Assay for Plasma 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3 by “High-Performance” Liquid Chromatography

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We describe a new, rapid, and simple column-chromatographic procedure for 25-OH vitamin D3 in plasma. The vitamin is extracted by use of Sep-pak C18 (octadecyl alkylated silicic acid), a short factory-packed reversed-phase column, and 25-OH vitamin D2 + 25-OH vitamin D3 fraction is eluted with methanol/water. The 25-OH D2 and 25-OH D3 are then well resolved on a high-resolution 3-μm silicic acid straight-phase liquid-chromatography column. The peaks are quantified against a 25-OH D3 standard by ultraviolet absorbance. Recovery was assessed by use of tritiated 25-OH D3. The within-assay coefficient of variation of the method was 5% and recovery 93%. The method was evaluated with 28 samples from control subjects and 17 samples from patients, seven with liver disease and 10 who had undergone ileo-ileostomy for hypercholesterolemia. The normal seasonal variation was observed for 25-OH D3 concentrations, and they correlated negatively and significantly with those of 25-OH D2. Post-ileo-ileostomy concentrations of 25-OH D3 in plasma were generally similar to those in normal individuals for the same season (winter), but 25-OH D2 concentrations were insignificantly lower. The patients with chronic liver disease had significantly lower 25-OH D3 concentrations than normal persons but higher 25-OH D2 concentrations, with a significantly higher 25-OH D2/25-OH D3 ratio, indicating poor storage of vitamin D3.

Additional Keyphrases: primary biliary cirrhosis · ileo-ileostomy · liver disease · seasonal and other influences · reference intervals · urgent assays · GC/MS

Concentrations of 25-hydroxyvitamin D2 (25-OH D2) and 25-hydroxyvitamin D3 (25-OH D3) in plasma are important indices of vitamin D status in health and disease 1. Although both vitamin D2 (D2) and vitamin D3 (D3) may be absorbed from food of plant and animal origin, D3 may also be made in the skin by photo-rearrangement of 7-dehydrocholesterol of metabolic or dietary origin.

Many competitive protein binding assays for 25-OH D2 and 25-OH D3 have been described (e.g., 1–3) but, as with other methods based on this principle, absolute values may not be reliably measured (4).

Liquid-chromatographic assays for these metabolites, either together or separately, have also been developed (e.g., 5–8). This approach measures absolute concentrations more reliably, but the extraction and prechromatography steps present problems in that interfering substances may be introduced or persist.

In this paper we describe a new, rapid column extraction with rapid, reliable prechromatography on the same prepacked short column. With this procedure, urgent samples can be assayed within 2.5 h in as little as 0.5 mL of plasma. Peaks for 25-OH D2 and 25-OH D3 are well resolved in straight-phase high-performance liquid chromatography (HPLC).

Materials and Methods

“Pro-analysis” grade hexane and methanol were obtained from Merck, Darmstadt, F.R.G. Isopropanol was supplied by Orion-Yhtymä Oy, Helsinki, Finland. Philips-Duphar B.V., Amsterdam, Holland, supplied the 25-OH D3 standard. [23,24(n)-3H]25-OH D3, specific activity 102 kCi/mol; [1α,2α(n)-3H]D3, specific activity 16 kCi/mol; [23,24(n)-3H]24,25-(OH)2D3, specific activity 62 kCi/mol; and [23,24(n)-3H]1,25-(OH)2 D3, specific activity 82 kCi/mol, were from the Radiochemical Centre, Amersham, England. Sep-pak C18-bonded silica columns, one Model M-45 solvent-delivery system, and one 254-nm fixed-wavelength ultraviolet absorbance detector, Model 440, were from Waters Associates, Milford, MA 01757. A zero dead-volume loop injector, Model 7125, was from Rheodyne, Inc., Cotati, CA 94710. A 25 cm × 5 mm silicic acid HPLC column packed with 3-μm particles was supplied by Kaukomarkkinat Oy, Espoo, Finland, as was a similar size column packed with C18-bonded 3-μm silicic acid particles. Both columns were from Shandon Southern, Runcorn, Cheshire, England. A Scintag SW 9120 chart recorder was from Scientific Computer and Instruments AG, Switzerland. The automated Decem liquid scintillation counter came from Wallac Oy, Turku, Finland.

Plasma Samples

Blood samples from ostensibly normal adults (12 men, 14 women, ages 17–54 years, with no history of vitamin D supplementation or diseases related to calcium or vitamin D metabolism) were collected into heparinized tubes and the plasma was separated and stored at −20 °C.

Samples were also obtained from six male and from four female patients (ages 32 to 58 years) who had undergone ileo-ileostomy as treatment for hypercholesterolemia. Further samples were obtained from seven patients (six women and one man) with chronic liver disease. Three had chronic active hepatitis and four primary biliary cirrhosis.

In one normal subject (J.T.D.) a 25-OH D2 dose–response study was performed by dosing with 150 μg of D2 for 16 days in November. Plasma samples were obtained before, during, and for some weeks after stopping D2 dosage.

Application of Samples to the Sep-pak Column and Elution of Fractions

We used 0.5- to 3-mL plasma samples. The capability of the Sep-pak C18 column to adsorb individual tritiated vitamin D3 metabolites differing in polarity was determined under different conditions of dilution with water, at different pH values, and at different methanol concentrations.

To provide a fraction pure enough for measurement by HPLC, we performed experiments using different concentrations of methanol in water to define conditions in which the polar metabolites—and therefore many other possible interfering substances—would be washed away from the Sep-pak column before elution of the (25-OH D2 + D3) fraction.
Likewise, conditions were sought in which vitamin D₃ would remain on the column together with substances of similar polarity, which therefore would not interfere in the later ultraviolet absorption measurements.

Liquid Chromatography

To obtain maximum sensitivity, columns of excellent resolving power were chosen. Thus both the straight-phase silicic acid column and the reversed-phase octadecyl bonded silicic acid column were packed with 3-μm particles. The detection limit for 25-OH D₃ standard at retention volumes similar to that finally used (about 16.5 mL) in the straight-phase column was about 1 pmol. The solvent flow rate generally used in the straight-phase chromatography was 1.5 mL/min. In the reversed-phase studies it was 1.2 mL/min. Pumping pressures in straight-phase runs were about 8.28 MPa (1200 psi) and in the reversed-phase about 22.1 MPa (3200 psi).

Samples were injected dissolved in 5 to 10 μL of eluent (hexane/isopropanol or methanol/water) and elution was followed on a pen recorder taking its input from the ultraviolet absorption detector set to give a full-scale deflection on the recorder equivalent to 0.002 A. Typical peak heights from 17.5 pmol standard were 9 to 10 cm (noise level <0.2 cm).

Gas Chromatography–Mass Spectrometry

For gas chromatography–mass spectrometry we used a Hewlett-Packard 5992 GC/MS system equipped with a 25-m methyl silicone fused-silica capillary column (OV-101, Carbowax deactivated) with an inner diameter of 0.31-0.32 mm. The carrier gas was helium (2 mL/min) and the selected-ion monitoring was carried out by using a temperature program. The temperature was kept at 60 °C for 1 min, then increased by 12 °C/min to 275 °C. The electron energy was 70 eV and the multiplier voltage 2200 V. The ions monitored for 25-OH D₃ were m/e 544, 529, and 454 and those for 25 OH D₂ were m/e 556, 466, and 451. The retention time of 25-OH D₃ was 24.2 min; that of 25-OH-D₂ was 25.1 min. Silylation was performed by using 200 μL of the mixture pyridine/hexamethyl disilazane/trimethylchlorosilazane, 9/3/1 by vol.

Statistics

We used the t-test according to De Jonge to assess significances (9).

Results

Optimization of Adsorption Conditions for the Sep-pak C18 Extraction

When tritiated D₃, 25-OH D₃, 24,25-dihydroxy vitamin D₃, or 1,25-dihydroxy vitamin D₃ was dissolved in an equimolar mixture of methanol and water, it was adsorbed quantitatively onto the Sep-pak C18 column. If, however, the proportion of methanol was decreased to 30/70 by vol or increased to 60/40 by vol the adsorption to the column was poor. When plasma was used in place of water, protein precipitation made it impossible to feed samples into the column. Chloroform added in various concentrations gave the same problem.

When the pH of the plasma sample was made more acidic with concentrated HCl, proteins no longer precipitated to the extent that the column would become blocked. Alkalization of the sample with concentrated NaOH also avoided the problem of precipitation of proteins on addition of methanol but, as in the case of plasma alone or plasma diluted with water, the alkyl chains of the column were unable to compete effectively with the plasma binding proteins, so that only up to half to two-thirds of added ³H-labeled metabolites were taken onto the column, even in the presence of methanol/water 1/1 by vol.

To study the effect of pH on adsorption we diluted four parts of plasma with three of water acidified with 6 mol/L HCl and finally rendered 55% methanolic, mixed well, and immediately applied the mixture to the Sep-pak C18 column. More than 95% of the label preincubated with the plasma was adsorbed onto the column at pH 2.

If we added several micrograms of dihydrotachysterol-2 to the plasma in the incubation step, there was no improvement in the adsorption percentage.

Optimization of Elution Conditions from the Sep-pak Column

When labeled D₃ or D₂ metabolites were applied to the Sep-pak C18 column as described above, the elution patterns were such that the less polar the steroid the slower it eluted as the methanol concentration in water was reduced from 100 to 50%. After many experiments we adopted the following system: The polar dihydroxylated metabolites, 1,25-(OH)₂ D₃ and 24,25(OH)₂ D₃, were eluted into a first fraction with methanol/water (69/31 by vol). The 25-(OH) D₃ containing fraction was eluted with 80/20 methanol/water, and the D₃ fraction then remained on the column. Figure 1 shows these results. Of the polar metabolites, 98% were removed in the first fraction, and 82% of the 25-(OH) D₃ was collected in the second fraction, whilst 8% of it passed into the first fraction. Vitamin D₃ itself comprises 5.2% in the second fraction.

Liquid Chromatography

Typical chromatograms are shown in Figure 2. The conditions we used were: silicic acid column (see Materials); eluent, hexane/isopropanol (1000/24); flow rate, 1.5 mL/min; paper speed, 0.5 cm/min; and sensitivity, 2 nA full-scale. The peak labeled "25-OH D₃" (retention time ~11.4 min) corresponded to the labeled metabolite initially added to the plasma, and by collecting the peak in fractions it was possible to show that the peaks of ultraviolet absorption and radioactivity coincided. The retention time for this peak was also identical with
is barely detectable, but after the subject receives 16 days of dosage with 150 \( \mu \text{g} \) of D\(_2\) per day it has grown to be much bigger than the 25-OH D\(_3\) peak. Further, 19 days and 29 days after cessation of dosage with D\(_2\) the peak shows a decline. In addition to this there is suppression of the 25-OH D\(_3\) peak.

The peak was quantified in proportion to the 25-OH D\(_3\) peak height per picomole at the same retention time.

The peak was also studied in a reversed-phase system, collected from that system, and restudied in the straight-phase system just described. The conditions in the reversed-phase were: C18 bonded silicic acid column (see Materials); eluent, methanol/water 85/15 (by vol); flow rate, 1.2 mL/min; paper speed, 0.25 cm/min; sensitivity, 2 mA full scale. The 25-OH D\(_2\) peak appeared after and close to the 25-OH D\(_3\) peak in this system. 25-OH D\(_2\) appeared at 13.5 mL (retention time, 11.3 min) and 25-OH D\(_3\) at 14.4 mL (retention time, 12 min). The same dose–response characteristics were obtained as in the straight-phase studies of D\(_2\)-dosed subject J.T.D.

When eluate corresponding to the peak was collected and re-injected into the straight-phase system it eluted coincidently with the 25-OH D\(_2\) peak independently identified in that system.

Additional evidence for the identity of the 25-OH D\(_2\) peak was that there was no other candidate peak showing a dose–response in the chromatogram. A patient receiving D\(_2\) for subclinical rickets showed the same peak, showing the same dose–response characteristic as in the normal subject.

To be still more certain of the identity of the 25-OH D\(_2\), we did select ion monitoring on silylated material collected in straight-phase HPLC from plasma of the subject who had been dosed with vitamin D\(_2\). The presence of 25-OH D\(_2\) was demonstrated by monitoring the molecular ion (M\(^+\)) similarly to a previous report (10) and peaks at M-90 and M-(90 + 15). The molecular ion and the two fragments occurred in the same relative intensity as found for 25-OH D\(_3\), suggesting identity of the steroid with 25-OH D\(_2\).

Scheme Finally Chosen for Routine Measurement

Half to three milliliters of plasma is diluted to 3 mL with distilled water, \([^{3}H]25\text{-OH} D_{3}\) (\(-\text{2000 cpm}\)) is added and mixed in. Four milliliters of water (adjusted to pH 1.0 with HCl) is added (giving pH 2.0), followed by 8.5 mL of methanol (final concn 5%). After mixing, the sample is aspirated slowly (e.g., 2 mL/min) through a Sep-pak C18 column and the effluent is discarded. The Sep-pak is washed with 30 mL of 69/31 methanol/water, then a 20-mL fraction containing 25-OH D\(_2\) + 25-OH D\(_3\) is eluted with 80/20 methanol/water, collected, and diluted with 12 mL of distilled water (to adjust back to 55% methanol). The column is quickly washed with 10 mL of methanol and then with 10 mL of distilled water. The fraction is then reapplyed to the column and, after washing with 0.5 mL of methanol, a 3-mL methanol fraction (containing the 25-OH D\(_2\) and 25-OH D\(_3\) metabolites) is collected and evaporated under nitrogen. The sample is dissolved in 25 \( \mu \text{L} \) of hexane/isopropanol (1000/24) and the metabolite concentrations are measured in straight-phase HPLC on a 25 cm \( \times \) 0.5 cm silicic acid column under the conditions given in Methods. The 25-OH D\(_3\) peak is collected and its radioactivity counted to give recovery. The results are calculated from peak height and standard peak height per picomole. For 25-OH D\(_2\) in the system we used, the sample peak height is first multiplied by 0.75 (to correct for the earlier elution of 25-OH D\(_3\)). This factor must be determined for each chromatographic system, or a 25-OH D\(_2\) standard must be used. The factor is obtained by injecting a known quantity of 25-OH D\(_3\) (a) under the usual running conditions and (b) with slightly more isopropanol in the eluent so that its retention volume is smaller and equal to that for 25-OH D\(_2\) under the usual running con-
conditions. The factor is then peak height under the usual conditions divided by peak height under the new conditions.

The within-assay coefficient of variation for measurement of 25-OH D$_2$ and 25-OH D$_3$ was 5% (n = 10). When small peaks <2 cm high were included this increased to 8% (n = 12). Accuracy, as assessed by adding from 0 to 300 nmol of 25-OH D$_2$ to 10 plasma samples and assaying, was 7% with precision (CV) 5%.

Concentrations of Metabolites in Plasma

Control subjects. Values obtained with the method for normal men and women are shown in Table 1. The month of sampling, age, sex, diagnosis, and 25-OH D$_2$ and 25-OH D$_3$ values are given.

The values for 25-OH D$_3$ in winter were significantly lower than in summer (p <0.001) and the values for 25-OH D$_2$ in winter were significantly higher than in summer (p <0.02). Also, the ratio 25-OH D$_2$/25-OH D$_3$ was significantly lower in summer (0.042) than in winter (0.10), p <0.01.

Patients. The mean concentrations and range for 25-OH D$_3$ (in nmol/L) and for 25-OH D$_2$ (in nmol/L) and the ratio of the latter to the former in the patients having had an ileo-ileostomy were 25.6 (12-38), 3.3 (<1 to 10) and 0.17 (0.015 to 0.39), respectively. 25-OH D$_2$ values tended to increase as 25-OH D$_3$ values decreased but, unlike this trend in the control subjects, not significantly. Values for 25-OH D$_2$ did not differ significantly from those for the control subjects for the winter period, and although 25-OH D$_2$ concentrations were lower than in the winter for control subjects, the difference did not reach statistical significance at the 95% confidence level. The ratios of metabolites also did not differ significantly from those in normals (Table 2).

In the patients with liver disease the mean (and range) for 25-OH D$_3$ and 25-OH D$_2$ (in nmol/L) were 28.9 (16-48) and 7.7 (2.7-21), respectively, and the ratio of the latter to the former was 0.28 (0.013-0.44). The correlation of 25-OH D$_2$ with 25-OH D$_3$ gave a correlation coefficient of 0.50, which was not statistically significant but was significantly different from that for the controls (p <0.01). The 25-OH D$_3$ values were significantly lower than those for the control group (p <0.001), and the mean 25-OH D$_2$ values were higher than in the controls, but this was not statistically significant. However, the mean ratio 25-OH D$_2$/25-OH D$_3$ was significantly higher than in the controls (p <0.01).

Discussion

The main advantages of the present method over earlier methods for measurement of 25-OH D$_2$ and 25-OH D$_3$ are its simplicity and rapidity. In 2.5 h a result can be available to the clinician. All previous methods have used solvent extraction usually followed by chromatography on columns that have to be made up in the laboratory, such as Sephadex LH-20 or sillicic acid. Because ultraviolet absorption detectors have become available that are both stable and very sensitive, it is possible to measure metabolites in as little as 0.5 mL of plasma or serum or less, depending on the sample concentration.
The column extraction procedure presented depends on the denaturing effect of methanol on proteins, just as in the solvent-extraction methods, and also on the solubility of vitamin D metabolites in 55/45 methanol/water. Thus the metabolites are freed into solution and taken up from solution onto the non-polar sites on the Sep-pak C18 column. The extraction efficiency is greater than 95%, only a small fraction of vitamin D or its metabolites passing through the short Sep-pak C18 column, presumably by a degree of binding to some plasma proteins. The need for acidity, pH 2, was to solubilize plasma proteins, which otherwise are precipitated by methanol in the concentrations used. Even so, the main problem appears not to be that precipitated would prevent vitamin D metabolites entering solution but simply that the column would become blocked by the proteins.

Preparatory chromatographic purification of fractions containing vitamin D metabolites may be complicated by the differing polarities of D2 and D3 metabolites. Thus separations on Sephadex LH-20 may lead, for example, to D3 metabolites eluting with D2 metabolites of a different degree of hydroxylation (11). In the present system of prechromatography on Sep-pak C18, this problem seems to have been avoided for 25-OH D2 and 25-OH D3. Even when the plate number of such a reversed-phase column is several thousand times greater than in the Sep-pak C18 columns (e.g., in reversed-phase HPLC on the 3-μm particle 25 cm × 5 mm column used in elution studies of the 25-OH vitamin D3 in this work) 25-OH D2 and 25-OH D3 eluted at nearly the same time, so that the assumption that it is possible to use one labeled metabolite to assess recovery of its pair seems justified in the case of Sep-pak C18. This is also a simplifying factor in methods aimed at measurement of both metabolites.

The possible conversion of 25-OH D3 to other forms such as isotechnysterol (12), investigated in aqueous acid over the pH range 1 to 7, was negligible. In strong aqueous acid (e.g., 6 mol/L HCl) some conversion did occur. Thus no loss of sensitivity due to loss of material occurs at pH 2.

The good and clean resolution of the peaks for 25-OH D2 and 25-OH D3 in the straight-phase HPLC is due to two factors: (a) the column we used was of good resolving power due to the smallness of its particles (3 μm), and (b) the prechromatography is in the reversed-phase while the HPLC is in the straight-phase. Thus, although the Sep-pak C18 is short and has few theoretical plates it allows the washing away of substances of higher polarity and the selection of a narrow polarity spectrum containing the 25-hydroxylated metabolites, whilst the straight-phase HPLC washes through substances of lower polarity quickly and by adjustment of conditions the resolving power of the column is exercised optimally in the elution zone of these metabolites.

In the control subjects the mean values during winter and summer for 25-OH D3 were different, as expected (13, 14). Although the same was true at a lower level of significance for 25-OH D2, the pattern was reversed, with higher values in winter than in summer. This has been interpreted to mean that dietary vitamin D3 is of somewhat greater importance in winter than in summer when D2 is much more freely available. This interpretation is also supported by our metabolic study of a normal volunteer (Figure 3), which shows that 25-hydroxylation of vitamin D2 depends on its availability relative to vitamin D3. A similar argument applies for D3. The significant negative correlation between 25-OH D2 and 25-OH D3 confirms this in the normals.

In the patients who had undergone an ileo-ileostomy operation for hypercholesterolemia and from whom samples had been taken in the winter period only, the values for the metabolites were similar to those in normals for the winter period, even though there was a tendency for the 25-OH D3 concentrations to be lower in the patients, perhaps suggesting malabsorption (13, 15). The results, however, suggest that vitamin
D 25-hydroxylation in these patients is undisturbed.

In the patients with chronic liver disease the subnormal 25-OH D₃ and somewhat higher than normal 25-OH D₂ values suggest a storage defect leading to some compensatory hydroxylation of D₂, also reflected in a 25-OH D₂/25-OH D₃ ratio significantly higher than normal. In some cases of liver disease, however, a defect of hydroxylation has been reported (15).

The method presented thus has advantages over earlier methods of the same type in terms of speed and simplicity and gives useful information on the two metabolites and their relations in health and disease.

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References