Assay of Vitamins D₂ and D₃, and 25-Hydroxyvitamins D₂ and D₃ in Human Plasma by High-Performance Liquid Chromatography

Glenville Jones

I describe a new assay that is capable of measuring vitamin D₂, vitamin D₃, 25-hydroxyvitamin D₂, and 25-hydroxyvitamin D₃ in 2 ml of plasma or serum. Plasma is extracted by the Bligh and Dyer technique [Can. J. Biochem. Physiol. 37, 911 (1959)], the lipid component is fractionated by two high-performance liquid-chromatographic systems based upon adsorption and reversed-phase chromatography, and each of the four vitamin D metabolites is measured by its absorbance at 254 nm. The method has a sensitivity limit of 0.5 μg/liter of plasma. The identity of metabolite peaks was confirmed by mass spectrometry, ultraviolet absorption spectrophotometry, and rechromatography, and there was good correlation (r = 0.84) between plasma 25-hydroxyvitamin D as measured by the present method and by a protein binding assay developed in our laboratory. Mean concentrations of vitamin D and 25-hydroxyvitamin D in normal adults (n = 25) in December were 2.2 ± 1.1 (SD) and 16 ± 3.9 (SD) μg/liter, respectively. 25-Hydroxyvitamin D₂ made up 31% of the total 25-hydroxyvitamin D. Patients receiving pharmacological doses of vitamin D had values for vitamin D and 25-hydroxyvitamin D that were 10- to 100-fold normal. This method provides a rapid, reliable physico-chemical assay that appears to have advantages over existing protein binding assays and can be used to measure circulating vitamin D.

Additional Keyphrases: vitamin D and its metabolites · normal values · mass spectra · protein-binding assay · ultraviolet spectrophotometry

Assays of 25-hydroxyvitamin D (25-OH-D) (1, 2) and 1,25-dihydroxyvitamin D (1,25-(OH)₂D) (3, 4) have proved to be useful aids in the study of vitamin D metabolism in man, but existing methods have certain drawbacks and limitations. Commonly used protein-binding assays offer poor reproducibility and are sensitive to nonspecific interfering substances, which must be removed by chromatography. Alternative physicochemical assays require time-consuming purification schemes and chemical derivatization before analysis. To overcome some of the problems met with in previously reported protein binding and physico-chemical assays, I have sought to develop an assay based upon high-performance liquid chromatography (HPLC). This technique resolves all the known metabolites of vitamin D₂ and vitamin D₃ in a synthetic mixture of the compounds (5). Because pure metabolites of vitamin D possessing the characteristic cis-tetraene structure were detectable in the low-nanogram range with an ultraviolet detector (5), we believed that it should be possible to measure simultaneously many of the metabolites present in a sample of human plasma. Here, I report an assay for vitamins D₂ and D₃ and 25-hydroxyvitamin D₂ and D₃ in 2 ml of human plasma or serum. The procedure consists of extracting the sample by the Bligh-Dyer technique (6) and separating the vitamin D metabolites by two HPLC steps on commercially available pre-packed columns. In addition to providing a convenient and rapid assay of 25-OH-D by a physicochemical technique, the procedure has an advantage over available competitive protein binding assays in that it provides information on the concentrations of both 25-OH-D₂ and 25-OH-D₃, and it can be used to measure the concentration of vitamin D — i.e., vitamins D₂ and D₃.

A preliminary summary of this work appears elsewhere (7).

Materials and Methods

Apparatus

The chromatograph I used in these studies was a Model LC 204 fitted with a Model 6000 A pumping system, U6K injection valve, and a Model 440 ultraviolet fixed wavelength (254 nm) detector (all from Waters Associates, Milford, Mass. 01757). In some experiments a Model SF 770 variable-wavelength (200-400 nm) detector (Schoeffel Instrument Corp., Westwood, N.J. 07675) was connected in series after the fixed-wavelength detector. In either case, dead space between...
the detector and the exit from the liquid chromatography was minimized by use of 0.009-inch (i.d.) stainless-steel tubing.

Stainless-steel columns (22 cm x 6.2 mm i.d.), pre-packaged with microparticulate Zorbax-SIL or Zorbax-ODS, were purchased from Du Pont Instruments, Wilmington, Del. 19898.

A Unicam SP 1800 Spectrophotometer (Pye-Unicam, Cambridge, England CB1 2PX) was used to measure concentrations of vitamin D compounds in solution (ε = 18 300). Mass spectra were obtained with a MAT CH-5 mass spectrometer coupled to a Model 620 i computer (both from Varian Instruments, Palo Alto, Calif. 94303). Samples were introduced by a direct-insertion probe, temperature-programmed from 50 to 300 °C during 500 s. Ionization voltage was 70 eV and background was subtracted by using a Varian module subtractor.

Scintillation counting was performed on a Model LS 355 (Beckman Instruments, Palo Alto, Calif. 94303) ambient-temperature scintillation counter, fitted with external standardization.

Materials

Solvents: All solvents were from Burdick & Jackson Labs., Inc., Muskegon, Mich. 49442, “distilled-in-glass” spectroscopic grade.

Vitamin D metabolites: Crystalline vitamin D2 and vitamin D3 were purchased from Sigma Chemical Co., St. Louis, Mo. 63178. Crystalline 25-OH-D2 and 25-OH-D3 were generous gifts from Drs. J. A. Campbell, Jack Hinson, and John Babcock of the Upjohn Co., Kalamazoo, Mich. 49001. [1,2-3H]Vitamin D3 (6 Ci/mm01) was obtained from Amersham/Searle Corp., Arlington Heights, Ill. 60005. [26,27-3H]25-OH-D3 (9.3 Ci/mm01) was supplied by New England Nuclear, Boston, Mass. 02118. [3α-3H]Vitamin D2 (3.5 Ci/mm01) was synthesized by the sodium [3H]bortohydride (11.25 Ci/mm01) reduction of 3α-acetoxyergosta-3,5,7,22-tetraene (8). [3α-3H]25-OH-D2 (3.5 Ci/mm01) was generated biologically from [3α-3H]vitamin D2 by use of liver homogenate incubations (9). All metabolites were purified by HPLC before use (5).

Sephadex LH20 was supplied by Pharmacia Inc., Piscataway, N.J. 08854. Hydroxyalkoxypropyl Sephadex (HAPS) was synthesized from Sephadex LH 20 and Nodox (a long-chain olefinic epoxide, carbon chain length, 15–18) (a gift of the Ashtabula Chemical Co., Columbus, Ohio 43216) by the method of Ellingboe et al. (10).

Plasma samples: For much of the developmental work on the assay I used a 300-ml pool of normal human plasma. Normal ranges for vitamin D, 25-OH-D2, and 25-OH-D3 were evaluated by using plasma from 25 apparently healthy adult laboratory workers at our hospital, sampled during December. Some studies involved the use of plasma obtained from children with disorders of calcium and phosphate metabolism before or after beginning therapy with graded pharmacological doses of vitamin D2 or D3 (1000–100 000 int. units/day).2

Procedures

Extraction of Blood Samples

To 2 ml of plasma were added 5000 cpm of [3H]D2 and 5000 cpm of [3H]25-OH-D3, each in 10 µl of ethanol (Figure 1). These labeled metabolites served to monitor analytical recoveries during various steps of the assay. Incubation of the plasma with radioactive metabolites at 4 °C for 30 min ensured a thorough equilibration of exogenous and endogenous vitamin D metabolites, as suggested by the work of Kida and Goodman (11). The lipids were extracted with 7.5 ml of methanol/chloroform (2/1) according to the method of Bligh and Dyer (6). To separate phases, I added 2.5 ml of saturated KCl and 2.5 ml of chloroform, followed by centrifugation at 1000 × g for 10 min. The aqueous (upper) layer was re-extracted with an additional 6-ml portion of chloroform. The combined chloroform layers were evaporated under reduced pressure to yield a yellow lipid extract. This occasionally appeared cloudy, because of the presence of white insoluble material, when the extract was redissolved in 1 ml of isopropanol/hexane (5.5/94.5 by vol). For this reason, before chromatography, the solution was filtered through a syringe fitted with a Swinney filter holder containing a Millipore 0.45 µm Teflon filter (organic sample clarification kit; Waters Associates). The clarified lipid extract was finally transferred to a 5-ml conical screw-capped vial (Pierce Chemical Co., Rockford, Ill. 61105) and evaporated in a stream of nitrogen gas. The residue was dissolved in 200 µl of the isopropanol/hexane mixture. Flocculent precipitates, sometimes observed after refrigeration of

2 1 International Unit of vitamin D = 25 ng.
these extracts, were removed by centrifugation at 2000 \( \times g \) for 2 min.

**Chromatography of Lipid Extracts**

As shown schematically in Figure 1, the first stage of fractionation of the lipid extract was done by HPLC on Zorbax-SIL (microparticulate silica), to separate the vitamin D and 25-OH-D fractions from most of the contaminating lipid. The total extract, containing about 20 mg of lipid dissolved in 200 \( \mu l \) of the isopropanol/hexane, was injected at a constant flow rate of 1.5 ml/min and normal operating pressure of 8.28 MPa (1200 psi) into a 22 cm \( \times \) 6.2 mm (i.d.) Zorbax-SIL column. Fractions corresponding to "vitamin D" (5.5–7 min) and "25-OH-D" (10.3–14 min) (represented by arrows in Figure 2) were collected, evaporated under a stream of nitrogen gas, and redissolved in 100 \( \mu l \) of the eluting solvent used for reversed-phase chromatography on Zorbax-ODS. Solvent mixtures employed were: "vitamin D," methanol/water (98.5/1.5 by vol); "25-OH-D," methanol/water (91/9 by vol). No significant ultraviolet-absorbing peaks eluted from the Zorbax-SIL column after 15 min; thus samples for assay were injected successively every 15 min. After running a series of 6 to 12 samples, the Zorbax-SIL column was washed with 10 ml of methanol, to remove any strongly adsorbed plasma components.

**HPLC of Vitamin D and 25-OH-D Fractions**

The "vitamin D" fraction, dissolved in 100 \( \mu l \) of methanol/water (98.5/1.5 by vol), was subjected to reversed-phase chromatography on a 22 cm \( \times \) 6.2 mm (i.d.) Zorbax-ODS column, to separate vitamin D2 (14.6 min) from vitamin D3 (15.7 min) and resolve residual contaminating impurities. A constant flow rate of 1.5 ml/min of the methanol/water solvent produced a normal operating pressure of 6.9 MPa (1000 psi). [\( ^3 \)H]Vitamin D3, which was eluted between 13.5 and 15.5 min in 3 ml of solvent, was collected and mixed with 10 ml of Aquasol (New England Nuclear), and the radioactivity was counted in a liquid scintillation counter with counting efficiency of 30%. A 10-\( \mu l \) aliquot of the stock [\( ^3 \)H]vitamin D, identical to that added to the original plasma sample, was counted in the same mixture (3 ml of methanol/10 ml of Aquasol), to assess the analytical recovery of [\( ^3 \)H]vitamin D2 through the overall procedure depicted in Figure 1.

The "25-OH-D" fraction, dissolved in 100 \( \mu l \) of methanol/water (91/9 by vol), was chromatographed on a 22 cm \( \times \) 6.2 mm (i.d.) Zorbax-ODS column, to separate 25-OH-D2 (14.0 min) from 25-OH-D3 (15.3 min) and resolve residual contaminating impurities. A constant solvent flow rate of 1.5 ml/min produced a normal operating pressure of 9.66 MPa (1400 psi). [\( ^3 \)H]25-OH-D3, which eluted between 13.0 and 15.0 min in 3 ml of effluent, was mixed directly with 10 ml of Aquasol and the radioactivity was counted in a liquid scintillation counter at a counting efficiency of 30%. A 10-\( \mu l \) aliquot of the stock [\( ^3 \)H]25-OH-D3, identical to that added to the original plasma sample, was counted in the same mixture (3 ml of methanol–H2O/10 ml of Aquasol), to assess the analytical recovery of [\( ^3 \)H]25-OH-D3 through the overall procedure.

**Rechromatography of Vitamin D Fraction from Normal Plasma**

To measure vitamin D in normal plasma samples it was often necessary to use an extra step of HPLC after eluting the vitamin D fraction from the Zorbax-ODS column. Effluent appearing between 13.5 and 16.5 min and containing both vitamin D2 and vitamin D3 was collected and evaporated under a stream of nitrogen gas. The purified extract was dissolved in 100 \( \mu l \) of isopropanol/hexane (1/99 by vol) and rechromatographed on Zorbax-SIL (22 cm \( \times \) 6.2 mm i.d.; 2 ml/min; 6.9 MPa) in the same solvent. [\( ^3 \)H]Vitamin D2, which was eluted between 12.5–14.5 min, was collected and evaporated under nitrogen. This fraction was counted in methanol/Aquasol as described above.

**Quantitation of Vitamin D Metabolites**

The triangulation method (12) was used to measure peaks that had appropriate retention times and no peak distortion by contaminants, obtained during the HPLC step on Zorbax ODS. In the case of "vitamin D" fractions from normal plasma, peaks were measured after

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3 Zorbax-ODS is a microparticulate silica with octadecyl silane residues bonded on to its surface.
rechromatography on Zorbax-SIL, which greatly improved the definition of the peaks. Peak area was related directly to the amount of vitamin D metabolite by the equation:

\[ Q = \left( \frac{M}{\epsilon_{254}} \right) \times P \times \frac{1}{2} \times B \times F \times 10^3 \]  

(1)

where

- \( Q \) = quantity of vitamin D metabolite in peak in ng,
- \( M \) = molecular weight of vitamin D metabolite,
- \( \epsilon_{254} \) = molar absorptivity of vitamin D metabolite at 254 nm \( = 16,500 \),
- \( P \) = peak height in absorbance units,
- \( B \) = peak base in minutes, and
- \( F \) = flow rate in ml/min.

The validity of this equation was checked during each run by injecting standards of known concentration.

Analytical recoveries of vitamin D metabolites were measured by comparing the radioactivity in the effluent from Zorbax-ODS chromatography with that added initially to the plasma sample. In the case of "vitamin D" peaks \(^3\)Hvitamin D\(_2\) was used to assess recovery of both vitamin D\(_2\) and vitamin D\(_3\) in plasma. Similarly, \(^3\)H25-OH-D\(_3\) was used to assess recovery of both 25-OH-D\(_2\) and 25-OH-D\(_3\) in plasma.

The concentration of the vitamin D metabolite in the original 2-ml plasma sample was calculated by the equation:

\[ C = \frac{Q \times \text{recovery factor}}{2} \]  

(2)

where

- \( C \) = concentration in plasma in \( \mu \text{g/liter} \),
- \( Q \) = quantity in peak in ng, and
- \( \text{recovery factor} = \frac{\text{original dpm added to plasma}}{\text{recovered dpm present in metabolite peak}} \).

The basic equations, 1 and 2, were combined into a simple program for a Model 9810A Calculator (Hewlett-Packard, Palo Alto, Calif. 94304).

Additional Methods Used to Validate the Procedure

To validate the normal procedure, I used several techniques as alternatives to (e.g., other purification schemes) or in addition to (e.g., rechromatography of vitamin D or 25-OH-D peaks) the steps of the basic method. Additional evidence of peak identity was provided by ultraviolet absorption spectrophotometry and mass spectrometry. Quantitative aspects of the procedure were assessed by vitamin D and 25-OH-D recovery experiments and by comparison of 25-OH-D results with those obtained using a competitive-binding assay.

Alternative purification steps. In some instances Sephadex LH 20 or HAPS chromatography was substituted for the initial HPLC on Zorbax-SIL used in the regular procedure (Figure 1). In these cases, only fractions containing 25-OH-D\(_3\) or 25-OH-D\(_2\) were collected and subjected to Zorbax-ODS chromatography.

(a) Sephadex LH 20 chromatography was performed as originally described by Holick and DeLuca (13) by using a 1 \( \times \) 60 cm gel bed swelled and eluted with an equivalent mixture of chloroform and hexane. Positions of \(^3\)H25-OH-D\(_2\) and \(^3\)H25-OH-D\(_3\) were monitored by taking aliquots of 5-ml fractions of effluent. Alternatively, 10-ml graduated pipets filled to the 2.7-ml mark with 7.3 ml of gel (3 g) were used for purification of lipid extracts. After applying to the column a sample dissolved in 250 \( \mu \text{l} \) of eluting solvent (chloroform/hexane) the first 9 ml of effluent was discarded. The next 10 ml of effluent, containing the \(^3\)H25-OH-D\(_3\) and 25-OH-D\(_2\), was collected for HPLC analysis on Zorbax-ODS.

(b) HAPS column chromatography was done by using a graduated pipet filled to the 2.7-ml mark with 3 g of HAPS (carbon chain length, 15–18), swelled and eluted with chloroform/hexane (1/9 by vol) (14). Lipid extracts were applied in 250 \( \mu \text{l} \) of eluting solvent and the first 18 ml of effluent was discarded. The next 7 ml contained 25-OH-D\(_2\) plus the \(^3\)H25-OH-D\(_3\) added as an indicator of recovery of 25-OH-D\(_2\), and the subsequent 11 ml contained 25-OH-D\(_3\) plus \(^3\)H25-OH-D\(_3\). Each fraction was subjected separately to HPLC on Zorbax-ODS.

Rechromatography of 25-OH-D fractions on Zorbax-SIL. Rechromatography of 25-OH-D fractions involved the collection of both 25-OH-D\(_2\) and 25-OH-D\(_3\) as a single fraction (13–16.5 min) from the Zorbax-ODS HPLC step of the regular procedure (Figure 1).

The sample was evaporated in a stream of nitrogen gas and redissolved in 100 \( \mu \text{l} \) of isopropanol/hexane (5.5/94.5 by vol). HPLC on Zorbax-SIL was performed at a constant flow rate of 2 \( \mu \)l/min. \(^3\)H25-OH-D\(_3\) was collected (9–10.5 min) and counted in methanol/Aquasol. \(^3\)H25-OH-D\(_3\) was used as an indicator of recovery for both 25-hydroxy metabolites.

Ultraviolet Absorption Spectra of HPLC Peaks

Twenty 2-ml aliquots of the normal plasma pool were purified by the regular scheme shown in Figure 1. Vitamin D and 25-OH-D fractions were collected from the Zorbax-ODS column. All the vitamin D and 25-OH-D fractions were pooled and evaporated in a stream of nitrogen. Each pooled fraction was redissolved in 200 \( \mu \text{l} \) of isopropanol/hexane and 10-\( \mu \)l aliquots were injected into the liquid chromatograph fitted with a Zorbax-SIL column, a fixed-wavelength (254 nm) detector, and a Schoeffel variable-wavelength detector set at wavelengths between 215 and 315 nm. Details of the HPLC were dictated by the metabolite under study and were described above.

Spectra were plotted after measuring the peak area at each setting of the variable wavelength detector. To ensure that injection volumes were identical for each wavelength setting of the variable-wavelength detector, we also compared peak areas obtained with use of the fixed-wavelength (254 nm) detector. Correction factors
were applied to the variable wavelength plot if there were discrepancies in the 254-nm peak area or the recovered radioactivity.

Mass Spectra of HPLC Peaks

Forty 2-ml aliquots of the normal plasma pool were purified according to the regular scheme shown in Figure 1. 25-OH-D fractions were then pooled and rechromatographed on Zorbax-SIL as described above. 25-OH-D2 and 25-OH-D3 fractions were collected separately, evaporated under nitrogen, dissolved in 10 μl of ethanol, and transferred to the direct probe of the mass spectrometer.

Modified Rat-Plasma Binding Protein Assay for 25-OH-D

The method used was essentially a modified version of that developed by Belsey et al. (1). Plasma samples (0.5 ml) were first extracted with methanol/chloroform according to Bligh and Dyer (6). Extracts were chromatographed on 15 cm × 6 mm columns of Sephadex LH 20 eluted with an equimolue mixture of chloroform and hexane. All the 25-OH-D (25-OH-D2 and 25-OH-D3) was present in the first 9 ml of effluent from this column. The 25-OH-D fraction was evaporated under nitrogen and assayed for 25-OH-D, the buffers and incubation conditions being those of Belsey et al. (15). Under these conditions we observed no interference by neutral lipid or vitamin D present in the 25-OH-D fraction. Interfering substances remained as a residue on the Sephadex columns used here.

Results

Extraction of Plasma Samples

Recovery during the chloroform/methanol extraction stage of the procedure was assessed by comparing radioactivity present in lipid extracts to radioactivity added to plasma samples. Recoveries were 89.1 ± 4.1% (n = 6)4 for [3H]25-OH-D3 and 90.5% ± 3.2% (n = 6) for [3H]vitamin D2.

Initial Step of Chromatography of Lipid Extracts

I found that chromatography of lipid extracts was necessary before chromatography on Zorbax-ODS, because vitamin D metabolite peaks were masked by overlapping impurities when this step was omitted.

Several types of chromatography were used as the initial purification step in an attempt to find a rapid, reliable method of preparing a 25-OH-D fraction suitable for analysis by HPLC on Zorbax-ODS. Table 1 summarizes the results obtained when a single plasma sample was analyzed by HPLC on Zorbax-ODS after one of four different chromatographic procedures. Although values for 25-OH-D3 determined by each alternative were similar, only two methods, HPLC on Zorbax-SIL and a lengthy “open-column” procedure with Sephadex LH-20, permitted the determination of 25-OH-D2. Furthermore, with Sephadex LH 20, the recovery of 25-OH-D2 could only be assessed by the use of [3H]25-OH-D2, whereas [3H]25-OH-D3 acted as a recovery marker for both 25-OH-D2 and 25-OH-D3 during HPLC on Zorbax-SIL. In addition to this disadvantage, I observed that all of the “open-column” procedures resulted in greater ultraviolet contamination of the 25-OH-D3 fraction than did HPLC on Zorbax-SIL. Sometimes 25-OH-D3 fractions from Sephadex columns were so contaminated as to make measurements impossible. Thus HPLC on Zorbax-SIL proved to be the most efficient and convenient method of pre-purification, and it was adopted as the method of choice in the scheme shown in Figure 1. A typical chromatogram of the purification of a plasma lipid extract on Zorbax-SIL is illustrated in Figure 2, frame b. Vitamin D2, vitamin D3, 25-OH-D2, and 25-OH-D3 were added to the lipid extract as internal standards. By comparison to Figure 2, frame a, which shows the chromatogram obtained with standards alone, one sees that the presence of 15–20 mg of lipid from the extraction of 2 ml of plasma does not significantly affect the chromatographic position of the vitamin D metabolites. The excellent reproducibility of retention time from one injection to the next facilitated the timed collection of fractions and in addition validated the assumption that [3H]25-OH-D3 could be used to assess the recovery of both 25-OH-D2 and 25-OH-D3.

Chromatography of “Vitamin D” Fractions on Zorbax-ODS

With use of a methanol/water (98.5/1.5) solvent system, the Zorbax-ODS column packing resolved vitamin D2 (14.6 min) and vitamin D3 (15.7 min) very well (Figure 3a). In plasma extracts from patients receiving pharmacological doses of vitamin D (Figure 3c and d) the peak corresponding to the type of therapy (D2 or D3) stands out from the background of ultraviolet-absorbing peaks. However, in normal individuals these vitamin D peaks are extremely small and are often immeasurable.

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4 All mean values are expressed ± 1 SD.

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Table 1. Determination of 25-OH-D3 and 25-OH-D2 Concentrations in the Normal Plasma Pool by the Present Method after Various Initial Purification Steps

<table>
<thead>
<tr>
<th>Initial purification step</th>
<th>Concns of vitamin D metabolite (nM μg/liter) as determined by chromatog. on Zorbax-ODS</th>
<th>25-OH-D2</th>
<th>25-OH-D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) 16 g Sephadex LH-20 col. chromatography</td>
<td>15.7 ± 2.8</td>
<td>3.9 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>(b) 3 g Sephadex LH-20 col. chromatography</td>
<td>16.2 ± 1.4</td>
<td>n.d. b</td>
<td></td>
</tr>
<tr>
<td>(c) 3 g HAPS column chromatography</td>
<td>15.9 ± 1.1</td>
<td>n.d. b</td>
<td></td>
</tr>
<tr>
<td>(d) 6.2 mm × 22 cm Zorbax-SIL HPLC</td>
<td>14.7 ± 0.9</td>
<td>3.3 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD, n = 4. b n.d. = not determined because of overlapping impurity peaks.
Fig. 3. HPLC of vitamin D fractions on Zorbax-ODS
(a) standards (D$_2$: 112 ng; D$_3$: 117 ng)
(b) plasma extract of normal adult
(c) plasma extract of child treated with 50,000 int. units of vitamin D$_3$ daily
(d) plasma extract of child treated with 100,000 int. units of vitamin D$_3$ daily
Frames b, c, and d represent material purified through the Zorbax-SIL chromatography stage of Figure 1. Figures above peaks represent retention times in minutes. Flow rate = 1.5 ml/min; solvent system methanol/water (98.5/1.5 by vol); K'$_{D_2}$ = 6.3; K'$_{D_3}$ = 6.85; $\alpha$ = 1.087; N = 4720; R$_S$ = 1.19

Fig. 4. Rechromatography of vitamin D fraction from normal plasma extract
Fraction from scheme shown in Figure 1 was subjected to additional Zorbax-SIL chromatography. Peak at 13.6 min represents a mixture of vitamin D$_2$ and vitamin D$_3$. Conditions as in Figure 8

owing to co-migration with impurity peaks (Figure 3b). As a result all "normal" vitamin D fractions were rechromatographed on Zorbax-SIL as depicted in Figure 4. The single peak shown at 13.6 min represents total vitamin D, because vitamin D$_2$ and vitamin D$_3$ migrate together in this system.

Chromatography of "25-OH-D" Fractions on Zorbax-ODS

With a methanol/water (91/9) solvent system, the Zorbax-ODS column well resolved 25-OH-D$_2$ (14.0 min) from 25-OH-D$_3$ (15.3 min) (Figure 5). Interestingly, the order of elution of the parent vitamins (vitamin D$_2$ before vitamin D$_3$) is reversed in the case of 25-hydroxy derivatives.

Contamination of 25-OH-D peaks with impurities was not a problem. 25-OH-D$_3$ and 25-OH-D$_2$ peaks were both observed in normal plasma (Figure 5b), whereas in patients receiving pharmacological doses of vitamin D the type of 25-OH-D observed in the plasma corresponded to the type of therapy administered (Figure 5c and d). In all cases, retention times of metabolite peaks in plasma extracts were identical to those observed for synthetic standards.

Overall Analytical Recovery of Tracers

Overall recovery of radioactive tracer, from its addition to the original plasma through the lipid extraction and the two-column procedure to its final elution from HPLC on Zorbax-ODS, averaged 68.8 ± 6.5% (n = 12) in the case of [³H]25-OH-D$_3$ and 65.4 ± 6.0% (n = 8) in the case of [³H]D$_2$.

Calibration Curve for the HPLC Detector

As described in Materials and Methods, vitamin D and its metabolites were measured by peak-area measurement by the triangulation method (equation 1). The calibration of the Waters 440 detector at 254 nm was checked by injecting solutions of pure crystalline vitamin D$_2$ and 25-OH-D$_3$ of known concentrations (as determined by ultraviolet spectrophotometry). Calibration curves (not shown) were constructed comparing the amounts of vitamin D$_2$ or 25-OH-D$_3$ injected and
the amounts determined by peak area measurements with the ultraviolet detector. There was good agreement between the two: for 18 such comparisons the amount of vitamin D metabolite detected was 97.8% ± 4% (n = 18) of that injected.

Precision and Accuracy of the HPLC Procedure for Plasma

The "within-run" coefficient of variation of the HPLC procedure for 25-OH-D determined as a single plasma sample, 25-OH-D = 11.9 ± 1.1 μg/liter, was 9% on six repeated determinations on the same day. The "between-run" coefficient of variation for the HPLC method for 25-OH-D determined on the same plasma sample over a period of six months was 16% (n = 20).

The accuracy of the method was assessed by adding known quantities of vitamin D₂ and 25-OH-D₃ (10–700 ng) to aliquots of a normal plasma sample containing, per liter, 2 μg of vitamin D and 16 μg of 25-OH-D and measuring the vitamin D and 25-OH-D by the regular two-stage procedure. There was good agreement between the observed and theoretical values for both D₂ and 25-OH-D₃ added to plasma (Figure 6).

Normal Ranges for 25-OH-D and Vitamin D

I have applied the new procedure to about 100 plasma samples thus far. Total 25-OH-D, determined by the HPLC procedure for a group of apparently healthy laboratory workers in December, was 16.0 ± 3.9 μg/liter (n = 25), with a range of 9.1–23.9 μg/liter. The total 25-OH-D was composed of 13.1 ± 10.6% 25-OH-D₂, the remainder appearing as 25-OH-D₃.

Total vitamin D determined by the HPLC procedure was 2.2 ± 1.1 μg/liter (n = 24) with a range of 0.8–4.7 μg/liter.

Comparison of Determinations of Total 25-OH-D by HPLC and the Modified Binding Assay

Values for total 25-OH-D concentration determined by the HPLC procedure and those determined by the modified binding assay described above (1, 15) correlated well (r = 0.84) (Figure 7a). The range of total 25-OH-D values determined by the modified binding assay in our group of 25 healthy laboratory workers was...
8.7 to 23.6 µg/liter (mean, 16.3 ± 4.4 µg/liter). The good correlation between the HPLC procedure and the protein-binding assay extends across the range of 25-OH-D values (10–500 µg/liter) observed in children receiving 0 to 100,000 int. units of vitamin D per day (Figure 7b).

Evidence That the Metabolic Peaks Were Correctly Identified

(a) Rechromatography on Zorbax-SIL. Representative samples from a variety of normal adults and treated patients were analyzed by the usual two-step procedure outlined in Figure 1. Then vitamin D and 25-OH-D fractions were separately rechromatographed on Zorbax-SIL.

The results of rechromatography of vitamin D fractions from normal plasma have already been given (Figure 4). Assay results for vitamin D-containing fractions from patients receiving pharmacological doses of vitamin D, rerun on Zorbax-SIL, are shown in Figure 8. Though vitamins D2 and D3 are not separated on Zorbax-SIL, they do have slightly different retention

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Table 2. Comparison of Vitamin D and 25-OH-D Concentrations as Determined by the Method Depicted in Figure 1a and by Methods Involving Further Purificationb, c

<table>
<thead>
<tr>
<th></th>
<th>Normal adult</th>
<th>Child receiving 50,000 int. units of D2/day</th>
<th>Child receiving 100,000 int. units of D2/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/liter</td>
<td>µg/liter</td>
<td>µg/liter</td>
</tr>
<tr>
<td>Regular 25-OH-D assay</td>
<td>1.6</td>
<td>12.9</td>
<td>14.5</td>
</tr>
<tr>
<td>Zorbax-SIL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rechromatography of 25-OH-D on Zorbax-SIL</td>
<td>1.6</td>
<td>11.9</td>
<td>13.5</td>
</tr>
<tr>
<td>Zorbax-ODS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rechromatography of 25-OH-D on Zorbax-ODS</td>
<td>1.9</td>
<td>13.1</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Comparison of Vitamin D and 25-OH-D Concentrations as Determined by the Method Depicted in Figure 1a and by Methods Involving Further Purificationb, c

<table>
<thead>
<tr>
<th></th>
<th>Regular D assay</th>
<th>Rechromatography of D on Zorbax-SIL</th>
<th>Further Rechromatography on Zorbax-SIL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D2</td>
<td>D3</td>
<td>Total D</td>
</tr>
<tr>
<td></td>
<td>µg/liter</td>
<td>µg/liter</td>
<td>µg/liter</td>
</tr>
<tr>
<td>Regular D assay</td>
<td>2.8</td>
<td>0</td>
<td>772</td>
</tr>
<tr>
<td>Rechromatography of D on Zorbax-SIL</td>
<td>1.4</td>
<td>0</td>
<td>790</td>
</tr>
<tr>
<td>Further Rechromatography on Zorbax-SIL</td>
<td>1.8</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* The two-column procedure as in the scheme in Figure 1. b Three-column procedure. c Four-column procedure. These data represent results obtained from chromatograms shown in Figures 3, 4, 5, 8, and 9.
times and in patients receiving pharmacological doses of either vitamin D$_2$ or D$_3$ it was possible to identify the type of vitamin the patient had received on the basis of the retention time of the vitamin D fraction rerun on Zorbax-SIL. In all cases this identification was consistent with that ascribed from Zorbax-ODS chromatography. On rechromatography of 25-OH-D fractions on Zorbax-SIL, 25-OH-D$_2$ (8.4 min) and 25-OH-D$_3$ (9.6 min) were well resolved (Figure 9). The theoretical plate count$^5$ (16) for this column was greater than that of the Zorbax-ODS system and, as a result, the sensitivity of the detection was increased. Again, identities obtained by rechromatography of the 25-OH-D peaks were completely consistent with those ascribed to the peaks on use of the two-column procedure.

Table 2 presents the quantitation of the peaks obtained by rechromatography of vitamin D and 25-OH-D fractions. The data confirm that the specific activity (ng/dpm) for each peak was constant after the regular two-stage procedure and that additional purification did not decrease the value obtained for the concentration of the metabolite in the original plasma. Had the “vitamin D metabolite” peak been contaminated with other ultraviolet-absorbing impurities the specific activity (ng/dpm), and hence the concentration of metabolite in plasma (μg/liter), would have been falsely high. It is most likely that these values would have decreased after rechromatography. For analysis of vitamin D in normal plasma, the three-column procedure was necessary to reach constant specific activity measurements.

(b) Ultraviolet spectra. Ultraviolet spectra of 25-OH-D fractions from representative normal plasma samples were plotted as were the spectra of the vitamin D and 25-OH-D fractions from patients treated with pharmacological doses of vitamin D$_2$ or D$_3$. Figure 10 depicts examples of the characteristic cis-triene ultraviolet spectrum (absorption maximum at 265 nm and minimum at 228 nm $\lambda_{max} : \lambda_{min} \sim 1.9:1$) obtained with 25-OH-D$_2$ and 25-OH-D$_3$ from normal plasma.

(c) Mass spectra of 25-OH-D$_2$ and 25-OH-D$_3$. The mass spectrum of 700 ng of 25-OH-D$_3$ (Figure 11) possessed the expected molecular ion (m/e 400) and all the major fragments observed previously (17): [382 (–H$_2$O); 367 (–CH$_3$ and H$_2$O); 341 (C$_{24}$-C$_{25}$ cleavage); 271 (loss of side chain); 253 (271 – H$_2$O): 158; 136 (cis-triene cleavage); and 118 (136 – H$_2$O)]. The mass spectrum of ~100 ng of 25-OH-D$_2$ (not illustrated) was not sufficiently intense to exhibit the molecular ion at 412, but showed the major fragments generated by most vitamin D compounds [m/e 136 and 118].

**Discussion**

I have described a procedure for using HPLC to simultaneously determine vitamin D$_2$, vitamin D$_3$, 25-OH-D$_2$, and 25-OH-D$_3$ in 2 ml of human plasma or serum. It differs from other physico-chemical assays that have been developed for 25-OH-D$_3$ (18, 19) in that it requires no derivatization of the metabolite under study, involves a much simpler purification scheme, and measures both 25-OH-D$_2$ and 25-OH-D$_3$. Furthermore, the assay is similar in terms of speed, sensitivity, and reproducibility to existing competitive protein-binding assays for estimation of total 25-OH-D, but it has several additional advantages. Only rarely (20, 21) have protein-binding assays been designed to measure more than a single variable, whereas the present assay pro-

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$^5$ In the legend to Figure 9, chromatographic parameters (16) are represented by the following symbols: $K'$ = relative retention time; $\alpha$ = selectivity; N = theoretical plates per column; $R_s$ = resolution.
vides information on the ratio of 25-OH-D$_2$/25-OH-D$_3$, and determines the concentration of vitamin D in plasma. Knowledge of the type of 25-OH-D in plasma provides an opportunity to determine the relative contributions of diet and exposure to ultraviolet light in experimental animals and the effects in humans who are receiving supplements of vitamin D$_2$, and it aids in assessing the response of human subjects to vitamin D therapy.

The procedure is simple in design; it consists of lipid extraction and two chromatographic steps. Extraction with chloroform/methanol was shown to be a reliable method for recovering lipid from plasma protein. Our results indicate that chloroform/methanol extraction recovers 90% of radioactively labeled vitamin D or 25-OH-D added to plasma. Recently, it was suggested that extraction with chloroform/methanol was inferior to a procedure involving dichloromethane (4) in its ability to extract radioactive vitamin D metabolites from plasma, but I believe that the reported lack of success with the chloroform/methanol procedure can be attributed to non-use of the proportions of chloroform/methanol/water (2/2/1.8) recommended by Bligh and Dyer in their original description of the method (6).

Prepurification of the lipid extract of plasma on a Zorbax-SIL column by HPLC proved to be far more satisfactory than any of the other alternatives we have tested. Eisman et al. (22) recently devised a method for determining 25-OH-D$_2$ and 25-OH-D$_3$ in 4 ml of human plasma, based upon a procedure involving two "open" Sephadex columns and a single HPLC step on silica. The misconceptions that complex puriprepurification is necessary before HPLC can be applied for assaying plasma and that the loading capacity of HPLC is insufficient are dispelled by these experiments. I demonstrated that a 22 cm x 6.2 mm microparticulate silica column can separate most of the extraneous ultraviolet-absorbing lipid from the vitamin D and 25-OH-D fractions of whole-lipid extracts of plasma. Use of HPLC as a preparative tool offers numerous advantages over open-column procedures. Precise sample injection, constant flow elution, and timed collection of each plasma extract permit better reproducibility. Detector monitoring of column effluent makes it feasible to regularly check elution positions by use of standards. The theoretical disadvantage that all samples must be run through the same HPLC column rather than simultaneously through a set of similar "open" columns is offset by the short time (15 min) required for analysis on Zorbax-SIL.

Use of Zorbax-ODS for HPLC of 25-OH-D$_3$ has recently been described by Koshy and VanDersSlik (23). I have demonstrated by using methanol/water mixtures that a 22 cm x 6.2 mm Zorbax-ODS column will adequately resolve 25-OH-D$_2$ from 25-OH-D$_3$ and improve the resolution of vitamin D$_2$ and vitamin D$_3$ reported by others (24). The use here of a highly efficient HPLC column (N$^2$ greater than 4000 plates per column) offering superior sensitivity and resolution to that employed by Eisman et al. (22) (1600 plates per column), makes possible the routine detection of metabolites in only 2 ml of plasma as opposed to the 4-ml sample used by Eisman et al. (22). It should be noted that the 6.2-mm (i.d.) column, the fixed injection volume, and the use of eluting solvent subsequently as the sample solvent were all measures that helped to minimize the variations often encountered in measurement of peak area and retention time. "Shoulders" on the peaks were not observed when analyzing 25-OH-D fractions and did not present problems with the analysis of vitamin D fractions if concentrations exceeded 10 &mu;g/liter. However, in fractions from plasmas with vitamin D concentrations <10 &mu;g/liter (all normal plasmas fell into this category) it was necessary to use a three-column method involving an additional chromatography step on Zorbax-SIL to obtain values for total vitamin D.

The combination of two different types of chromatography, adsorption on Zorbax-SIL and reversed-phase liquid–liquid chromatography on Zorbax-ODS, probably accounts for the successful purification of nanogram quantities of vitamin D metabolites from a matrix consisting of milligram quantities of lipid. Furthermore, though the procedure for plasma involves an extraction and two HPLC steps, it recovers about two-thirds of the original amount of vitamin D metabolites. Losses were probably due to problems of manipulating small amounts of lipid extract and semipurified fractions. The precision of the present assay is comparable to that of existing protein-binding assays (25).

Much evidence from recovery experiments, reinchromatography, ultraviolet absorption, and mass spectral studies indicates that the HPLC assay procedure for 25-OH-D and vitamin D actually measures the ascribed metabolite correctly and accurately. Our mean value of 16 &mu;g/liter for the concentration of total 25-OH-D in normal adult human plasma is somewhat lower than that obtained by some workers using protein-binding techniques in which chromatography is omitted (15, 26, 27), but agrees well with that obtained by others using protein-binding techniques involving chromatography (2, 28). The value is in excellent agreement with our own protein-binding assay, which includes an initial chromatographic step. We found that such a purification step was essential before carrying out the competitive protein binding, to remove interfering substances that increase the reaction value by two- to three-fold above the value obtained when the chromatographic step is incorporated into the protein binding assay. Although seasonal (29), racial (30), and geographical (28) factors can influence serum 25-OH-D concentrations in the population, I believe that some of the disparities in the results obtained in various laboratories can be accounted for by omission or inadequate use of chromatography by some workers (15, 26, 27). There are no published assays for vitamin D in human plasma with which to compare our results by HPLC. However, our range for the concentration of vitamin D in normal adult human plasma, 0.9 to 4.7 &mu;g/liter, is similar to that obtained by others with a protein-binding assay (E. Delvin
and F. Glorieux, personal communication) and a gas chromatography/selected ion monitoring procedure (31). Because of the low concentration of total vitamin D in the samples, it was impossible to apply many of the analytical tools at our disposal to establish the identity and purity of the vitamin D in normal plasma. One must accept the measured value of vitamin D in normal plasma (2.2 ng/ml) as the maximum possible concentration. The true value could be lower if further refinements in methodology revealed contamination of the small peak with extraneous ultraviolet-absorbing substances. Nevertheless, these observations support the view that little vitamin D circulates in plasma, but rather is transported rapidly to the liver for storage (32, 33).

My observation that there is a dramatic increase in the plasma concentration of vitamin D during administration of pharmacological doses of vitamin D suggests that the assay will be useful in assessing the response of patients to therapy. I accept the suggestion that the high concentration of vitamin D in the plasma of treated patients may be the result of mobilization of extrahepatic stores of vitamin D (34). The fact that the concentration of vitamin D in plasma remains high despite discontinuation of therapy may help explain the apparent lack of inhibition of the liver vitamin D 25-hydroxylase that is seen in patients with very high (200–500 ng/ml) concentrations of 25-OH-D in their plasma. Fukushima et al. (35) have shown that 25-hydroxylation of vitamin D can be carried out by two different liver enzymes: a tightly regulated specific vitamin D-25-hydroxylase (36) and a non-regulated unspecific 25-hydroxylase that can also use synthetic 1α-hydroxyvitamin D3 and dihydrotachysterol as a substrate. It is possible that this non-regulated 25-hydroxylase uses vitamin D as a substrate to generate 25-OH-D in an uncontrolled manner when plasma concentrations of vitamin D are increased by high-dosage therapy with vitamin D. I thank Beth Brynes and David Duthie for their technical help, and Drs. Donald Fraser and Sang Whay Koo for the blood samples used in this study and their encouragement and helpful discussion during the development of the assay. Drs. Ingeborg Radde and Graham Ellis provided useful suggestions during the preparation of the manuscript. Dr. Louis Marai operated the mass spectrometer.

References

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