

Sample	Concentration (ng/ml)		Recovery[b] (%)	Intra-assay R.S.D. (%)	Inter-assay[c] R.S.D. (%)
	Added	Observed[a]			
1	0	16.8±0.6		3.4	
2	0	18.7±1.0		5.5	
3	0	18.8±0.8		4.4	
4	0	15.5±0.4		2.7	
1	10	27.4±0.7	106.5±7.2	6.8	
2	10	27.7±0.7	90.5±7.2	8.0	
3	10	28.3±0.6	94.8±5.9	6.2	
4	10	26.1±0.5	106.3±4.7	4.4	8.2
1	20	37.1±0.9	101.7±4.9	4.5	
2	20	37.9±1.5	96.0±7.8	8.1	
3	20	39.0±3.5	93.6±8.4	9.0	
4	20	36.6±1.0	105.5±4.8	4.5	5.4

[a] Mean±S.D., $n=5$.
[b] Net recovery.
[c] R.S.D. of recovery rates of samples 1–4.

was found to be twice as high as that of the free form. In contrast, the free form is predominant in the plasma of the patients, and the sulfate was not detected in four patients.

4. Discussion

The determination of $25(OH)D_3$ using HPLC was usually performed using a radioactive tracer as an I.S. and/or a normal-phase column [2,5,6], which is not always sufficiently versatile for routine analysis. Our proposed method using the non-radioactive I.S. and reversed-phase HPLC overcomes these problems and is satisfactory in its accuracy and precision.

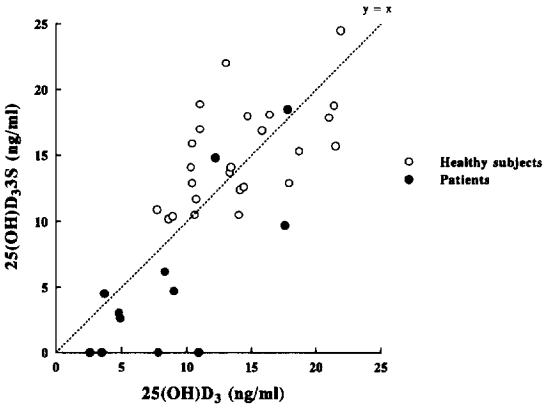

Fig. 3. Correlation between the concentration of $25(OH)D_3$ and $25(OH)D_33S$ in plasma from healthy subjects and from patients with chronic renal failure.

The determination of $25(OH)D_3$ and $25(OH)D_33S$ in human plasma was done with the newly developed and previously reported [4] methods, respectively. Nearly equal amounts of $25(OH)D_3$ and $25(OH)D_33S$ were detected in the plasma of healthy subjects, which is consistent with the previous data [6]. In contrast, the free form was predominant in the plasma of the patients with chronic renal failure. This is the first report of the determination of these metabolites in the plasma of patients with the disease. Although the biological role of sulfated $25(OH)D_3$ is not known, the non-toxic sulfate could be a storage form of biologically active metabolites of D_3, as previously suggested [5,6].

The biosynthesis, metabolism and biological significance of conjugated metabolites of D_3, including the sulfate and glucuronide, remain to be determined.

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References

[1] K. Shimada and N. Kobayashi, Trends Anal. Chem., 10 (1991) 103.
[2] M. Shimizu, Y. Iwasaki, H. Ishida and S. Yamada, J. Chromatogr. B, 672 (1995) 63.
[3] K. Shimada, K. Mitamura, M. Mukouyama, T. Okumura and K. Sugaya, J. Chromatogr. Sci., 33 (1995) 82.
[4] K. Shimada, K. Mitamura and N. Kitama, Biomed. Chromatogr., 9 (1995) 229.
[5] M. Axelson, FEBS Lett., 191 (1985) 171.
[6] M. Axelson and N.J. Christensen, J. Steroid Biochem., 31 (1988) 35.
[7] M. Tsuji, S. Yokoyama and Y. Tachibana, Bull. Chem. Soc. Jpn., 62 (1989) 3132.
[8] K. Shimada and T. Mizuguchi, J. Chromatogr., 606 (1992) 133.