Determination of Vitamin D and its Metabolites in Plasma from Normal and Anephric Man

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A multiple assay capable of reliably determining vitamins D2 and D3 (ergocalciferol and cholecalciferol), 25(OH)D2 (25-hydroxyvitamin D2) and 25(OH)D3 (25-hydroxyvitamin D3), 24,25(OH)2D (24,25-dihydroxyvitamin D), 25,26(OH)2D (25,26-dihydroxyvitamin D) and 1,25(OH)2D (1,25-dihydroxyvitamin D) in a single 3–5 ml sample of human plasma was developed. The procedure involves methanol/methylene chloride extraction of plasma lipids followed by separation of the metabolites and purification from interfering contaminants by batch elution chromatography on Sephadex LH-20 and Lipidex 5000 and by h.p.l.c. (high-pressure liquid chromatography). Vitamins D2 and D3 and 25(OH)D2 and 25(OH)D3 are quantified by h.p.l.c. by using u.v. detection, comparing their peak heights with those of standards. 24,25(OH)2D and 25,26(OH)2D are measured by competitive protein-binding assay with diluted plasma from vitamin D-deficient rats. 1,25(OH)2D is measured by competitive protein-binding assay with diluted cytosol from vitamin D-deficient chick intestine. Values in normal human plasma samples taken in February are: vitamin D 3.5 ± 2.5 ng/ml; 25(OH)D 31.6 ± 9.3 ng/ml; 24,25(OH)2D 3.5 ± 1.4 ng/ml; 25,26(OH)2D 0.7 ± 0.5 ng/ml; 1,25(OH)2D 31 ± 9 pg/ml (means ± s.b.). Values in two normal human plasma samples taken in February after 1 week of high sun exposure are: vitamin D 27.1 ± 7.9 ng/ml; 25(OH)D 56.8 ± 4.2 ng/ml; 24,25(OH)2D 4.3 ± 1.6 ng/ml; 25,26(OH)2D 0.5 ± 0.2 ng/ml. Values in anephric-human plasma are: vitamin D 2.7 ± 0.8 ng/ml; 25(OH)D 36.4 ± 16.5 ng/ml; 24,25(OH)2D 1.9 ± 1.3 ng/ml; 25,26(OH)2D 0.6 ± 0.3 ng/ml; 1,25(OH)2D was undetectable.

It has been well established that vitamin D must be metabolized before its biological activity can be expressed. Vitamin D is hydroxylated to 25(OH)D in the liver (Blunt & DeLuca, 1969) and then further hydroxylated in the kidney to either 1,25(OH)2D or 24,25(OH)2D (DeLuca & Schnoes, 1976). 1,25(OH)2D is now recognized as the active form of vitamin D in bone-mineral mobilization and is exclusively responsible for the initiation of active intestinal absorption of calcium and phosphorus (DeLuca & Schnoes, 1976). In contrast, a role for 24,25(OH)2D or 25,26(OH)2D [another known metabolite of 25(OH)D (Suda et al., 1970b)] has yet to be established. The 25-hydroxylation reaction of vitamin D is partially feedback-regulated (Bhattacharyya & DeLuca, 1973), whereas the metabolism of 25(OH)D to 1,25(OH)2D or 24,25(OH)2D is strictly modulated directly or indirectly by serum calcium, serum phosphorus and parathyroid hormone (DeLuca & Schnoes, 1976). Further, the pathogenesis of several metabolic bone diseases is attended by disturbances in the vitamin D metabolism system (DeLuca & Schnoes, 1976). In view of the above, a need developed for a multiple assay capable of measuring vitamin D and its metabolites in a single small sample of plasma.

Separate ligand-binding assays for the measurement of 25(OH)D (Belsey et al., 1971; Haddad & Chyu, 1971; Bayard et al., 1972; Edelstein et al., 1974; Preece et al., 1974; Bouillon et al., 1976; Garcia-Pascual et al., 1976), 24,25(OH)2D (Haddad et al., 1976b, 1977; Taylor et al., 1976), and 1,25(OH)2D (Brumbaugh et al., 1974; Eisman et al., 1976) in human plasma have been reported, but these are capable of measuring just one variable and many suffer from inadequate chromatography. Two metabolite assays have also been published (Hughes et al., 1976; Jones, 1978), but these, too, are limited.

Abbreviations used: vitamin D2, ergocalciferol; vitamin D3, cholecalciferol; 25(OH)D, 25-hydroxyvitamin D2; 25(OH)D3, 25-hydroxyvitamin D3; 24,25(OH)2D, 24,25-dihydroxyvitamin D2; 24,25(OH)2D3, 24,25-dihydroxyvitamin D3; 25,26(OH)2D, 25,26-dihydroxyvitamin D; 1,25(OH)2D, 1,25-dihydroxyvitamin D; h.p.l.c., high-pressure liquid chromatography.

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The methodology outlined below describes a multiple assay capable of quantifying vitamins D$_2$ and D$_3$, 25(OH)D$_2$ and 25(OH)D$_3$, 24,25(OH)$_2$D$_3$, 25,26(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ in a single 3–5 ml sample of plasma. Concentrations of each of these metabolites in normal and anephric-human plasma were determined with this method.

**Materials and Methods**

**Apparatus**

All h.p.l.c. was carried out with a model LC 204 chromatograph fitted with a model 6000A pumping system, U6K injection valve and a model 440 u.v. fixed-wavelength (254 nm) detector (all from Waters Associates, Milford, MA, U.S.A.). A model 24 spectrophotometer (Beckman Instruments, Irvine, CA, U.S.A.) was used to measure concentrations of vitamin D compounds in solution (ε 18 200 litre·mol$^{-1}$·cm$^{-1}$) at 265 nm. Scintillation counting was performed at room temperature with a model LS-100C liquid-scintillation system (Beckman Instruments, Fullerton, CA, U.S.A.) fitted with external standardization.

**Materials**

**Solvents.** All solvents used for extractions and conventional column chromatography were Fisher Certified ACS grade (Fisher Scientific Co., Pittsburgh, PA, U.S.A.) and distilled once. All solvents used for h.p.l.c. were Fisher h.p.l.c. grade.

**Vitamin D metabolites.** Crystalline vitamin D$_2$, vitamin D$_3$ and 25(OH)D$_3$ were obtained from Phillips-Duphar, Amsterdam, The Netherlands. Crystalline 25(OH)D$_2$ was a gift from the Upjohn Co., Kalamazoo, MI, U.S.A. Crystalline 24,25-(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ were gifts from Hoffman–La Roche, Nutley, NJ, U.S.A. 25,26-(OH)$_2$D$_3$ was synthesized in this laboratory (Lam et al., 1975). [3α-3H]Vitamin D$_3$ (1.9 Ci/mmol) was synthesized in this laboratory by L. LeVan by the method of S. Yamada (S. Yamada, H. K. Schnoes & H. F. DeLuca, unpublished work). [3α-3H]Vitamin D$_3$ (15 Ci/mmol) was synthesized in this laboratory by S. Yamada (S. Yamada, H. K. Schnoes & H. F. DeLuca, unpublished work). This method involves the formation of an iron–carbonyl complex with the vitamin, oxidation of the hydroxy group to the 3-ketone followed by reduction with B$_2$H$_4$. Both compounds were purified on a column (1 cm x 60 cm) of Lipidex 5000 (Packard Instruments, Downers Grove, IL) with hexane/chloroform (9:1, v/v) as eluent. 25(OH)[3α-3H]D$_2$ (1.9 Ci/mmol) was generated biologically by L. LeVan from [3α-3H]Vitamin D$_2$ (Bhattacharyya & DeLuca, 1974) and purified on a column (1 cm x 60 cm) of Sephadex LH-20 eluted with chloroform/hexane (1:1, v/v) and a column (1 cm x 60 cm) of Lipidex 5000 eluted with hexane/chloroform (9:1, v/v). 25(OH)[26,27-3H]D$_3$, (80 Ci/mmol) was synthesized in this laboratory by J. Napoli and M. Fivizzani (J. L. Napoli, M. A. Fivizzani & H. F. DeLuca, unpublished work) and purified on a column (1 cm x 60 cm) of Lipidex 5000 eluted with hexane/chloroform (9:1, v/v). 24,25-(OH)$_2$[26,27-3H]D$_3$ (80 Ci/mmol) was generated biologically from 25(OH)[26,27-3H]D$_3$ by the method of Tanaka et al. (1975), and 25,26(OH)$_2$[23,24-3H]D$_3$ (78 Ci/mmol) was generated biologically from 25(OH)[23,24-3H]D$_3$ (78 Ci/mmol; prepared by S. Yamada (Yamada et al., 1978)) by the method of Tanaka et al. (1975). Both of these compounds were purified on a column (2 cm x 40 cm) of Sephadex LH-20 eluted with hexane/chloroform/methanol (9:1:1, by vol.) and then a column (1 cm x 60 cm) of Sephadex LH-20 eluted with chloroform/hexane (13:7, v/v). 1,25(OH)$_2$[26,27-3H]D$_3$ (80 Ci/mmol) was generated biologically from 25(OH)[26,27-3H]D$_3$ by the method of Tanaka et al. (1975) and purified on a column (1 cm x 30 cm) of Sephadex LH-20 eluted with chloroform/hexane (13:7, v/v) and then a column (2 cm x 40 cm) of Sephadex LH-20 eluted with hexane/chloroform/methanol (9:1:1, by vol.). Radioactive metabolites that served as internal standards in the assay procedure were further purified on a h.p.l.c. Zorbax-SIL column by using the following solvent systems: for [3H]vitamin D$_3$, propan-2-ol/hexane (1:99, v/v); for 25(OH)[3H]D$_3$, propan-2-ol/hexane (1:24, v/v); for 24,25(OH)$_2$[3H]D$_3$, 25,26(OH)$_2$[3H]D$_3$ and 1,25(OH)$_2$[3H]D$_3$, propan-2-ol/hexane (1:9, v/v).

**Chromatography materials.** Sephadex LH-20 was purchased from Pharmacia, Piscataway, NJ, U.S.A. Lipidex 5000 (similar to hydroxyalkoxypropyl–Sephadex or HAPS) was obtained from Packard Instruments Co., Downers Grove, IL, U.S.A. Stainless-steel columns (25 cm x 4.6 mm internal diam.), prepacked with microparticulate Zorbax-SIL or Zorbax-ODS, were supplied by DuPont Instruments, Wilmington, DE, U.S.A.

**Animals.** Male weanling rats, purchased from the Holtzman Co., Madison, WI, U.S.A., were fed on an adequate-calcium adequate-phosphorus vitamin D-deficient diet for 3 weeks (Suda et al., 1970a) and served as a source of plasma binding protein. White Leghorn chickens (1-day-old) obtained from Northern Hatcheries, Beaver Dam, WI, U.S.A., were fed on an adequate-calcium vitamin D-deficient diet for 4 weeks (Eisman et al., 1976) and served as a source of intestinal cytosol binding protein.

Dextran (no. D-4751; clinical grade) and neutralized activated charcoal were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. $^3$H in fractions from column samples was counted with 35% counting efficiency in toluene scintillation solution (2 g of POPOP (2,5-diphenyloxazole) and 100 mg of dimethyl-POPOP (1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene) per litre of toluene), and that in binding
assays was counted with 28% counting efficiency in aqueous scintillation solution (5.5 g of PPO and 70 mg of dimethyl-POPOP per litre of 33% Triton X-100 in toluene).

**Plasma samples.** Much of the developmental work on the assay was done on a pool of normal human plasma from the local American Red Cross blood bank. Normal concentrations of vitamin D and its metabolites were determined by using plasma from healthy adult laboratory workers sampled in February. Anephric-human plasma samples were kindly supplied by Dr. E. Slatopolsky, Department of Medicine, Washington University, St. Louis, MO, U.S.A. and by Dr. C. Gallagher, Department of Internal Medicine, Creighton University, Omaha, NB, U.S.A.

**Procedures**

**Extraction of plasma samples.** All glassware used at this step and throughout the entire assay procedure was cleaned by rinsing with methanol and chloroform. To 3-5 ml of plasma and to counting vials in triplicate were added the following radioactive internal standards, each in 25 μl of ethanol, to monitor the analytical recoveries of the assay: 3000 c.p.m. of [3H]vitamin D₃, 25(OH)[3H]D₃ and 1,25(OH)₂[3H]D₃, and 2000 c.p.m. of 24,25(OH)₂-[3H]D₃ and 25,26(OH)₂[3H]D₃. After vortex-mixing the plasma samples and equilibrating the radioactive metabolites for 30 min, the lipids were extracted by adding 3.75 vol. of methanol/methylene chloride (2:1, v/v), shaking vigorously and venting, and then shaking for 5 min on a horizontal shaker at 3 oscillations/s. After leaving the samples for 15 min, the phases were separated by adding 1.25 vol. of methylene chloride and shaking them for 1 min, followed by centrifugation at 1500 g for 10 min. The lower methylene chloride layer was collected and the upper aqueous layer re-extracted with another 1.25 vol. of methylene chloride. The combined methylene chloride layers were evaporated under reduced pressure on a rotary evaporator, adding a small amount of ethanol to clear the solution, and the yellow lipid residue was solubilized in 0.5 ml of hexane/chloroform/methanol (9:1:1, by vol.).

**Chromatography of lipid extracts.** As indicated in Scheme 1, the lipid extracts were chromatographed on a column (0.7 cm x 12 cm) of Sephadex LH-20 in hexane/chloroform/methanol (9:1:1, by vol.). The plasma lipid extract was applied in 0.5 ml of solvent, followed by two rinses of 0.5 ml. Then 3.5 ml of solvent was added and the first 5.0 ml was collected for the vitamin D fraction. An additional 5.5 ml was added to the column and the 5.0–10.5 ml fraction collected for the 25(OH)D determination. Finally, 16.5 ml was added and the 10.5–27.0 ml fraction collected for the three dihydroxyvitamin D metabolites.

**Analysis of vitamin D₂ and vitamin D₃.** The vitamin D fraction from the initial Sephadex LH-20 column was further purified on a column (0.7 cm x 18 cm) of Lipidex 5000 eluted with hexane/chloroform (19:1, v/v). The sample was evaporated under N₂ and applied in 0.5 ml of solvent, followed by two 0.5 ml rinses. Then 9.0 ml was added, and the 0.0–10.5 ml fraction was discarded. An additional 7.0 ml was added and the 10.5–17.5 ml portion was collected for vitamin D analysis. The fraction was evaporated under N₂ and the residue redissolved in 50 μl of propan-2-ol/hexane (1:99, v/v).

Final purification of the vitamin D fraction was carried out by h.p.l.c. on a Zorbax-SIL column equilibrated in propan-2-ol/hexane (1:99, v/v). At a constant flow rate of 2.0 ml/min (6210 kPa), the sample was injected in 50 μl of solvent followed by a 50 μl rinse. The elution region corresponding to vitamin D₂ and vitamin D₃ (8.3–10.8 min, peak at 9.3 min) was collected and the eluate evaporated under N₂ and redissolved in 50 μl of methanol/water (49:1, v/v). The fraction collected was determined previously by using a crystalline vitamin D₃ standard.

Final quantification of plasma vitamin D₂ and vitamin D₃ was accomplished by reversed-phase h.p.l.c. on a Zorbax-ODS column equilibrated in methanol/water (49:1, v/v) at a constant flow rate of 1.5 ml/min (8280 kPa). A solution of known concentration (5 ng of vitamin D₂ plus 250 c.p.m. of [3H]vitamin D₃ per 5 μl) was prepared in methanol/ water (49:1, v/v). Amounts of 5, 10, 20 and 40 ng were injected and the [3H]vitamin D₃ fraction (11.2–13.7 min, peak at 12.2 min) was collected in counting vials, evaporated and counted for radioactivity in toluene scintillation solution along with equivalent portions of the standard solution. The peak heights at 0.002 or 0.005 absorbance unit (full scale) were divided by the percentage recovery to yield a standard curve relating corrected peak height to ng of vitamin D₃. A standard of vitamin D₂ was injected to determine its elution position (peak at 11.2 min). The plasma samples were injected in 50 μl of solvent with a 50 μl rinse and the vitamin D₃ fraction was collected, evaporated and counted for radioactivity in toluene scintillant along with the initial sample of [3H]-vitamin D₃. The recoveries of vitamins D₂ and D₃ were assumed to be the same. The peak heights of vitamin D₂ and vitamin D₃ in the sample were divided by the percentage recovery to yield corrected peak heights. Correcting standard and sample peak heights for recovery after h.p.l.c. avoids the assumption that standard and sample preparations are recovered with the same efficiency from the h.p.l.c. column. The sample peak heights were related to the standard curve to arrive at the total amount of vitamin D₂ and vitamin D₃ in the original plasma sample. Dividing by the sample volume yielded the concentration in ng/ml.
Human plasma (3-5 ml) + tritium-labelled metabolites

→ Methanol/methylene chloride extraction

→ Hexane/chloroform/methanol (9:1:1, by vol.); Sephadex LH-20

→ Vitamin D fraction

→ Hexane/chloroform (19:1, v/v); Lipidex 5000

→ Straight-phase h.p.l.c. on Zorbax-SIL; propan-2-ol/hexane (1:99, v/v)

→ Reversed-phase h.p.l.c. on Zorbax-ODS; water/methanol (1:49, v/v)

→ Quantification by u.v. absorbance (vitamin D2 and D3 forms)

→ 25(OH)D fraction

→ Hexane/chloroform (9:1, v/v); Lipidex 5000

→ Straight-phase h.p.l.c. on Zorbax-SIL; propan-2-ol/hexane (1:24, v/v)

→ 24,25(OH)2D

→ 25,26(OH)2D

→ 1,25(OH)2D

→ Quantification by competitive binding assay using rat plasma binding protein (total vitamin D)

→ Quantification by competitive binding assay using chick intestinal cytosol binding protein (total vitamin D)

Scheme 1. Outline of the multiple assay procedure for the analysis of vitamins D2 and D3, 25(OH)D2 and 25(OH)D3, 24,25(OH)2D, 25,26(OH)2D and 1,25(OH)2D in human plasma.
VITAMIN D METABOLITES

Analysis of 25(OH)D₂ and 25(OH)D₃. The 25(OH)D fraction from the initial Sephadex LH-20 column was evaporated under N₂ and chromatographed on a column (0.7 cm x 15 cm) of Lipidex 5000 eluted with hexane/chloroform (9:1, v/v). The sample was applied in 0.5 ml of solvent, followed by two 0.5 ml rinses. Then 12.5 ml was added and the 0.0-14.0 ml fraction was discarded. An additional 20.0 ml was added and the 14.0-34.0 ml fraction contained 25(OH)D. The fraction was evaporated under N₂ and the residue redissolved in 50 μl of propan-2-ol/hexane (1:24, v/v).

Final quantification of plasma 25(OH)D₂ and 25(OH)D₃ was accomplished by straight-phase h.p.l.c. on a Zorbax-SIL column equilibrated in propan-2-ol/hexane (1:24, v/v) at a constant flow rate of 2.0 ml/min (6210 kPa). A solution of known concentration [25ng of 25(OH)D₂ plus 250 c.p.m. of 25(OH)[³H]D₃ per 5 μl] was prepared in propan-2-ol/hexane (1:24, v/v). Amounts of 25, 50, 100 and 200 ng were injected, and the 25(OH)[³H]D₃ fraction (10.4-12.9 min, peak at 11.4 min) was collected in counting vials, evaporated and counted for radioactivity in toluene scintillant along with equivalent portions of the standard solution. The peak heights at 0.005 or 0.01 absorbance unit (full scale) were divided by the percentage recovery to yield a standard curve relating corrected peak height to ng of 25(OH)-D₃. A standard of 25(OH)D₂ was injected to determine its elution position (peak at 9.4 min). The plasma samples were injected in 50 μl of solvent with a 50 μl rinse, and the region containing 25(OH)D₃ was collected, evaporated and counted for radioactivity in toluene scintillant along with the initial portion of 25(OH)[³H]D₃. The recoveries of 25(OH)D₂ and 25(OH)D₃ were assumed to be the same. The peak heights of 25(OH)D₂ and 25(OH)D₃ in the sample were divided by the percentage recovery to yield corrected peak heights. The sample peak heights were related to the standard curve to arrive at the total amounts of 25(OH)D₂ and 25(OH)D₃ in the original plasma sample. Total amounts of 25(OH)D₂ determined from a 25(OH)D₃ standard curve are multiplied by 0.76. Dividing by the sample volume yielded the concentration in ng/ml.

Separation of dihydroxyvitamin D metabolites. The dihydroxyvitamin D-metabolite-containing fraction from the initial Sephadex LH-20 column was subjected to h.p.l.c. on a Zorbax-SIL column equilibrated in propan-2-ol/hexane (1:9, v/v) at a flow rate of 2.0 ml/min (6210 kPa). Elution positions were determined by injecting 10 μl of a standard ethanolic solution containing 5 ng of 24,25(OH)₂D₃/μl, 5 ng of 25,26(OH)₂D₃/μl and 7.5 ng of 1,25(OH)₂D₃/μl. The plasma samples were evaporated under N₂ and redissolved in 50 μl of propan-2-ol/hexane (1:9, v/v). They were injected with a 50 μl rinse and the following regions were collected separately, allowing room for vitamin D₂ metabolites: 24,25(OH)₂D₃, 5.0-7.5 min (peak at 6.0 min); 25,26(OH)₂D₃, 9.2-11.7 min (peak at 10.2 min); 1,25(OH)₂D₃, 14.4-18.7 min (peak at 16.4 min).

Analysis of 24,25(OH)₂D and 25,26(OH)₂D. Both were quantified by a rat plasma protein competitive binding assay modified from the method of Haddad et al. (1977). The 24,25(OH)₂D and 25,26(OH)₂D regions from the h.p.l.c. Zorbax-SIL column were evaporated under N₂ and the samples redissolved in 140 μl of ethanol. Duplicate 25 μl portions of each were counted for radioactivity along with the initial samples of 24,25(OH)₂[³H]D₃ and 25,26(OH)₂-[³H]D₃ in toluene scintillant to assess percentage recovery. Triplicate 25 μl portions of the 24,25-(OH)₂D and 25,26(OH)₂D samples were pipetted into separate 12 mm x 75 mm glass tubes. A standard curve was prepared with the following amounts of 25(OH)D₃ in triplicate in 25 μl of ethanol: 0.0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng plus 1 μg (22000-fold excess to determine non-specific binding). 25(OH)-[³H]D₃ (6000 c.p.m.) was added in 20 μl of ethanol to all standard and sample tubes, followed by 0.5 ml of diluted rat plasma (1:5000 dilution in 0.05 M sodium phosphate, pH 7.4) on ice and the contents were vortex-mixed and incubated for at least 1 h or overnight at 4°C. In a 5 min period, 0.2 ml of cold 5% charcoal/0.5% dextran suspension in the same buffer was added to all tubes on ice, and their contents were vortex-mixed. After 30 min on ice, the tubes were centrifuged at 4500 rev./min for 20 min at 4°C. Portions (0.5 ml) of the supernatant were mixed with 3.5 ml of aqueous counting solution to determine protein-bound radioactivity. The amounts of 24,25-(OH)₂D or 25,26(OH)₂D in each sample tube were determined by relating the bound radioactivity to the standard curve. The plasma concentration of 24,25(OH)₂D or 25,26(OH)₂D was then calculated by the following equation:

\[
\text{ng/ml} = \frac{\text{ng in sample tube}}{(25 \mu l / 140 \mu l) \times (\text{ml of plasma sample}) \times (\% \text{ recovery})}
\]

Analysis of 1,25(OH)₂D. This metabolite was assayed by a modification of the method of Eiseman et al. (1976), by using freeze-dried cytosol binding protein prepared from chick intestinal mucosa. The 1,25(OH)₂D fraction from the h.p.l.c. Zorbax-SIL column was evaporated under N₂ and redissolved in 210 μl of ethanol. Duplicate 25 μl samples were counted for radioactivity along with the initial portion of 1,25(OH)₂[³H]D₃ in toluene scintillant to assess percentage recovery. Triplicate 50 μl samples were pipetted into separate 12 mm x 75 mm
glass tubes. A standard curve was prepared with the following amounts of 1,25(OH)2D3 in quadruplicate in 50 μl of ethanol: 0.0, 1.5, 3.0, 6.0, 12.0, 24.0, 48.0 and 96.0 pg plus 7.7 ng (330-fold excess to determine non-specific binding). 1,25(OH)2[3H]D3 (approx. 400 c.p.m.) was added to each standard tube to compensate for the recovered radioactivity in the samples. Then 1,25(OH)2[3H]D3 (3000 c.p.m.) was added in 20 μl of ethanol to all standard and sample tubes. Reconstituted intestinal cytosol binding protein was diluted with cold buffer (0.025 M potassium phosphate, 0.1 M-KCl and 1 mM-dithiothreitol, pH 7.4) to 0.8 mg of protein/ml, and 0.5 ml of diluted cytosol was added to all tubes on ice and vortex-mixed. Protein was determined by the biuret method, with bovine serum albumin as standard. After 10 min on ice, the tubes were incubated for 1 h at 25°C in a water bath at 120 oscillations/min and returned to the ice. Cold 0.5% charcoal/0.05% dextran suspension in the same buffer (0.2 ml) was added in a 5 min period to all tubes on ice, and their contents were vortex-mixed. After 10 min on ice, the tubes were centrifuged at 4500 rev./min for 20 min at 4°C. Portions (0.5 ml) of the supernatant were mixed with 3.5 ml of aqueous scintillant to determine protein-bound radioactivity. The amount of 1,25-(OH)2D in each sample tube was determined by relating the bound radioactivity to the standard curve. The plasma concentration of 1,25(OH)2D was then calculated by the following equation:

\[
\text{pg/ml} = \frac{\text{pg in sample tube}}{(50 \mu l/210 \mu l) \times (\text{ml of plasma sample}) \times (\% \text{ recovery})}
\]

**Results**

*Extraction of plasma samples*

Repetitive extractions with diethyl ether, methylene chloride or ethyl acetate alone yielded unsatisfactory recoveries for some of the iritiated metabolites of vitamin D added to plasma. Total lipid extraction, by the method of Bligh & Dyer (1959), with the use of either chloroform or methylene chloride (Bouillon et al., 1976), yielded superior recoveries of 90% or more after extraction of vitamin D, 25(OH)D2, 24,25(OH)2D, 25,26(OH)2D and 1,25(OH)2D.

*Initial chromatography of lipid extracts*

The initial chromatographic step, batch elution from Sephadex LH-20 in hexane/chloroform/methanol (9:1:1, by vol.), removed the bulk of the interfering lipids from 25(OH)D and the dihydroxyvitamin D metabolites and separated the compounds into three fractions: the vitamin D fraction, the 25(OH)D fraction and the dihydroxyvitamin D fraction. A typical chromatogram illustrating the profile of the various vitamin D standards is shown in Fig. 1(a).

No separation of the vitamins D2 and D3 forms of the various metabolites was observed with this column.

**Analysis of vitamin D2 and vitamin D3**

Adequate removal of interfering u.v.-absorbing substances in the Sephadex LH-20 vitamin D-containing fraction was accomplished by batch elution on Lipidex 5000 eluted with hexane/chloroform (19:1, v/v), and by h.p.l.c. on a Zorbax-SIL column eluted with propan-2-ol/hexane (1:99, v/v). Vitamins D2 and D3 were collected together in both systems, as no separation was observed (Figs. 1b and 2a). They were nearly resolved by h.p.l.c. on a Zorbax-ODS column eluted with methanol/water (49:1, v/v) (Fig. 2b). Standard curves of vitamins D2 and D3, relating peak heights corrected for recovery losses to ng applied, were virtually identical and linear (Fig. 3). A typical h.p.l.c. profile of a normal human plasma sample (Fig. 4a) shows two peaks co-eluted with vitamin D2 and vitamin D3 standards, and the effective removal of all interfering 254 nm-absorbing compounds from this region of the chromatogram. Also shown are h.p.l.c. profiles from anephric-human plasma and from plasma sampled immediately after the normal subject returned from 1 week of swimming in the Caribbean during midwinter (Figs. 4b and 4c). No u.v.-absorbing peaks in the vitamin D2- and vitamin D3-containing regions were observed in plasma from vitamin D-deficient chicks or rats.

**Analysis of 25(OH)D2 and 25(OH)D3**

Interfering u.v.-absorbing contaminants in the 25(OH)D-containing fraction eluted from Sephadex LH-20 were removed by batch elution on Lipidex 5000 eluted with hexane/chloroform (9:1, v/v). 25(OH)D2 and 25(OH)D3 were slightly resolved by using this column (Fig. 1c), but were collected together. They were completely resolved by h.p.l.c. on a Zorbax-SIL column eluted with propan-2-ol/hexane (1:24, v/v) (Fig. 5). Peak heights of the standards of 25(OH)D2 and 25(OH)D3, corrected for recovery losses, bore a linear relationship to the amount applied (Fig. 6). Since the 25(OH)D2-containing peak is slightly sharper than that containing 25(OH)D3 on the Zorbax-SIL column, the amount of 25(OH)D2 represented by a given peak height was only 76% of the amount of 25(OH)D3 represented (Fig. 6). Therefore 25(OH)D2 determinations are multiplied by 0.76 when 25(OH)D3 is used...
Fig. 1. Purification of vitamin D metabolites by conventional column chromatography
(a) Elution of vitamin D and its major metabolites from a column (0.7 cm x 12 cm) of Sephadex LH-20 developed with a solvent system of hexane/chloroform/methanol (9:1:1, by vol.). (b) Elution of vitamin D2 and vitamin D3 from a column (0.7 cm x 18 cm) of Lipidex 5000 developed with a solvent system of hexane/chloroform (19:1, v/v). (c) Elution of 25(OH)D2 and 25(OH)D3 from a column (0.7 cm x 15 cm) of Lipidex 5000 developed with a solvent system of hexane/chloroform (9:1, v/v).

Fig. 2. H.p.l.c. with detection by measuring absorbance at 254 nm of vitamins D2 and D3
(a) Co-elution on a column (0.46 cm x 25 cm) of Zorbax-SIL silicic acid developed with a solvent system of propan-2-ol/hexane (1:99, v/v) at a flow rate of 2.0 ml/min. (b) Separation on a column (0.46 cm x 25 cm) of reversed-phase Zorbax-ODS developed with a solvent system of water/methanol (1:49, v/v) at a flow rate of 1.5 ml/min.

Fig. 3. Standard curve of vitamins D2 (○) and D3 (●), relating corrected peak height to amount (ng) of vitamin D2 or vitamin D3
as a standard. A typical Zorbax-SIL profile of a normal human plasma sample (Fig. 7a) shows two peaks that were co-eluted with 25(OH)D₂ and 25(OH)D₃ standards and the effective removal of all interfering 254 nm-absorbing compounds from this region. Also shown are h.p.l.c. profiles from anephri-
human plasma and from plasma sampled immediately after the normal subject returned from 1 week of swimming in the Caribbean during midwinter (Figs. 7b and 7c). No peaks of u.v.-absorbing material in the regions containing 25(OH)D$_2$ and 25(OH)D$_3$ were observed in plasma from vitamin D-deficient chicks or rats. When the 25(OH)D$_2$- and 25(OH)D$_3$-containing peaks were collected from the Zorbax-SIL column and rechromatographed on a reversed-phase Zorbax-ODS system, homogeneous peaks were observed that were exactly co-eluted with their respective standards. A water blank, supplemented with 100.0 ng of 25(OH)D$_3$ and analysed, was found to contain 100.4 ng with an overall recovery of 53.9%. Previous work with a very similar h.p.l.c. assay of 25(OH)D gave plasma concentrations in good agreement with values obtained from a competitive protein-binding assay of the same samples (Eisman et al., 1977).

**Analysis of 24,25(OH)$_2$D and 25,26(OH)$_2$D**

Separation of 24,25(OH)$_2$D, 25,26(OH)$_2$D and 1,25(OH)$_2$D and purification from interfering binding contaminants was achieved by h.p.l.c. on a Zorbax-SIL column eluted with propan-2-ol/hexane (1:9, v/v) (Fig. 8). Since 24,25(OH)$_2$D$_2$ and 1,25-(OH)$_2$D$_2$ are eluted slightly before their vitamin D$_3$ analogues in this system (Jones & DeLuca, 1975), collection of these and 25,26(OH)$_2$D is timed to allow for isolation of both analogues. H.p.l.c. using u.v. detection did not prove sensitive enough for routine measurement of 24,25(OH)$_2$D or 25,26-(OH)$_2$D, so a rat plasma competitive-protein binding assay was developed.

Since unlabelled 25(OH)D$_3$, 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$ were equipotent in their displacement of 25(OH)$_3$[H]D$_3$ from rat plasma binding protein (Fig. 9), 25(OH)$_3$[H]D$_3$ and unlabelled 25(OH)D$_3$ were used to construct a standard curve for the convenient common assay of 24,25(OH)$_2$D and 25,26(OH)$_2$D. 25(OH)D$_2$ and 25(OH)D$_3$ are equally recognized by the rat binding protein (Preece et al., 1974; Haddad et al., 1976), so it seems likely to be true for 24,25(OH)$_2$D$_2$ and 25,26(OH)$_2$D$_2$ as well.

After Sephadex LH-20 chromatography of the lipid extracts, the dihydroxyvitamin D fraction was chromatographed on two Zorbax-SIL columns in series eluted with propan-2-ol/hexane (13:87, v/v) at 2.0 ml/min, and 1 ml fractions were collected and analysed by the rat-plasma-protein-binding assay. As shown in the binding profile in Fig. 10, normal

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**Fig. 8. H.p.l.c. with u.v. detection at 254 nm of the major vitamin D metabolites on a column (0.46 cm × 25 cm) of Zorbax-SIL silicic acid developed with a solvent system of propan-2-ol/hexane (1:9, v/v) at a flow rate of 2.0 ml/min**
human plasma is resolved into at least four peaks of binding activity present in the dihydroxyvitamin D-containing fraction from the Sephadex LH-20 column. Whereas peaks II [apparently present because of incomplete resolution of the 25(OH)D- and dihydroxyvitamin D-containing fractions from the Sephadex LH-20 column], III and IV are co-eluted with standards of 25(OH)D₃, 24,25(OH)₂D₃ and 25,26(OH)₃D₃, the unidentified peak I is eluted in the region of very non-polar compounds. A similar analysis of anephric-human plasma (Fig. 10) shows the same four peaks, but there is considerably more peak I and less 24,25(OH)₂D (peak III)-binding activity than in normal human plasma.

To determine whether peak I is related to vitamin D or is introduced by the solvents used, deionized water, vitamin D-deficient chick plasma and plasma from vitamin D₃-repleted chicks and rats were extracted and their dihydroxyvitamin D fractions collected from the Sephadex LH-20 column and chromatographed on a single Zorbax-SIL column. As shown in Figs. 11(a) and 11(b), peak I binding activity is not observed in the water blank, but is observed in vitamin D-deficient chick plasma; peaks II, III and IV were not observed. Plasma from both vitamin D-repleted chicks and rats (Figs. 11c and 11d) is resolved into peaks I, II [25(OH)D₃], III [24,25(OH)₂D₃] and IV [25,26(OH)₂D₃], as observed in human plasma. The peak I binding activity is much lower in vitamin D-repleted than in vitamin D-deficient chick plasma. Also present in both vitamin D-repleted chick and rat plasma is another peak (peak X) of binding activity, which is eluted immediately before 24,25(OH)₂D₃.

The analysis of 24,25(OH)₂D in chick and rat plasma after h.p.l.c. on Zorbax-SIL would be interfered with by the presence of peak X. It was found, however, that reversed-phase h.p.l.c. on Partisil-ODS (Whatman column, 0.46 cm × 25 cm) eluted with water/methanol (3:7, v/v) at 2.0 ml/min is capable of completely resolving peak X and 24,25(OH)₂D (Fig. 12). Rechromatography of both the human 24,25(OH)₂D- and 25,26(OH)₂D-binding peaks from the Zorbax-SIL system by reversed-phase Partisil-ODS h.p.l.c. yielded single homogeneous binding peaks that were co-eluted with authentic 24,25(OH)₂D₃ and 25,26(OH)₂D₃, and confirmed that there is no interference with the assay of 24,25(OH)₂D and 25,26(OH)₂D by peak X or other binding contaminants.

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**Fig. 10.** H.p.l.c. Zorbax-SIL with detection by assay with rat plasma binding protein of the dihydroxyvitamin D-containing fraction from Sephadex LH-20 isolated from plasma from normal (-----) and anephric (——) humans

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Fig. 11. H.p.l.c. on Zorbax-SIL with detection by assay with rat plasma binding protein of the dihydroxyvitamin D-containing fraction from Sephadex LH-20 isolated from (a) a water blank, (b) vitamin D-deficient chick plasma, (c) vitamin D₃-repleted chick plasma and (d) vitamin D₃-repleted rat plasma

Fig. 12. Reversed-phase h.p.l.c. on Partisil-ODS with detection by assay with rat plasma binding protein of the peak X through the 1,25(OH)₂D₃-containing region isolated from vitamin D₃-repleted chick plasma from Zorbax-SIL column
Analysis of 1,25(OH)\textsubscript{2}D

1,25(OH)\textsubscript{2}D was separated from 24,25(OH)\textsubscript{2}D, 25,26(OH)\textsubscript{2}D and interfering binding contaminants by h.p.l.c. as described above (Fig. 8). H.p.l.c. with u.v. detection was not sensitive enough to measure 1,25(OH)\textsubscript{2}D, so a modified competitive binding assay with chick intestinal cytosol protein was used. Shown in Fig. 13 is a typical standard binding curve of the displacement of 1,25(OH)\textsubscript{2}[\textsuperscript{3}H]D\textsubscript{3} by unlabelled 1,25(OH)\textsubscript{2}D\textsubscript{3}. 1,25(OH)\textsubscript{2}D\textsubscript{2} and 1,25(OH)\textsubscript{2}D\textsubscript{3} are equally recognized by this binding protein and can be assayed together (Eisman et al., 1976).

After Sephadex LH-20 chromatography of the lipid extracts, the dihydroxyvitamin D fraction was chromatographed on two Zorbax-SIL columns in series as described above, and 1ml fractions were collected and analysed by the chick-intestinal-cytosol-protein-binding assay. The binding profile of normal human plasma (Fig. 14) had small peaks of binding activity that were co-eluted with 25(OH)D\textsubscript{3}, 24,25-(OH)\textsubscript{2}D\textsubscript{3} and 25,26(OH)\textsubscript{2}D\textsubscript{3} standards plus a substantial peak (peak I) of binding activity eluted at the void volume. Also present were two homogeneous binding peaks that were co-eluted with authentic 1,25(OH)\textsubscript{2}D\textsubscript{2} and 1,25(OH)\textsubscript{2}D\textsubscript{3}. A similar analysis of anephric-human plasma (Fig. 14) showed the same small peaks of binding activity that were co-eluted with 25(OH)D\textsubscript{3}, 24,25(OH)\textsubscript{2}D\textsubscript{3} and 25,26-(OH)\textsubscript{2}D\textsubscript{3} standards plus a much greater amount of binding activity appearing in peak I. No 1,25(OH)\textsubscript{2}D...
peaks were detectable in anephric-human plasma nor in vitamin D-deficient chick or rat plasma.

**Assay characteristics**

The overall recoveries (means ± s.d.) of 3H-labelled internal standards added to the plasma after the final purification steps of the procedure for vitamin D, 25(OH)D3, 24,25(OH)2D3, 25,26(OH)2D3 and 1,25(OH)2D3 were respectively 50.1 ± 7.2% (n = 25), 74.4 ± 5.0% (n = 20), 71.9 ± 6.2% (n = 25), 75.2 ± 5.6% (n = 25) and 58.4 ± 14.8% (n = 25). For the determinations of 25(OH)D, 24,25(OH)2D, 25,26(OH)2D and 1,25(OH)2D, the intra-assay coefficients of variation were respectively 8% (n = 5), 12% (n = 6), 9% (n = 6) and 17% (n = 7), and the interassay coefficients of variation were 10% (n = 8), 13% (n = 3), 19% (n = 3) and 26% (n = 9). The routine sensitivities for the vitamin D and 25(OH)D assays by h.p.l.c. with u.v. detection were 2 and 5 ng, whereas the sensitivity for the combined 24,25(OH)2D- and 25,26(OH)2D-binding assay was 0.1 ng/tube and that for the 1,25(OH)2D-binding assay was 5 pg/tube. Therefore the limits of detection in plasma for vitamin D, 25(OH)D, 24,25(OH)2D or 25,26(OH)2D and 1,25(OH)2D were respectively 0.5 ng/ml, 1 ng/ml, 0.2 ng/ml and 7 pg/ml.

**Plasma concentrations of vitamin D and metabolites (see Table 1)**

Total vitamin D determined by the h.p.l.c. procedure in plasma samples taken from a group of normal laboratory workers in February had a range of 0.9–7.2 ng/ml (n = 8). Vitamin D2 in these samples was 0.5–4.6 ng/ml, and vitamin D3 was 0.7–5.7 ng/ml. Total 25(OH)D in normal samples ranged from 20.6 to 45.7 ng/ml (n = 19). 25(OH)D2 and 25(OH)D3 had respective ranges of 1.0–15.9 ng/ml and 19.6–41.9 ng/ml. The ranges of 24,25(OH)2D, 25,26(OH)2D and 1,25(OH)2D respectively were 1.6–5.8 ng/ml (n = 12), 0.3–1.6 ng/ml (n = 12) and 20–39 pg/ml (n = 20).

Plasma samples taken from two normal subjects in February immediately upon returning from 1 week of swimming in the Caribbean were also analysed. The vitamin D2 concentrations were 1.5 and 0.5 ng/ml, and vitamin D3 was 31.2 and 21.0 ng/ml. 25(OH)D2 was 1.6 and 1.0 ng/ml, and 25(OH)D3 was 58.2 and 52.8 ng/ml. Total 24,25(OH)2D was 3.2 and 5.4 ng/ml, and total 25,26(OH)2D was 0.3 and 0.6 ng/ml.

Plasma samples from anephric patients were also subjected to the multiple assay. Total vitamin D had a range of 1.8–4.1 ng/ml (n = 6), with ranges of 0.7–2.4 ng/ml and 0.5–1.9 ng/ml for vitamin D2 and vitamin D3 respectively. Total 25(OH)D had a range of 18.6–75.9 ng/ml (n = 9), with ranges of 2.2–34.4 ng/ml and 9.5–27.7 ng/ml for 25(OH)D2 and 25(OH)D3 respectively. Total 24,25(OH)2D and 25,26(OH)2D had ranges of 0.5–4.2 ng/ml (n = 9) and 0.5–1.3 ng/ml (n = 9) respectively. There was no detectable 1,25-(OH)2D.

**Discussion**

The method outlined in this paper represents a new procedure for the quantification of vitamin D and its metabolites in a single small sample of human plasma. It is reasonably fast, accurate and reproducible. Before the vitamin D compounds can be analysed, the plasma lipid extract must be purified with separation of the metabolites to remove contaminants that interfere with measurement by h.p.l.c. using u.v. detection or by binding assay. Small-batch columns of Sephadex LH-20 (Holick & DeLuca, 1971) or Lipidex 5000 (Ellingboe et al., 1970) have proved useful in providing suitable samples for h.p.l.c. with good recoveries. With batch elution, several samples (routinely 10–12) can be run simultaneously and quickly. The method is simple and quite reproducible from column to column and from batch to batch of Sephadex LH-20 or Lipidex 5000.

The physicochemical analysis of vitamin D and 25(OH)D by h.p.l.c. with u.v. detection overcomes

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### Table 1. Vitamin D metabolite concentrations in plasma from normal and anephric man

For details of determinations see the text. Results are means ± s.d. for the numbers of determinations given in the text.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Normal</th>
<th>Normal with high exposure to sun</th>
<th>Anephric</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D2</td>
<td>1.2 ± 1.4</td>
<td>1.0 ± 0.7</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>2.3 ± 1.6</td>
<td>26.1 ± 7.2</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Total vitamin D</td>
<td>3.5 ± 2.5</td>
<td>27.1 ± 7.9</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>25(OH)D2</td>
<td>3.9 ± 3.1</td>
<td>1.3 ± 0.4</td>
<td>18.7 ± 9.0</td>
</tr>
<tr>
<td>25(OH)D3</td>
<td>27.6 ± 9.2</td>
<td>55.5 ± 3.8</td>
<td>17.7 ± 12.2</td>
</tr>
<tr>
<td>Total 25(OH)D</td>
<td>31.6 ± 9.3</td>
<td>56.8 ± 4.2</td>
<td>36.4 ± 16.5</td>
</tr>
<tr>
<td>24,25(OH)2D</td>
<td>3.5 ± 1.4</td>
<td>4.3 ± 1.6</td>
<td>1.9 ± 1.3</td>
</tr>
<tr>
<td>25,26(OH)2D</td>
<td>0.7 ± 0.5</td>
<td>0.5 ± 0.2</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>1,25(OH)2D</td>
<td>0.031 ± 0.009</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>
the inherent variability and sensitivity to interfering compounds associated with competitive binding assays. Furthermore, this method allows the individual measurement of vitamins D$_3$ and D$_2$ and 25(OH)D$_2$ and 25(OH)D$_3$. Attempts to analyse normal plasma samples for vitamin D on the Zorbax-T3 column directly after purification on Lipidex 5000 eluted with hexane/chloroform (19:1, v/v) did not succeed because the peaks produced are extremely small and often immeasurable in the presence of impurities, although samples with much higher concentrations of vitamin D could be measured in this manner.

The equal recognition of 24,25(OH)$_2$D and 25,26-(OH)$_2$D by the rat plasma binding protein allows the convenient use of a common assay to quantify the two steroids, with 25(OH)D$_3$ and 25(OH)[3H]D$_3$ as the standard and tracer. Thus only small amounts of biosynthetically prepared 24,25(OH)$_2$[3H]D$_3$ and 25,26(OH)$_2$[3H]D$_3$ are needed to serve as internal standards. However, the binding protein used reacts with compounds other than the known vitamin D metabolites. Rat-plasma-protein-binding analysis of fractions from Sephadex LH-20 chromatography, containing dihydroxyvitamin D metabolites, shows that at least two compounds exhibiting binding activity [peak I, unknown, and peak II, probably 25(OH)D] are removed from this region by h.p.l.c. Furthermore, the incomplete resolution of peak X and 24,25(OH)$_2$D in rat and chick plasma shows the need for an additional reversed-phase h.p.l.c. system. Thus, extensive purification of 24,25(OH)$_2$D and 25,26(OH)$_2$D isolated from plasma by h.p.l.c. is necessary before meaningful competitive binding analysis is possible.

The 1,25(OH)$_2$D isolated by h.p.l.c. on Zorbax-SIL appears to be homogeneous and reliably measured by the chick-intestinal-cytosol competitive protein-binding assay. As with the rat plasma protein, purification by Zorbax-SIL h.p.l.c. is necessary to remove interfering binding substances. The modifications of the method of Eisman et al. (1976), i.e., use of dextran-coated charcoal instead of polyethylene glycol precipitation, allow for a more sensitive less time-consuming binding assay.

Our mean values for the concentrations of vitamin D and 25(OH)D in normal human plasma (see Table 1) generally agree with previously reported values as determined by either h.p.l.c. or competitive binding-protein techniques (Jones, 1978; Eisman et al., 1977; Haddad & Chyu, 1971; Preece et al., 1974). These observations support the view that vitamin D is rapidly stored, with little circulating in the plasma (Ponchon & DeLuca, 1969), whereas 25(OH)D is the major circulating form of vitamin D. The total vitamin D and 25(OH)D concentrations in anephric-human plasma were similar to our normal values, but the vitamin D$_2$ analogue comprised a greater proportion of both compounds in anephric-human plasma, whereas the vitamin D$_3$ analogue predominated in normal plasma. Of note are the much higher concentrations of vitamin D and 25(OH)D in plasma from people recently exposed to much sunlight. Although their vitamin D$_2$ and 25(OH)D$_2$ concentrations were normal, their vitamin D$_3$ and 25(OH)D$_3$ concentrations were much higher than normal, resulting in higher concentrations of total vitamin D and 25(OH)D, as reported for life-guards (Haddad & Chyu, 1971).

Our mean value for the normal plasma concentration of 24,25(OH)$_2$D was similar to that reported by Haddad et al. (1977), but higher than that by Taylor et al. (1976). This may be a reflection of lower vitamin D intakes in the U.K., as compared with the U.S.A. Our mean value for the 24,25(OH)$_2$D concentration in anephric-human plasma was considerably lower than that measured by Haddad et al. (1977), suggesting that the contaminating peak I-binding activity (much higher in anephric-human than in normal plasma) that we have observed is interfering in their assay (Horst et al., 1979). The inability of Taylor et al. (1976) to detect 24,25(OH)$_2$D in anephric-human plasma is apparently due to their lower assay sensitivity and to lower values in the U.K. because of a difference in vitamin D status. The presence of 24,25(OH)$_2$D in anephric-human plasma suggests that an extrarenal 25(OH)D$_2$ 24-hydroxylase is present in man; this is supported by work demonstrating the existence of this enzyme in rat intestine (Kumar et al., 1978; Tanaka et al., 1977).

The plasma concentrations of 25,26(OH)$_2$D do not change from our normal values upon nephrectomy or exposure to sunlight. Although 25,26(OH)$_2$D is synthesized by chick kidney homogenates (Tanaka et al., 1978), there is apparently an extrarenal 25(OH)D 26-hydroxylase as well.

The normal plasma concentration of 1,25(OH)$_2$D is in good agreement with that previously reported (Eisman et al., 1976; Brumbaugh et al., 1974). The lack of 1,25(OH)$_2$D in anephric-human plasma also agrees with Eisman et al. (1976) and Brumbaugh et al. (1974) and confirms the removal of interfering binding substances by the purification procedure.

Multiple assays have been reported by Caldas et al. (1978) and Lambert et al. (1977a,b) for human plasma. The former measures total 25(OH)D, 24,25(OH)$_2$D and 1,25(OH)$_2$D by competitive protein-binding assays with normal values in good agreement with ours, but it does not measure vitamin D and 25,26(OH)$_2$D nor 25(OH)D$_2$ and 25(OH)D$_3$ individually by h.p.l.c. It also makes use of h.p.l.c. for purification. The method of Lambert et al. (1977b) measures total vitamin D, 25(OH)D and 24,25(OH)$_2$D by h.p.l.c., and 1,25(OH)$_2$D by competitive protein-binding assay, but its reliability seems suspect. Their normal value for vitamin D
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(Lambert et al., 1977b) is much higher than that reported by us or Jones (1978) and lower still than in their original abstract (Lambert et al., 1977a). Also, their original normal value for 24,25(OH)\textsubscript{2}D (Lambert et al., 1977a) was much higher than reported by us or others. Yet their final value for 24,25(OH)\textsubscript{2}D (Lambert et al., 1977b) is more reasonable, but we find that this concentration produces an h.p.l.c. peak too small to be reliably measured. As a result, we were forced to use the more sensitive competitive protein-binding assay. Thus their assay is probably reliable only for total 25(OH)D and 1,25(OH)\textsubscript{2}D and is unable to measure vitamins D\textsubscript{2} and D\textsubscript{3} or 25(OH)D\textsubscript{2} and 25(OH)D\textsubscript{3} individually.

In summary, we report a multiple assay procedure that can measure, with sensitivity and accuracy, vitamin D\textsubscript{2} and vitamin D\textsubscript{3}, 25(OH)D\textsubscript{2} and 25(OH)D\textsubscript{3}, 24,25(OH)\textsubscript{2}D, 25,26(OH)\textsubscript{2}D and 1,25(OH)\textsubscript{2}D in a single 3–5 ml sample of plasma. It is possible to streamline the procedure and measure metabolites of particular interest, e.g. 25(OH)D\textsubscript{2}, 25,25(OH)\textsubscript{2}D and 1,25-(OH)\textsubscript{2}D. The method has been put to routine use in our laboratory to measure metabolite concentrations in plasma samples from humans, rats, chicks and cows. In the development of this multiple assay, we realized that chromatographic purification of plasma lipid extracts with separation of vitamin D metabolites and removal of interfering contaminants is extremely important in obtaining accurate measurements of vitamin D and its metabolites, whether detected by h.p.l.c. or competitive protein-binding assays. Thus values in earlier literature of metabolites obtained by methods that used inadequate chromatography are suspect. The multiple assay procedure outlined in this paper should prove to be a useful tool in the study of clinical disease states related to vitamin D in humans and in studies on vitamin D metabolism in laboratory research animals.

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