Specific Estimation of 24,25-Dihydroxyvitamin D in Plasma by Gas Chromatography–Mass Spectrometry

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This paper describes a specific mass-fragmentographic method, involving a stable-isotope-labeled internal standard, for measurement of 24,25-dihydroxyvitamin D in human plasma. Vitamin D metabolites were rapidly extracted from plasma by using Sep-Pak C18 cartridges and separated into fractions on Sep-Pak SIL cartridges. The polar fraction, containing the dihydroxylated metabolites, was further purified by "high-performance" liquid chromatography on Zorbax SIL. The fraction containing 24,25-dihydroxyvitamin D was collected, evaporated, and converted to the 24:25-cyclic n-butyl boronate–3-trimethylsilyl ether derivative before analysis by gas chromatography–mass spectrometry. The intensity of the mass fragment (m/z 449, m/z 455 for the hexadecaterated internal standard) arising from the loss of one of the angular methyls and the 3-silan group ([M-90-15]+) was monitored. The minimum limit of detection for this method is about 0.1 μg/L. Inter- and intra-assay reproducibility was acceptable, and analytical recovery of added 24,25-dihydroxyvitamin D3 over the concentration range 1.0 to 5.0 μg/L was quantitative. Concentrations of 24,25-dihydroxyvitamin D3 in plasma of 21 apparently healthy volunteers were between 0.55 and 5.3 μg/L, higher values being obtained after prolonged exposure to the sun. No 24,25-dihydroxyvitamin D2 could be detected in any plasma sample examined.

Additional Keyphrases: possible reference method · effects of exposure to sunlight · vitamin D metabolites

For biological activity, vitamin D is successively hydroxylated at C25 in the liver, and at C1α, in the kidney, to produce the active calcium homeostatic hormone, 1α,25-dihydroxyvitamin D.4 As an alternative to 1α-hydroxylation of 25-hydroxyvitamin D, hydroxylation can occur at C24 in the kidney, to produce 24,25-dihydroxyvitamin D (I, 2). This 24-hydroxylation may represent merely a catabolic pathway (2) or 24,25(OH)2D may have a physiological role. Although receptors for 24,25(OH)2D have been reported in several tissues (2), whether or not 24,25(OH)2D has a physiological role is still a matter of controversy.

Various methods are now available for estimating 24,25(OH)2D in biological fluids (reviewed in 4). The most common methods are competitive protein binding assays in which vitamin D binding globulin is used; they rely on extensive HPLC separation before assay to provide adequate specificity. The kidney, however, produces several dihydroxylated vitamin D metabolites, some of which co-migrate in many of the HPLC systems used, and thus the specificity of these procedures is suspect. Although radioimmunoassays for 24,25(OH)2D have been reported (5), no details were published, and it thus is not clear precisely how specific these assays are. Two methods of 24,25(OH)2D assay involving ultraviolet absorption after HPLC have been described (6, 7), but their minimum limit of sensitivity approximates the concentrations of 24,25(OH)2D found in human plasma. Clearly, many of the published methods for 24,25(OH)2D in human plasma lack specificity, although to resolve the question of whether 24,25(OH)2D has a physiological role requires specific methods for its measurement. Gas chromatography–mass spectrometry would appear to be suitable, and preliminary studies with use of radiolabeled internal standards have been reported (8). Here we report a more specific and sensitive method for 24,25(OH)2D in human plasma. Hexadeuterated 24,25(OH)2D3 is the internal standard. This method could be considered as a definitive reference against which other assays might be evaluated.

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4 Systematic and trivial names of vitamin D and its metabolites used in this paper are as follows: vitamin D2 (9,10-seco-cholesta-5,7,10(19)-22-tetrahydrofuran-3α,8β-diol); D3; vitamin D3 (9,10-seco-cholesta-5,7,10(19)-triene-3β,8β-diol); D2; 25-hydroxyvitamin D; 24,25-OH; 24,25-dihydroxyvitamin D; 24,25(OH)2D; and 25,26-dihydroxyvitamin D; 25,26(OH)2D. The term D is used when there is no need to distinguish between D2 and D3. Other nonstandard abbreviations used in this paper are: "high-performance" liquid chromatography: HPLC; gas chromatography: GC; mass spectrometry: MS; bis(tri-methylsilyl)trifluoroacetamide: BSTFA; trimethylsilyl: TMSi; and n-butyldiboronate: nBBA. Structures and interrelationships of some of the steroidal derivatives mentioned in the text are given in Figure 1.

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No standard 24,25(OH)2D2 was available, and thus it has not been possible to measure this metabolite.

Materials and Methods

Materials

24R,25-Dihydroxy[26,27-methyl-2H]vitamin D3 (specific activity: 158 kCi/mol) was obtained from Amersham International plc (Amersham, Bucks., U.K.). 24,25-Dihydroxy[26,27-2H]vitamin D3 was synthesized as described previously (9). Standard 24,25-dihydroxyvitamin D3 was synthesized (9) or was a generous gift from Dr. P. St. John-Smith (Roche Products Ltd., Welwyn Garden City, Herts., U.K.). Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ) kindly provided standard 25,26(0H)2D3. Labeled and unlabeled standards were purified by HPLC on receipt and re-purified monthly thereafter. Concentrations of 5,7-diene steroids were determined from their ultraviolet absorbance at 264 nm, a molar absorptivity at this wavelength of 18 300 being assumed (10). Extraction solvents (55 grade wherever possible, from BDH Chemicals Ltd., Poole, Dorset, U.K.) and HPLC solvents (Rathburn Chemicals Ltd., Peebleshire, Scotland, U.K.) were re-distilled before use unless otherwise specified. Acetonitrile was shaken with activated charcoal (Sigma London Chemical Co., Ltd., Poole, Dorset, U.K.) and filtered before use. Sep-Pak C18 and Sep-Pak SiL cartridges were purchased from Waters Associates, Northwich, Cheshire, U.K., and were used as described previously (11). We found it necessary to wash each cartridge two or three times before use with the most polar eluting solvent. n-Butylboronic acid and bis(trimethylsilyl)trifluoracetamide (BSTFA) (Fierce & Warriner (UK) Ltd., Cheshire, U.K.) were used without further purification.

HPLC was as described previously (12, 13) with use of a Model 7500A pump (Applied Chromatography Systems Ltd., Luton, Beds., U.K.), a Rhodyne 7125 injection valve, and a Model SPF70 (Schoeffel Instruments) variable-wavelength detector. A straight-phase Zorbax-SiL column (10 μm particle size, 250 x 4.7 mm; HPLC Technology Ltd., Macclesfield, Cheshire, U.K.) was eluted with isopropanol/methanol/hexane solvent, as described in the Methods section.

Mass-fragmentography was done as previously described (8) with a 2901 gas chromatograph–mass spectrometer (LKB Instruments Ltd., Croydon, Surrey, U.K.). Silanized glass GC columns, 2.5 m x 0.5 cm (i.d.) were packed with 2% OV1 on dimethylidichlorosilane-treated, acid-washed Celite 545, 100/120 mesh. The flow rate was 25 mL of helium per minute. The oven temperature was 300 °C.

All glassware was silanized by soaking overnight in a 10 mL/L solution of dimethylidichlorosilane in toluene and washed with methanol.

Blood was collected from apparently healthy laboratory workers into heparinized containers, and the plasma was separated. Unless analyzed without delay, plasma was stored at −20 °C under nitrogen.

For liquid scintillation counting we used a Model SL3000 (Kontron Intertechnique, St. Alban's, Herts., U.K.). We used 5 mL of NE225 liquid-scintillation fluid, collecting at least 10 000 counts per sample. Counting efficiency in this system was about 40%, with a background count rate of around 0.5 counts per second.

Methods

Sample extraction: Approximately 100 ng of [2H3]24,25(OH)2D3 was added to 5 mL of plasma, and the mixture was incubated at room temperature for 1 h. The plasma was then extracted with an equal volume of acetonitrile and centrifuged (10 000 x g, 10 min). The pellet was discarded and 2.5 mL of acetate buffer (0.2 mol/L, pH 5.6) was added to the supernatant fluid and applied to a Sep-Pak C18 cartridge that had been prewashed once with 10 mL of water and three times with 10-mL portions of methanol. The Sep-Pak cartridge was washed with 3 mL of methanol: water (60/40 by vol) and the vitamin D metabolites were eluted with 4 mL of acetonitrile.

HPLC purification: The acetonitrile extract was dried under a gentle stream of nitrogen, at 40 °C. The residue was dissolved in a 1/99 (by vol) mixture of isopropanol and petroleum ether (bp 80–100 °C) with use of two 0.2-mL portions, followed by 1.6 mL. The solution was applied at 4 °C to a Sep-Pak SiL cartridge pre-washed with the same solvent. The cartridge was successfully eluted at 4 °C with 13 mL of isopropanol/petroleum ether (1/99, by vol), to remove nonpolar metabolites and 10 mL of isopropanol/light petroleum ether (3/97, by vol), to remove 25-OHD, and finally with 10 mL of isopropanol/petroleum ether (30/70, by vol), to elute dihydroxylated vitamin D metabolites. This fraction contained 24,25(OH)2D3. The solvent was evaporated away at 40 °C in a vacuum oven, and the residue was dissolved in 100 μL of the HPLC mobile phase (isopropanol/methanol/hexane, 13/1/86 by vol) and injected onto a Zorbax SiL HPLC column that had been previously equilibrated with mobile phase. With a flow rate of 2 mL/min, the retention time of standard 24,25(OH)2D3 is about 4.5 min—this was checked at the beginning and end of each run. The fraction eluting between 4 and 5 min was collected and evaporated at 40 °C in a vacuum oven.

Derivatization and assay: The dried extracts and a series of standards [100 ng of deuterated 24,25(OH)2D3 + 0, 1, 3, 8, and 15 ng of 24,25(OH)2D3] were converted into 3,24,25-n-butylboronate cyclic ester–3-trimethylsilyl ether derivatives, ready for gas chromatography–mass spectrometry (GC-MS). These derivatives were formed by incubating with 40 μL of n-butylboronic acid in tetrahydrofuran (1 mg/mL) for 30 min at room temperature. Excess reagent was evaporated away in a vacuum oven at 40 °C, and the residue was redissolved in 50 μL of BSTFA. After about 30 min, 5–10 μL was injected into the GC-MS. We monitored the m/z 449 peak (m/z 455 for the deuterated standard), which is the base peak of the mass spectrum and represents the fragment (M-90-15)+. The peak heights of the pyro-isomer of 24,25(OH)2D3 and the deuterated internal standard were measured and, after correcting for the different amplification of each channel, a ratio of the peak height of 24,25(OH)2D3 to the peak height of [2H3]24,25(OH)2D3 was calculated. The same process was used for the standards, and the peak height ratio was plotted vs the amount of non-deuterated standard (Figure 2). Using this standard curve, we could convert peak height ratios for the samples to the mass of 24,25(OH)2D3 in each sample.

Results

Gas chromatography–mass spectrometry: A previous publication (8) reported use of trimethylsilyl ethers of the isochromanol isomer of 24,25(OH)2D3 for GC-MS. At that time, no standard 25,25(OH)2D3 was available to us, and we suggested that these two metabolites were separated in the GC system used. However, when 25,26(OH)2D3 became available, we found that the trimethylsilyl ethers of 24,25(OH)2D3 and 25,26(OH)2D3 ran very close to each other. Table 1 gives the GC retention times of trimethylsilyl ethers and n-BBA-TMSi derivatives of 24,25(OH)2D3, 25,26(OH)2D3, and their isochromanol isomers. On non-selective GC stationary phases such as OV1, the D3 metabolites have longer retention times than do the equivalent D3
metabolites. Use of cyclic boronates, which form derivatives only with compounds having two hydroxyls in close proximity, not only improved the specificity of this method but also gave well-separated cyclic n-butyl boronate-3trimethylsilyl (nBBA-TMSI) ether derivatives of 25,26(OH)\textsubscript{2}D\textsubscript{3} and 24,25(OH)\textsubscript{2}D\textsubscript{3}. A mass spectrum of the nBBA-TMSI derivative from standard 24,25(OH)\textsubscript{2}D\textsubscript{3} pyro-isomer is illustrated in Figure 3. The largest peak in this spectrum is the ion of m/z 449, which represents (M-90-15\textsuperscript{+}). In contrast to the behavior of TMSI ethers, where formation of isotachysterol isomers increased the intensity of the molecular ion (8), formation of the isotachysterol isomer of 24,25(OH)\textsubscript{2}D\textsubscript{3}--nBBA-TMSI significantly decreased the intensity of the ions of high m/z seen in the spectrum of the pyro-isomer. The intensity of the ion m/z 253, for example, which represents (M-90-C\textsubscript{8} side chain\textsuperscript{+}), was greatly increased in the spectrum from the isotachysterol isomer. However, this

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Table 1. Gas-Chromatographic Retention Times of Various Derivatives of 24,25(OH)\textsubscript{2}D\textsubscript{3} and 25,26(OH)\textsubscript{2}D\textsubscript{3}

<table>
<thead>
<tr>
<th>Derivative\textsuperscript{a}</th>
<th>Isomer\textsuperscript{b}</th>
<th>Retention time\textsuperscript{c} (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24,25(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>Pyro-</td>
<td>2.5208</td>
</tr>
<tr>
<td>Tri-TMSI</td>
<td>isoPyro-</td>
<td>3.4028</td>
</tr>
<tr>
<td>3-TMSI-nBBA</td>
<td>isoT</td>
<td>3.2402</td>
</tr>
<tr>
<td>3-TMSI-nBBA</td>
<td>isoT</td>
<td>3.2402</td>
</tr>
<tr>
<td>3-TMSI-nBBA</td>
<td>isoT</td>
<td>3.2402</td>
</tr>
<tr>
<td>25,26(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>Pyro-</td>
<td>2.5342</td>
</tr>
<tr>
<td>Tri-TMSI</td>
<td>isoPyro-</td>
<td>3.4178</td>
</tr>
<tr>
<td>Tri-TMSI</td>
<td>isoT</td>
<td>3.3611</td>
</tr>
<tr>
<td>3-TMSI-nBBA</td>
<td>isoT</td>
<td>3.2962</td>
</tr>
<tr>
<td>3-TMSI-nBBA</td>
<td>isoT</td>
<td>3.2962</td>
</tr>
<tr>
<td>3-TMSI-nBBA</td>
<td>isoT</td>
<td>3.2962</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Tri-TMSI: 3,24,25-trimethylsilyl ether; 3-TMSI-nBBA: 3-trimethylsilyl ether-24,25-cyclic n-butyl boronate ester.

\textsuperscript{b}Pyro-: the pyro-isomer, isoPyro-: the isopyro-isomer, isoT: isotachysterol.

\textsuperscript{c}Relative to that of the pyro- peak of vitamin D\textsubscript{2}-3-TMSI (approx. 1.8 min).

Samples were run on OV1 columns at 300 °C as described in text.

Fig. 2. Mass-fragmentography standard curve relating peak height ratio (peak height of 24,25(OH)\textsubscript{2}D\textsubscript{3} [m/z 449]; peak height of [\textsuperscript{2}H\textsubscript{6}]24,25(OH)\textsubscript{2}D\textsubscript{3} [m/z 455]) and mass of standard 24,25(OH)\textsubscript{2}D\textsubscript{3}, after correction of peak heights for differences in amplification. Each point represents the mean (\#) and SD (bars) of five separate experiments. The equation of the regression line is y = 0.01x + 0.005 (r = 0.99967, p < 0.001)

Fig. 3. Mass spectrum of the pyro-isomer of 24,25(OH)\textsubscript{2}D\textsubscript{3}--nBBA-TMSI, normalized with respect to the base peak (m/z 449, 100%) Peaks with intensities less than 5% of the base peak are not recorded. The nBBA-TMSI derivative was run in the GC-MS and the emerging GC peak was scanned from m/z 200 to 600 at the maximum point intensity at m/z 253 could not be used for mass-fragmentography, because the internal standard [\textsuperscript{2}H\textsubscript{6}]24,25(OH)\textsubscript{2}D\textsubscript{3} loses the deuterium label that is located on C26 and C27. Studies on these derivatives, to determine the relative intensities of these ions during mass fragmentography, are summarized in Figure 4. The ions at m/z 449, 464 ([M-90]+), and 554 (M\textsuperscript{+}) from the isotachysterol-nBBA-TMSI isomer were all of similar intensity but were not as intense as the (M-90-15)+ ion from the pyro-nBBA-TMSI isomer. We therefore decided that, to preserve reasonable sensitivity while retaining the deuterated side chain, pyro-isomers would have to be used and the ion at m/z 449 (m/z 455 for the deuterated internal standard) would be used for mass-fragmentography. Use of this ion gives enhanced sensitivity over that of the previous procedure (8) where the m/z 632 (the molecular ion) of 24,25(OH)\textsubscript{2}--isotachysterol--TMSI was monitored. Typical mass-fragmentograms from two normal plasma extracts are illustrated in Figure 5 together with the fragmentogram from standard [\textsuperscript{2}H\textsubscript{6}]24,25(OH)\textsubscript{2}D\textsubscript{3}.
Table 2. Reproducibility Studies

<table>
<thead>
<tr>
<th>Mean value, µg/L</th>
<th>SD</th>
<th>CV, %</th>
<th>No. assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-batch precision</td>
<td>1.46</td>
<td>0.08</td>
<td>5.6%</td>
</tr>
<tr>
<td>0.59</td>
<td>0.05</td>
<td>8.7%</td>
<td>5</td>
</tr>
<tr>
<td>Between-batch precision*</td>
<td>2.58</td>
<td>0.20</td>
<td>7.8%</td>
</tr>
<tr>
<td>0.40</td>
<td>0.08</td>
<td>20.0%</td>
<td>5</td>
</tr>
</tbody>
</table>

*Samples were stored at −20 °C and analyzed over a period of six weeks.

Table 3. Analytical Recoveries of Standard 24,25(OH)₂D₃ Added to Plasma

<table>
<thead>
<tr>
<th>Added µg/L</th>
<th>Recovered (mean ± SD)</th>
<th>% recovery (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.01 ± 0.12</td>
<td>101 ± 12</td>
</tr>
<tr>
<td>1.5</td>
<td>1.52 ± 0.17</td>
<td>101 ± 11</td>
</tr>
<tr>
<td>3.0</td>
<td>2.99 ± 0.29</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>5.0</td>
<td>4.98 ± 0.20</td>
<td>100 ± 4</td>
</tr>
</tbody>
</table>

Table 4. Concentration of 24,25(OH)₂D₃ in Plasma of 30 Apparently Normal Individuals

<table>
<thead>
<tr>
<th>Mean (and SD)</th>
<th>Range</th>
<th>Month plasma collected</th>
<th>No. plasmas</th>
</tr>
</thead>
<tbody>
<tr>
<td>24,25(OH)₂D₃ µg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 1.61 (0.60)</td>
<td>0.55–2.83</td>
<td>June to October</td>
<td>17</td>
</tr>
<tr>
<td>2. 1.33 (0.42)</td>
<td>0.55–2.07</td>
<td>June to July</td>
<td>11*</td>
</tr>
<tr>
<td>3. 2.13 (0.53)</td>
<td>1.51–2.83</td>
<td>September to October</td>
<td>6*</td>
</tr>
<tr>
<td>4. 3.17 (1.29)</td>
<td>1.32–5.73</td>
<td>December to January</td>
<td>9*</td>
</tr>
<tr>
<td>5. 4.82 (0.46)</td>
<td>4.10–5.39</td>
<td>September to October</td>
<td>4*</td>
</tr>
</tbody>
</table>

*These values were obtained by subdividing data in line 1 into those values obtained from June to July (line 2) and those obtained from September to October (line 3).

Values from Australia; all other samples collected in the U.K.

These four individuals had spent periods (two to three weeks) in the sun while on holiday outside the U.K. and are not included in lines 1–5.

The mean value in line 2 is significantly different from the mean value in line 3 (p < 0.005).

Normal values: Plasma was obtained during July to November from normal, apparently healthy laboratory staff and medical students. None were taking any vitamin D supplementation. In four cases, plasma was obtained from staff who had recently returned from summer holidays in the sun (three weeks in Barbados, or two weeks in Southern France or Spain). Nine plasma samples, collected during January in Australia from individuals who had maximum exposure to the sun, were kindly provided by Professor S. Posen and Miss Dianne Lissner (Royal North Shore Hospital, New South Wales). All plasma samples were analyzed in duplicate and the mean values are recorded in Table 4.

Specificity: Metabolites of vitamin D that reportedly interfere with competitive protein binding assays for 24,25(OH)₂D₃ are 25,26(OH)₂D₂ and 25-OHD₃-26,23-lactone. Both of these co-migrate with 24,25(OH)₂D₃ in the Zorbax-SIL straight-phase HPLC system used in most pre-assay separation procedures (14). Unfortunately, neither of these two metabolites were available and it was not possible to assess directly their effects on this assay. However, Jones (14), using a Zorbax CN HPLC system, completely separated 24,25(OH)₂D from 25-OHD₃-26,23-lactone. We therefore re-analyzed some plasma samples, particularly those with high 24,25(OH)₂D₃ concentrations, by the method described here, except we used Zorbax-CN as described by Jones (14) instead of Zorbax-SIL. Mass fragmentography of the appropriate fraction (identified by running standards) was done. We compared the results for plasmas separately assayed by using HPLC on Zorbax-CN and on Zorbax-SIL by least-squares regression analysis. The equation of the regression line was found to be: y(Zorbax-CN) = 1.091x(Zorbax-SIL) + 0.017 (n = 11; standard deviation of the slope: 0.261; standard deviation of the intercept: 0.886; correlation coefficient: 0.9935). Neither the slope nor the correlation coefficient differed significantly from 1.000 (p > 0.5), and the intercept was not significantly different from 0.000 (p > 0.5).

Because no D₃ metabolites were available, we could only assess indirectly the effect of these potentially interfering metabolites. A normal volunteer therefore took vitamin D₂, 12 000 int. units per day, for nine days. Plasma was collected and analyzed. No standard 24,25(OH)₂D₃ was available and thus no quantitative measurements could be made, but in the event, when the appropriate (M-90-15)⁺ ion for 24,25(OH)₂D₂ (m/z 449 + 12) was monitored, no 24,25(OH)₂D₂ could be detected. The results of this experi-
ment are given in Figure 6. Although 25-OHD$_3$ concentrations were increased, no increase in 24,25(OH)$_2$D$_3$ was observed; if metabolites of D$_2$ were formed under these conditions, they evidently did not interfere with this assay.

**Discussion**

Except for two HPLC methods (6, 7), all published procedures for estimation of 24,25(OH)$_2$D are based on competitive binding assays, usually involving the use of binding protein from rat plasma (4). 24,25(OH)$_2$D is the metabolite of vitamin D that is the most difficult to purify sufficiently for specific protein binding assay to be carried out (15). Several other metabolites of vitamin D, particularly 25-hydroxyvitamin D$_3$-26,23-lactone and 25,26(OH)$_2$D$_3$, comigrate in the HPLC systems commonly used (14) and may thus interfere in the final assay. In addition, metabolites of D$_2$ may not necessarily have the same binding affinity for the rat plasma protein as do their D$_3$ counterparts (15, 16). One example of the consequences of such possible interference is the report of seasonal variations in the level of 24,25(OH)$_2$D$_3$ (17). These results were not confirmed in a subsequent study in children, and it was suggested that the increased concentrations in summer were in fact due to inadequate methodology, leading to interference from 25-hydroxyvitamin D$_2$-26,23-lactone (18). However, the preliminary results reported here (Table 4) do tend to support the suggestion (17) of a seasonal variation of 24,25(OH)$_2$D$_3$ in plasma.

There is still uncertainty as to whether 24,25(OH)$_2$D has a physiological role or represents a catabolic pathway in the presence of increased 25-OHD concentrations, or both. Workers using 24,24-difluoro-25-OHD (3) have suggested that formation of 24,25(OH)$_2$D is not obligatory for the maintenance of adequate calcium transport and mobilization in the vitamin D-deficient rat. On the other hand, there appears to be evidence that 24,25(OH)$_2$D, perhaps together with 1,25(OH)$_2$D$_3$, may have a physiological role, and receptors for 24,25(OH)$_2$D have been found in a wide variety of tissues (2).

Therefore specific and accurate methods for measuring 24,25(OH)$_2$D in human plasma and other body fluids are clearly needed, to establish whether 24,25(OH)$_2$D is of physiological importance. Present methods for its estimation are usually suspect in terms of specificity unless extensive chromatography is carried out before assay. HPLC methods in which ultraviolet absorbance is used for quantification have not proved successful in our hands and are not widely used. Absorbance at 264 nm is in any case not specific; all 5,7-dienes absorb at this wavelength. Specificity therefore still depends on adequate chromatography, and sensitivity is only just sufficient.

Mass-fragmentographic assays for D (19), 25-OHD (20), and 1,25(OH)$_2$D (21) have been described previously, but so far no gas chromatographic–mass spectrometric method for 24,25(OH)$_2$D has been described, apart from the preliminary studies by Seamark et al. (4) in which trimethylsilyl ether derivatives of the isotachysterol isomer were used. This method subsequently proved to be inadequate, because the trimethylsilyl ethers of the isotachysterol isomers of 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$ co-migrated in the gas-chromatographic system used. This problem has been overcome in the method described here by the use of cyclic n-butyl boronates (22), which allows resolution of these two isomeric metabolites of vitamin D. Because these cyclic derivatives will only form across hydroxyls that are close to each other, their formation provides a further degree of specificity. Subsequent formation of trimethylsilyl ether groups on the remaining hydroxyls removes linear boronate esters (23). With the LKB 2091 mass spectrometer used in these studies, the electron impact fragmentation of these derivatives has led to severely decreased intensities of the molecular ion of n-butyl boronate derivatives of the isotachysterol isomer of 24,25(OH)$_2$D$_3$. It has not therefore proved possible to use isotachysterol isomers in this method, although preliminary studies in which other, more modern mass spectrometers were used have not encountered this problem. Because isotachysterol isomers have not been used, two gas-chromatographic peaks (pyro- and isopyro-isomers) are formed for each secosteroid injected, and in this method as in others (e.g., 21), the pyro-peak has been used for quantitation.

The use of Sep-Pak cartridges for extraction (11, 24) and fractionation (25), which have been described previously, provide a very simple and rapid procedure for the preliminary fractionation of these metabolites. Sensitivity is more than adequate for 24,25(OH)$_2$D assay in the volume of plasma used, and analytical recovery has been satisfactory. Specificity can never be claimed to be absolute until standards for potentially interfering metabolites are available. It has, however, been shown that the use of Zorbax-CN columns instead of Zorbax-SIL for HPLC does not affect the values found in plasma, thus indicating that 25-hydroxyvitamin D$_3$-26,23-lactone does not interfere with this assay (14).

Oral D$_2$ (12 000 int. units per day) has not significantly altered the values of 24,25(OH)$_2$D$_3$ measured, and thus it would appear that D$_2$ metabolites do not interfere with this assay. Such interference is unlikely, because the relative molecular mass of the D$_2$ metabolites is 12 mass units higher than that of their D$_3$ counterparts. In addition, the pyro-nBBA-TMSi derivative of 25,26(OH)$_2$D$_2$ will not co-migrate with pyro-nBBA-TMSi derivative of 24,25(OH)$_2$D$_3$, because D$_2$ metabolites have longer retention times than do the corresponding D$_3$ metabolites in the GC system we used. It would have been preferable to demonstrate that the method was unaffected by addition of potentially interfering metabolites, but no standards were available—and until they are it will not be possible to establish beyond doubt that they do not interfere. Although appropriate mass fragments, m/z 461, representing the (M-90 + 15)$^+$ ion, and m/z 446, representing the (M-90 + 15)$^+$ ion, which might be expected to arise from 24,25(OH)$_2$D$_3$, were monitored throughout,

![Fig. 6](image-url)  
Fig. 6. Changes in plasma concentrations of 25-OHD$_3$, 25-OHD$_2$, and 24,25(OH)$_2$D$_2$ in plasma of a normal woman taking oral D$_2$ (12 000 int. units daily, day 1 through day 9).
no peaks with these masses have been detected.

We believe that by using capillary-column chromatography and modern mass spectrometers with more sensitive electron multipliers it should be possible to detect and measure 24,25(OH)2D in much smaller volumes of plasma than described here. Use of chemical ionization techniques and negative-ion mass spectrometry may prove to be of value in further decreasing the limits of detection. Although mass spectrometry will not be used for routine assay, it could provide a reference method against which other, possibly less specific, methods can be evaluated.

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