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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 D_2 and D_3 (ergocalciferol and cholecalciferol), 25(OH)D_2 (25-hydroxyvitamin D_2) and 25(OH)D_3 (25-hydroxyvitamin D_3), 24,25(OH)$_2$D (24,25-dihydroxyvitamin D), 25,26(OH)$_2$D (25,26-dihydroxyvitamin D) and 1,25(OH)$_2$D (1,25-dihydroxyvitamin D) in a single 3–5ml 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 D_2 and D_3 and 25(OH)D_2 and 25(OH)D_3 are quantified by h.p.l.c. by using u.v. detection, comparing their peak heights with those of standards. 24,25(OH)$_2$D and 25,26(OH)$_2$D are measured by competitive protein-binding assay with diluted plasma from vitamin D-deficient rats. 1,25(OH)$_2$D 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 ± 2.5ng/ml; 25(OH)D 31.6 ± 9.3ng/ml; 24,25(OH)$_2$D 3.5 ± 1.4ng/ml; 25,26(OH)$_2$D 0.7 ± 0.5ng/ml; 1,25(OH)$_2$D 31 ± 9pg/ml (means ± S.D.). Values in two normal human plasma samples taken in February after 1 week of high sun exposure are: vitamin D 27.1 ± 7.9ng/ml; 25(OH)D 56.8 ± 4.2ng/ml; 24,25(OH)$_2$D 4.3 ± 1.6ng/ml; 25,26(OH)$_2$D 0.5 ± 0.2ng/ml. Values in anephric-human plasma are: vitamin D 2.7 ± 0.8ng/ml; 25(OH)D 36.4 ± 16.5ng/ml; 24,25(OH)$_2$D 1.9 ± 1.3ng/ml; 25,26(OH)$_2$D 0.6 ± 0.3ng/ml; 1,25(OH)$_2$D 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)$_2$D or 24,25(OH)$_2$D (DeLuca & Schnoes, 1976). 1,25-(OH)$_2$D 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)$_2$D or 25,26(OH)$_2$D [another known metabolite of 25(OH)D (Suda *et al.*, 1970*b*)] 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)$_2$D or 24,25(OH)$_2$D 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 metabolic 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)$_2$D (Haddad *et al.*, 1976*b*, 1977; Taylor *et al.*, 1976), and 1,25-(OH)$_2$D (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 D_2, ergocalciferol; vitamin D_3, cholecalciferol; 25(OH)D_2, 25-hydroxyvitamin D_2; 25(OH)D_3, 25-hydroxyvitamin D_3; 24,25-(OH)$_2D_2$, 24,25-dihydroxyvitamin D_2; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26(OH)$_2$D, 25,26-dihydroxyvitamin D; 1,25(OH)$_2D_2$, 1,25-dihydroxyvitamin D_2; 1,25(OH)$_2D_3$, 1,25-dihydroxyvitamin D_3; 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, 25,26$(OH)_2$D and 1,25$(OH)_2$D in a single 3–5ml 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 (254nm) 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 18200 litre·mol^{-1}·cm^{-1} at 265nm). 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-$(OH)_2D_3$ and 1,25$(OH)_2D_3$ were gifts from Hoffman–La Roche, Nutley, NJ, U.S.A. 25,26-$(OH)_2D_3$ was synthesized in this laboratory (Lam *et al.*, 1975). [3α-^{3}H]Vitamin D_2 (1.9Ci/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-^{3}H]Vitamin D_3 (15Ci/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^3H_4. Both compounds were purified on a column (1cm×60cm) of Lipidex 5000 (Packard Instruments, Downers Grove, IL) with hexane/chloroform (9:1, v/v) as eluent. 25(OH)[3α-^{3}H]D_2 (1.9Ci/mmol) was generated biologically by L. LeVan from [3α-^{3}H]vitamin D_2 (Bhattacharyya & DeLuca, 1974) and purified on a column (1cm×60cm) of Sephadex LH-20 eluted with chloroform/hexane (1:1, v/v) and a column (1cm×60cm) of Lipidex 5000 eluted with hexane/chloroform (9:1, v/v). 25(OH)[26,27-^{3}H]D_3 (80Ci/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 (1cm×60cm) of Lipidex 5000 eluted with hexane/chloroform (9:1, v/v). 24,25-$(OH)_2$[26,27-^{3}H]D_3 (80Ci/mmol) was generated biologically from 25(OH)[26,27-^{3}H]D_3 by the method of Tanaka *et al.* (1975), and 25,26$(OH)_2$[23,24-^{3}H]D_3 (78Ci/mmol) was generated biologically from 25(OH)[23,24-^{3}H]D_3 [78Ci/mmol; prepared by S. Yamada (Yamada *et al.*, 1978)] by the method of Tanaka *et al.* (1978). Both of these compounds were purified on a column (2cm×40cm) of Sephadex LH-20 eluted with hexane/chloroform/methanol (9:1:1, by vol.) and then a column (1cm×60cm) of Sephadex LH-20 eluted with chloroform/hexane (13:7, v/v). 1,25$(OH)_2$[26,27-^{3}H]D_3 (80Ci/mmol) was generated biologically from 25(OH)[26,27-^{3}H]D_3 by the method of Tanaka *et al.* (1975) and purified on a column (1cm×30cm) of Sephadex LH-20 eluted with chloroform/hexane (13:7, v/v) and then a column (2cm×40cm) 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 [^{3}H]vitamin D_3, propan-2-ol/hexane (1:99, v/v); for 25(OH)[^{3}H]D_3, propan-2-ol/hexane (1:24, v/v); for 24,25$(OH)_2$-[^{3}H]D_3, 25,26$(OH)_2$[^{3}H]D_3 and 1,25$(OH)_2$[^{3}H]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 (25cm×4.6mm 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.*, 1970*a*) 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 {2g of PPO (2,5-diphenyloxazole) and 100mg 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 (5.5g of PPO and 70mg 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

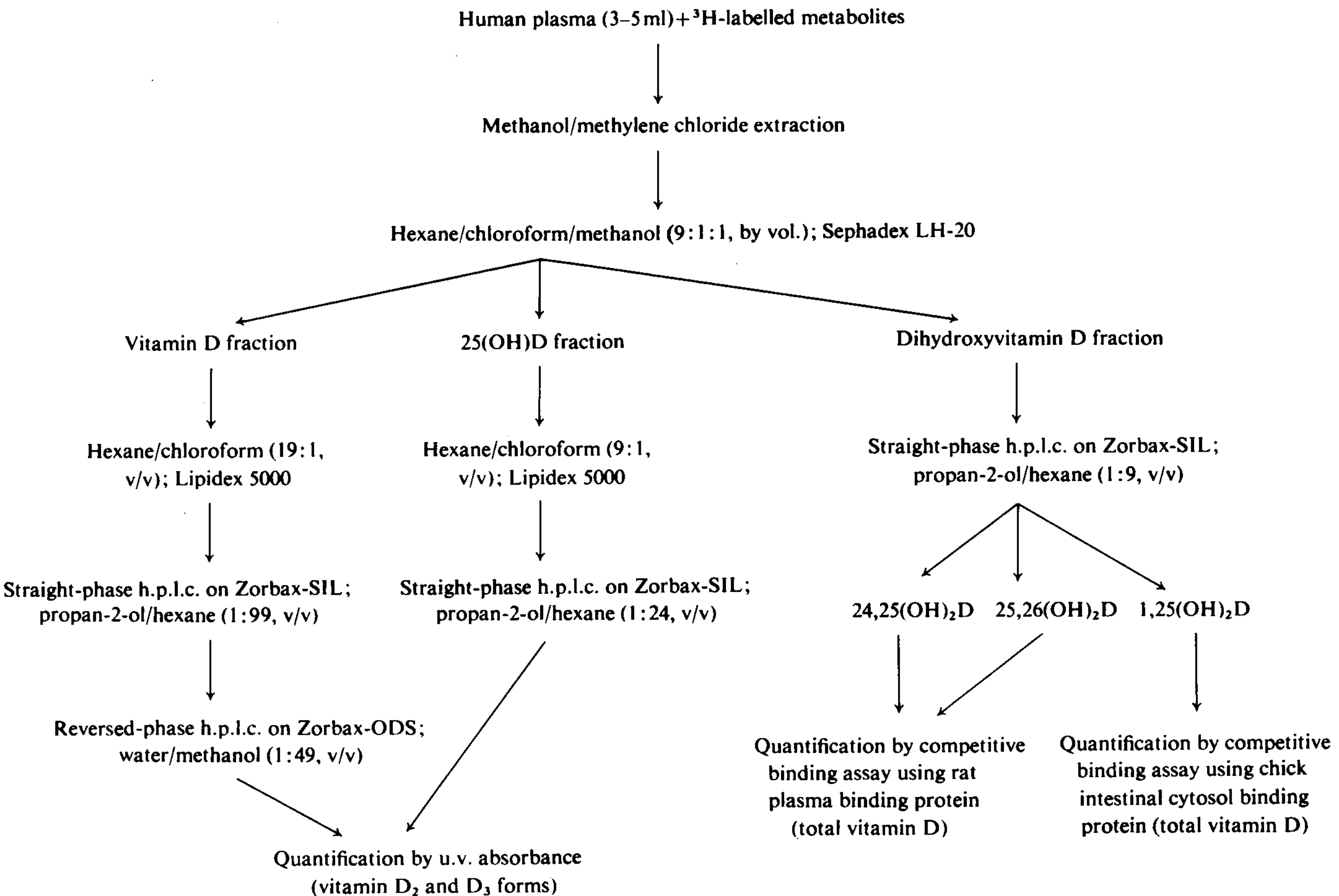
Extraction of plasma samples. All glassware used at this step and throughout the entire assay procedure was cleaned by rinsing with methanol and chloroform. To 3–5ml of plasma and to counting vials in triplicate were added the following radioactive internal standards, each in 25μl of ethanol, to monitor the analytical recoveries of the assay: 3000c.p.m. of [^{3}H]vitamin D_3, 25(OH)[^{3}H]D_3 and 1,25(OH)$_2$[^{3}H]D_3, and 2000c.p.m. of 24,25(OH)$_2$-[^{3}H]D_3 and 25,26(OH)$_2$[^{3}H]D_3. After vortex-mixing the plasma samples and equilibrating the radioactive metabolites for 30min, the lipids were extracted by adding 3.75vol. of methanol/methylene chloride (2:1, v/v), shaking vigorously and venting, and then shaking for 5min on a horizontal shaker at 3 oscillations/s. After leaving the samples for 15min, the phases were separated by adding 1.25vol. of methylene chloride and shaking them for 1min, followed by centrifugation at 1500g for 10min. The lower methylene chloride layer was collected and the upper aqueous layer re-extracted with another 1.25vol. 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 0.5ml 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 (0.7cm×12cm) of Sephadex LH-20 in hexane/chloroform/methanol (9:1:1, by vol.). The plasma lipid extract was applied in 0.5ml of solvent, followed by two rinses of 0.5ml. Then 3.5ml of solvent was added and the first 5.0ml was collected for the vitamin D fraction. An additional 5.5ml was added to the column and the 5.0–10.5ml fraction collected for the 25(OH)D determination. Finally, 16.5ml was added and the 10.5–27.0ml fraction collected for the three dihydroxyvitamin D metabolites.

Analysis of vitamin D_2 and vitamin D_3. The vitamin D fraction from the initial Sephadex LH-20 column was further purified on a column (0.7cm×18cm) of Lipidex 5000 eluted with hexane/chloroform (19:1, v/v). The sample was evaporated under N_2 and applied in 0.5ml of solvent, followed by two 0.5ml rinses. Then 9.0ml was added, and the 0.0–10.5ml fraction was discarded. An additional 7.0ml was added and the 10.5–17.5ml portion was collected for vitamin D analysis. The fraction was evaporated under N_2 and the residue redissolved in 50μl 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 2.0ml/min (6210kPa), the sample was injected in 50μl of solvent followed by a 50μl rinse. The elution region corresponding to vitamin D_2 and vitamin D_3 (8.3–10.8min, peak at 9.3min) was collected and the eluate evaporated under N_2 and redissolved in 50μl of methanol/water (49:1, v/v). The fraction collected was determined previously by using a crystalline vitamin D_3 standard.

Final quantification of plasma vitamin D_2 and vitamin D_3 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 1.5ml/min (8280kPa). A solution of known concentration (5ng of vitamin D_3 plus 250c.p.m. of [^{3}H]vitamin D_3 per 5μl) was prepared in methanol/water (49:1, v/v). Amounts of 5, 10, 20 and 40ng were injected and the [^{3}H]vitamin D_3 fraction (11.2–13.7min, peak at 12.2min) 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 D_3. A standard of vitamin D_2 was injected to determine its elution position (peak at 11.2min). The plasma samples were injected in 50μl of solvent with a 50μl rinse and the vitamin D_3 fraction was collected, evaporated and counted for radioactivity in toluene scintillant along with the initial sample of [^{3}H]-vitamin D_3. The recoveries of vitamins D_2 and D_3 were assumed to be the same. The peak heights of vitamin D_2 and vitamin D_3 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 D_2 and vitamin D_3 in the original plasma sample. Dividing by the sample volume yielded the concentration in ng/ml.



Scheme 1. *Outline of the multiple assay procedure for the analysis of vitamins D_2 and D_3, 25(OH)D_2 and 25(OH)D_3, 24,25(OH)$_2$D, 25,26(OH)$_2$D and 1,25(OH)$_2$D in huma plasma*

Analysis of 25(OH)D_2 and 25(OH)D_3. The 25(OH)D fraction from the initial Sephadex LH-20 column was evaporated under N_2 and chromatographed on a column (0.7cm × 15cm) of Lipidex 5000 eluted with hexane/chloroform (9:1, v/v). The sample was applied in 0.5ml of solvent, followed by two 0.5ml rinses. Then 12.5ml was added and the 0.0–14.0ml fraction was discarded. An additional 20.0ml was added and the 14.0–34.0ml fraction contained 25(OH)D. The fraction was evaporated under N_2 and the residue redissolved in 50μl of propan-2-ol/hexane (1:24, v/v).

Final quantification of plasma 25(OH)D_2 and 25(OH)D_3 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 2.0ml/min (6210kPa). A solution of known concentration {25ng of 25(OH)D_3 plus 250c.p.m. of 25(OH)[^{3}H]D_3 per 5μl} was prepared in propan-2-ol/hexane (1:24, v/v). Amounts of 25, 50, 100 and 200ng were injected, and the 25(OH)[^{3}H]D_3 fraction (10.4–12.9min, peak at 11.4min) 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)-D_3. A standard of 25(OH)D_2 was injected to determine its elution position (peak at 9.4min). The plasma samples were injected in 50μl of solvent with a 50μl rinse, and the region containing 25(OH)D_3 was collected, evaporated and counted for radioactivity in toluene scintillant along with the initial portion of 25(OH)[^{3}H]D_3. The recoveries of 25-(OH)D_2 and 25(OH)D_3 were assumed to be the same. The peak heights of 25(OH)D_2 and 25(OH)D_3 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)D_2 and 25(OH)D_3 in the original plasma sample. Total amounts of 25(OH)D_2 determined from a 25(OH)D_3 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 2.0ml/min (6210kPa). Elution positions were determined by injecting 10μl of a standard ethanol solution containing 5ng of 24,25(OH)$_2$$D_3$/μl, 5ng of 25,26(OH)$_2$$D_3$/μl and 7.5ng of 1,25(OH)$_2$$D_3$/μl. The plasma samples were evaporated under N_2 and redissolved in 50μl of propan-2-ol/hexane (1:9, v/v). They were injected with a 50μl rinse and the following regions were collected separately, allowing room for vitamin D_2 metabolites: 24,25(OH)$_2$$D_3$, 5.0–7.5min (peak at 6.0min); 25,26(OH)$_2$$D_3$, 9.2–11.7min (peak at 10.2min); 1,25(OH)$_2$$D_3$, 14.4–18.7min (peak at 16.4min).

Analysis of 24,25(OH)$_2$D and 25,26(OH)$_2$D. Both were quantified by a rat plasma protein competitive binding assay modified from the method of Haddad *et al.* (1977). The 24,25(OH)$_2$D and 25,26(OH)$_2$D regions from the h.p.l.c. Zorbax-SIL column were evaporated under N_2 and the samples redissolved in 140μl of ethanol. Duplicate 25μl portions of each were counted for radioactivity along with the initial samples of 24,25(OH)$_2$[^{3}H]D_3 and 25,26(OH)$_2$-[^{3}H]D_3 in toluene scintillant to assess percentage recovery. Triplicate 25μl portions of the 24,25-(OH)$_2$D and 25,26(OH)$_2$D samples were pipetted into separate 12mm × 75mm glass tubes. A standard curve was prepared with the following amounts of 25(OH)D_3 in triplicate in 25μl of ethanol: 0.0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4ng plus 1μg (22000-fold excess to determine non-specific binding). 25(OH)-[^{3}H]D_3 (6000c.p.m.) was added in 20μl of ethanol to all standard and sample tubes, followed by 0.5ml of diluted rat plasma (1:5000 dilution in 0.05M-sodium phosphate, pH7.4) on ice and the contents were vortex-mixed and incubated for at least 1h or overnight at 4°C. In a 5min period, 0.2ml 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 30min on ice, the tubes were centrifuged at 4500rev./min for 20min at 4°C. Portions (0.5ml) of the supernatant were mixed with 3.5ml of aqueous counting solution to determine protein-bound radioactivity. The amounts of 24,25-(OH)$_2$D or 25,26(OH)$_2$D in each sample tube were determined by relating the bound radioactivity to the standard curve. The plasma concentration of 24,25(OH)$_2$D or 25,26(OH)$_2$D was then calculated by the following equation:

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

Analysis of 1,25(OH)$_2$D. This metabolite was assayed by a modification of the method of Eisman *et al.* (1976), by using freeze-dried cytosol binding protein prepared from chick intestinal mucosa. The 1,25(OH)$_2$D fraction from the h.p.l.c. Zorbax-SIL column was evaporated under N_2 and redissolved in 210μl of ethanol. Duplicate 25μl samples were counted for radioactivity along with the initial portion of 1,25(OH)$_2$[^{3}H]D_3 in toluene scintillant to assess percentage recovery. Triplicate 50μl samples were pipetted into separate 12mm × 75mm

glass tubes. A standard curve was prepared with the following amounts of $1,25(OH)_2D_3$ 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). $1,25(OH)_2[^3H]D_3$ (approx. 400 c.p.m.) was added to each standard tube to compensate for the recovered radioactivity in the samples. Then $1,25(OH)_2[^3H]D_3$ (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:

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

No separation of the vitamins D_2 and D_3 forms of the various metabolites was observed with this column.

Analysis of vitamin D_2 and vitamin D_3

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 D_2 and D_3 were collected together in both systems, as no separation was observed (Figs. 1*b* and 2*a*). They were nearly resolved by h.p.l.c. on a Zorbax-ODS column eluted with methanol/water (49:1, v/v) (Fig. 2*b*). Standard curves of vitamins D_2 and D_3, 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. 4*a*) shows two peaks co-eluted with vitamin D_2 and vitamin D_3 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. 4*b* and 4*c*). No u.v.-absorbing peaks in the vitamin D_2- and vitamin D_3-containing regions were observed in plasma from vitamin D-deficient chicks or rats.

Results

Extraction of plasma samples

Repetitive extractions with diethyl ether, methylene chloride or ethyl acetate alone yielded unsatisfactory recoveries for some of the tritiated 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)D, $24,25(OH)_2D$, $25,26(OH)_2D$ 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*).

Analysis of $25(OH)D_2$ and $25(OH)D_3$

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). $25(OH)D_2$ and $25(OH)D_3$ were slightly resolved by using this column (Fig. 1*c*), 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 $25(OH)D_2$ and $25(OH)D_3$, corrected for recovery losses, bore a linear relationship to the amount applied (Fig. 6). Since the $25(OH)D_2$-containing peak is slightly sharper than that containing $25(OH)D_3$ on the Zorbax-SIL column, the amount of $25(OH)D_2$ represented by a given peak height was only 76% of the amount of $25(OH)D_3$ represented (Fig. 6). Therefore $25(OH)D_2$ determinations are multiplied by 0.76 when $25(OH)D_3$ is used

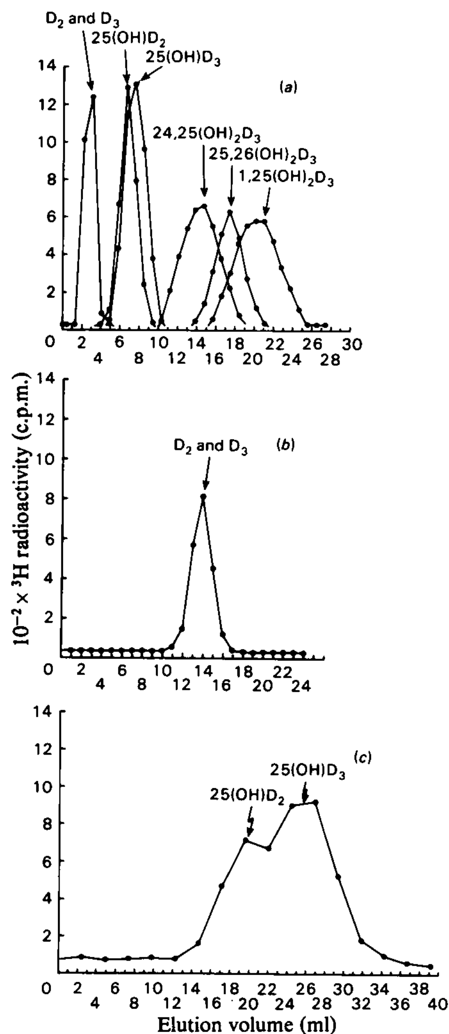


Fig. 1. *Purification of vitamin D metabolites by conventional column chromatography*

(*a*) Elution of vitamin D and its major metabolites from a column (0.7cm × 12cm) of Sephadex LH-20 developed with a solvent system of hexane/chloroform/methanol (9:1:1, by vol.). (*b*) Elution of vitamin D_2 and vitamin D_3 from a column (0.7cm × 18cm) of Lipidex 5000 developed with a solvent system of hexane/chloroform (19:1, v/v). (*c*) Elution of $25(OH)D_2$ and $25(OH)D_3$ from a column (0.7cm × 15cm) of Lipidex 5000 developed with a solvent system of hexane/chloroform (9:1, v/v).

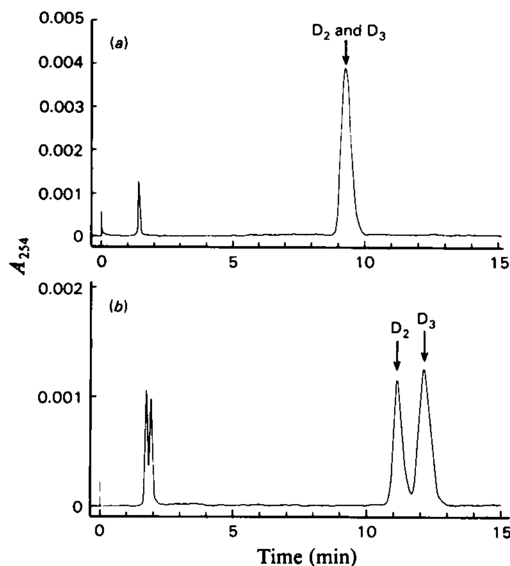


Fig. 2. *H.p.l.c. with detection by measuring absorbance at 254nm of vitamins D_2 and D_3*

(*a*) Co-elution on a column (0.46cm × 25cm) of Zorbax-SIL silicic acid developed with a solvent system of propan-2-ol/hexane (1:99, v/v) at a flow rate of 2.0ml/min. (*b*) Separation on a column (0.46cm × 25cm) of reversed-phase Zorbax-ODS developed with a solvent system of water/methanol (1:49, v/v) at a flow rate of 1.5ml/min.

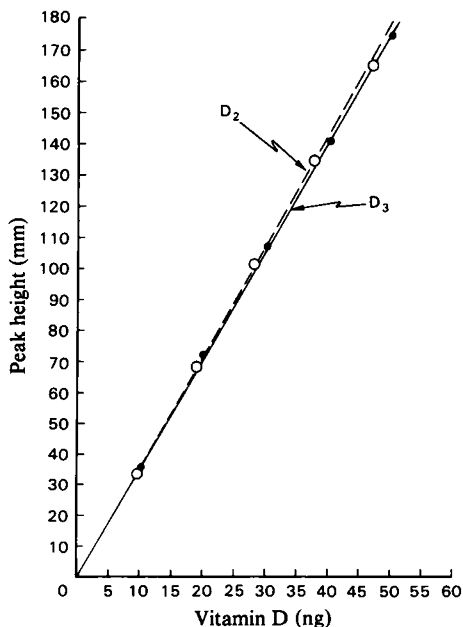


Fig. 3. *Standard curve of vitamins D_2 (○) and D_3 (●), relating corrected peak height to amount (ng) of vitamin D_2 or vitamin D_3*

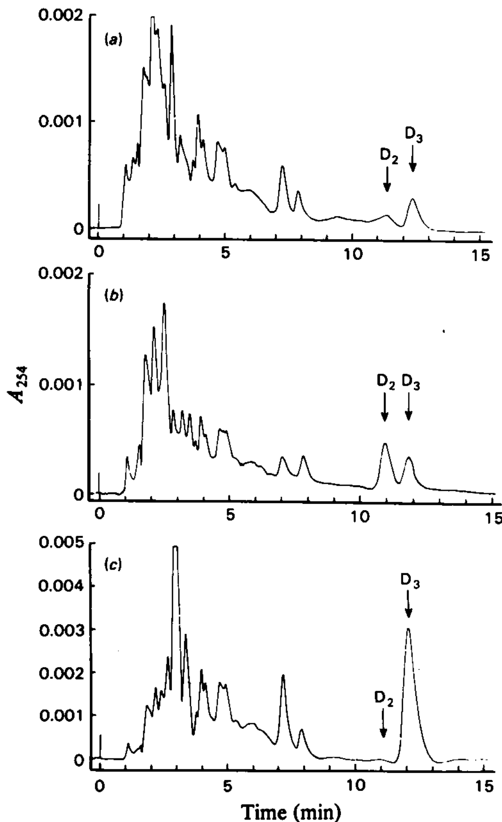


Fig. 4. *H.p.l.c. with u.v. detection at 254nm of vitamins D_2 and D_3 isolated from plasma of* (*a*) *normal humans,* (*b*) *anephric humans and* (*c*) *normal humans exposed to much sunlight*

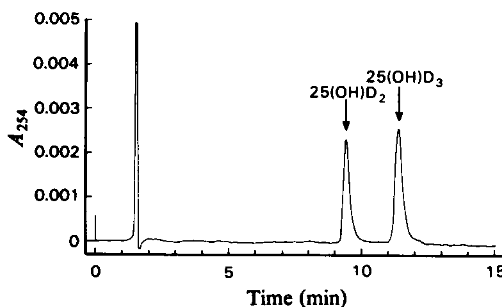


Fig. 5. *H.p.l.c. with u.v. detection at 254nm of 25(OH)D_2 and 25(OH)D_3 standards on a column* (0.46cm × 25cm) *of Zorbax-SIL silicic acid developed with a solvent system of propan-2-ol/hexane* (1:24, *v/v*) *at a flow rate of* 2.0*ml/min*

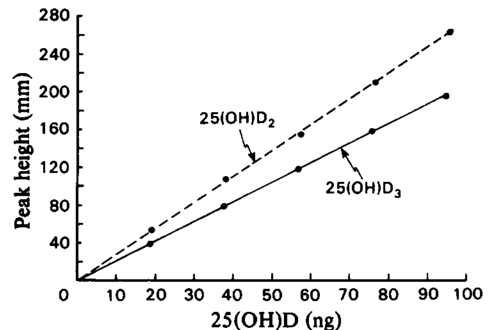


Fig. 6. *Standard curve of 25(OH)D_2 and 25(OH)D_3, relating corrected peak height to amount* (*ng*) *of metabolite*
The amount of 25(OH)D_2 represented by a given peak height was 76% of 25(OH)D_3.

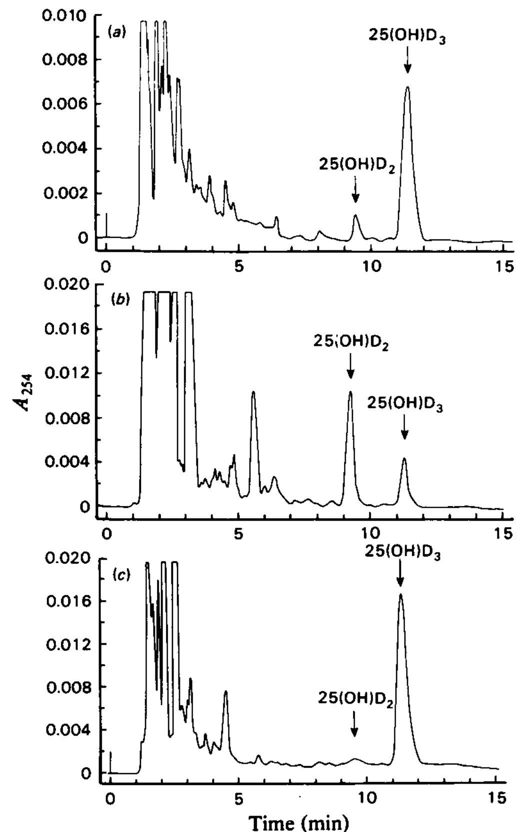


Fig. 7. *H.p.l.c. with u.v. detection at 254nm of 25(OH)D_2 and 25(OH)D_3 isolated from plasma of* (*a*) *normal humans,* (*b*) *anephric humans and* (*c*) *normal humans exposed to much sunlight*

as a standard. A typical Zorbax-SIL profile of a normal human plasma sample (Fig. 7*a*) shows two peaks that were co-eluted with 25(OH)D_2 and 25(OH)D_3 standards and the effective removal of all interfering 254nm-absorbing compounds from this region. Also shown are h.p.l.c. profiles from anephric-

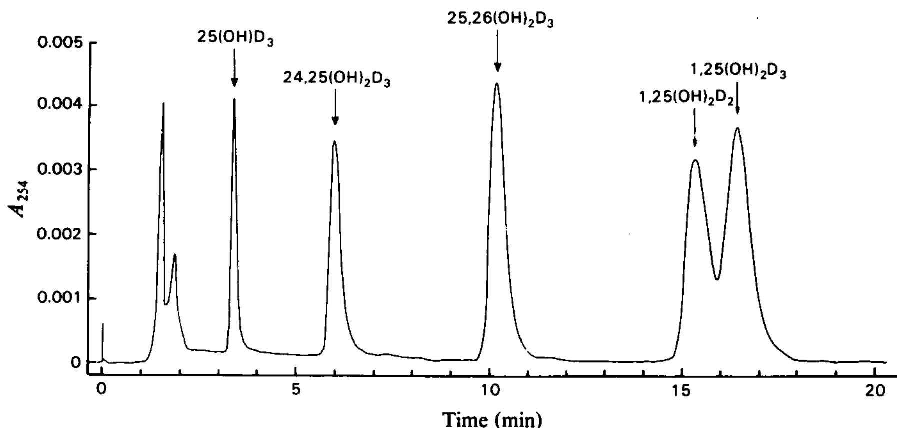


Fig. 8. *H.p.l.c. with u.v. detection at* 254*nm of the major vitamin D metabolites on a column* (0.46*cm*×25*cm*) *of Zorbax-SIL silicic acid developed with a solvent system of propan-2-ol/hexane* (1:9, *v/v*) *at a flow rate of* 2.0*ml/min*

human plasma and from plasma sampled immediately after the normal subject returned from 1 week of swimming in the Caribbean during midwinter (Figs. 7*b* and 7*c*). 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 100.0ng of $25(OH)D_3$ and analysed, was found to contain 100.4ng 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 $24,25(OH)_2D$ *and* $25,26(OH)_2D$

Separation of $24,25(OH)_2D$, $25,26(OH)_2D$ and $1,25(OH)_2D$ 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 $24,25(OH)_2D_2$ and $1,25(OH)_2D_2$ are eluted slightly before their vitamin D_3 analogues in this system (Jones & DeLuca, 1975), collection of these and $25,26(OH)_2D$ 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 $24,25(OH)_2D$ or $25,26(OH)_2D$, so a rat plasma competitive-protein binding assay was developed.

Since unlabelled $25(OH)D_3$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ were equipotent in their displacement

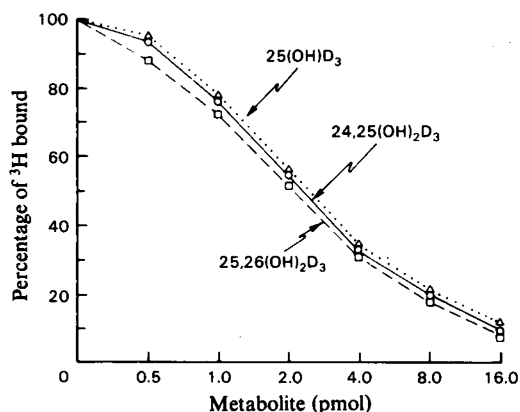


Fig. 9. *Competitive displacement of* $25(OH)[^3H]D_3$ *from rat plasma binding protein by* $25(OH)D_3$ (△), $24,25(OH)_2D_3$ (○) *and* $25,26(OH)_2D_3$ (□)

Each point represents the mean of three replicates.

of $25(OH)[^3H]D_3$ from rat plasma binding protein (Fig. 9), $25(OH)[^3H]D_3$ and unlabelled $25(OH)D_3$ were used to construct a standard curve for the convenient common assay of $24,25(OH)_2D$ and $25,26(OH)_2D$. $25(OH)D_2$ and $25(OH)D_3$ are equally recognized by the rat binding protein (Preece *et al.*, 1974; Haddad *et al.*, 1976*a*), so it seems likely to be true for $24,25(OH)_2D_2$ and $25,26(OH)_2D_2$ 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 2.0ml/min, and 1ml fractions were collected and analysed by the rat-plasma-protein-binding assay. As shown in the binding profile in Fig. 10, normal

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)D_3, 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$, 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 D_3-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. 11*c* and 11*d*) is resolved into peaks I, II [25(OH)D_3], III [24,25$(OH)_2D_3$] and IV [25,26$(OH)_2D_3$], 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$(OH)_2D_3$.

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, 0.46cm×25cm) eluted with water/methanol (3:7, v/v) at 2.0ml/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$(OH)_2D_3$ and 25,26$(OH)_2D_3$, 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.

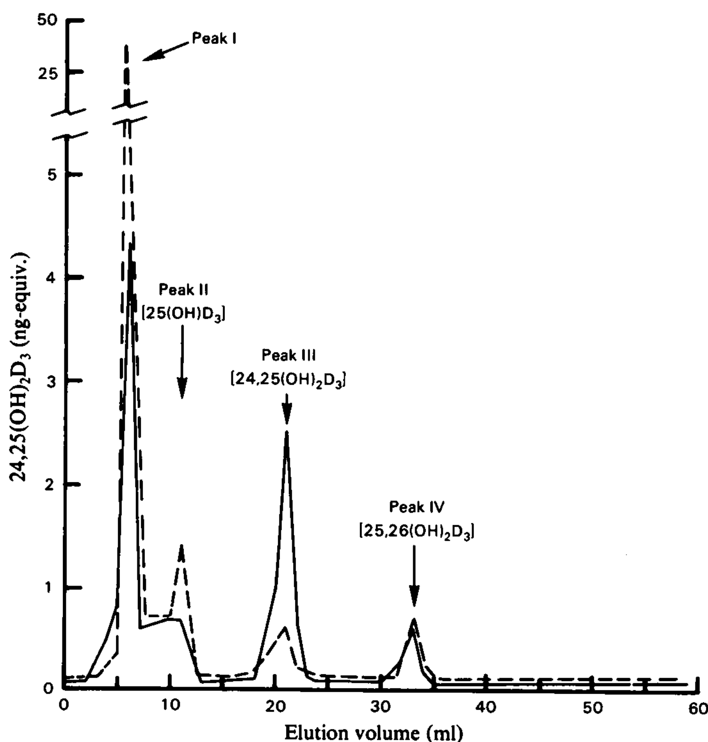


Fig. 10. *H.p.l.c. Zorbax-SIL with detection by assay with rat plasma binding protein of the dihydroxyvitamin D-containing fraction from Sephadex LH-20 isolated from plasma from normal (——) and anephric (----) humans*

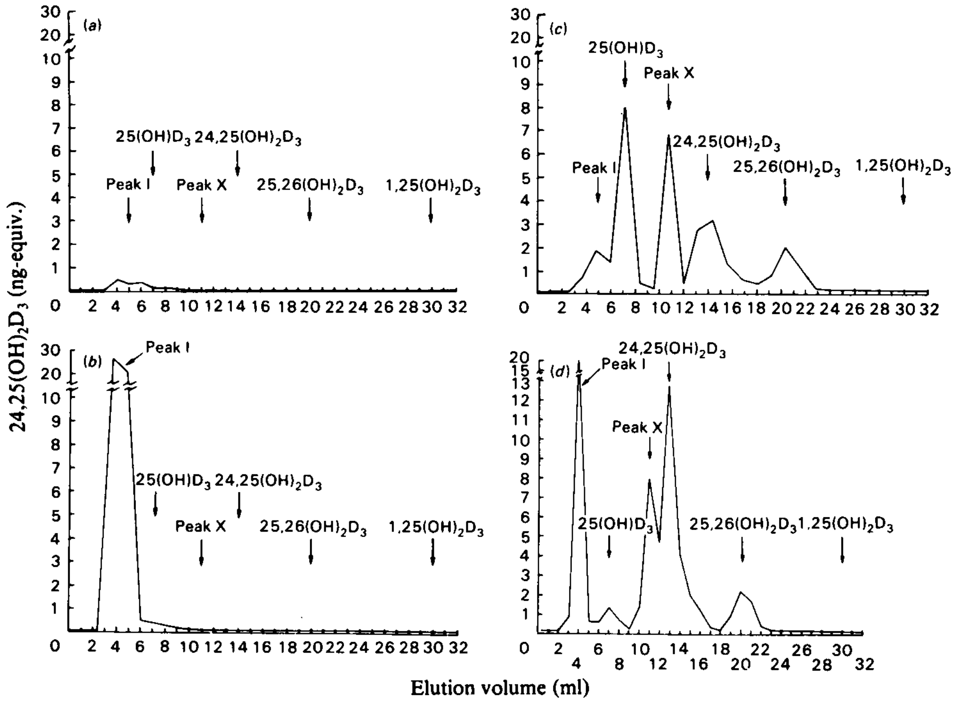


Fig. 11. *H.p.l.c. on Zorbax-SIL with detection by assay with rat plasma binding protein of the dihydroxyvitamin D-containing fraction from Sephadex LH-20 isolated from (a) a water blank, (b) vitamin D-deficient chick plasma, (c) vitamin D_3-repleted chick plasma and (d) vitamin D_3-repleted rat plasma*

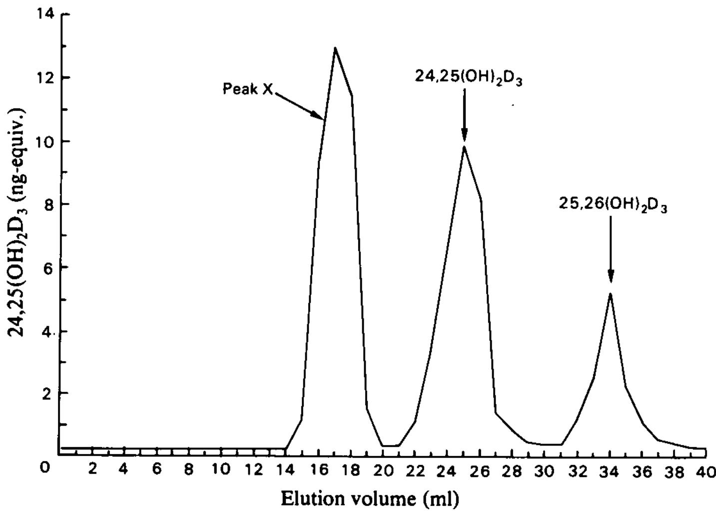


Fig. 12. *Reversed-phase h.p.l.c. on Partisil-ODS with detection by assay with rat plasma binding protein of the peak X through the 1,25(OH)$_2$D$_3$-containing region isolated from vitamin D_3-repleted chick plasma from Zorbax-SIL column*

Analysis of $1{,}25(OH)_2D$

$1{,}25(OH)_2D$ was separated from $24{,}25(OH)_2D$, $25{,}26(OH)_2D$ 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 $1{,}25(OH)_2D$, 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 $1{,}25(OH)_2[^3H]D_3$ by unlabelled $1{,}25(OH)_2D_3$. $1{,}25(OH)_2D_2$ and $1{,}25(OH)_2D_3$ are equally recognized by this binding protein and can be assayed together (Eisman *et al.*, 1976).

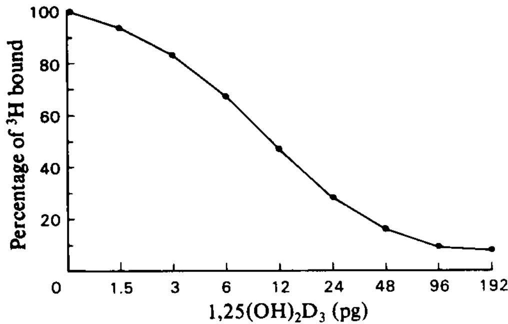


Fig. 13. *Competitive displacement of* $1{,}25(OH)_2[^3H]D_3$ *from chick intestinal cytosol binding protein by* $1{,}25(OH)_2D_3$
Each point represents the mean of four replicates.

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 1 ml 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 $25(OH)D_3$, $24{,}25(OH)_2D_3$ and $25{,}26(OH)_2D_3$ 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 $1{,}25(OH)_2D_2$ and $1{,}25(OH)_2D_3$. A similar analysis of anephric-human plasma (Fig. 14) showed the same small peaks of binding activity that were co-eluted with $25(OH)D_3$, $24{,}25(OH)_2D_3$ and $25{,}26(OH)_2D_3$ standards plus a much greater amount of binding activity appearing in peak I. No $1{,}25(OH)_2D$

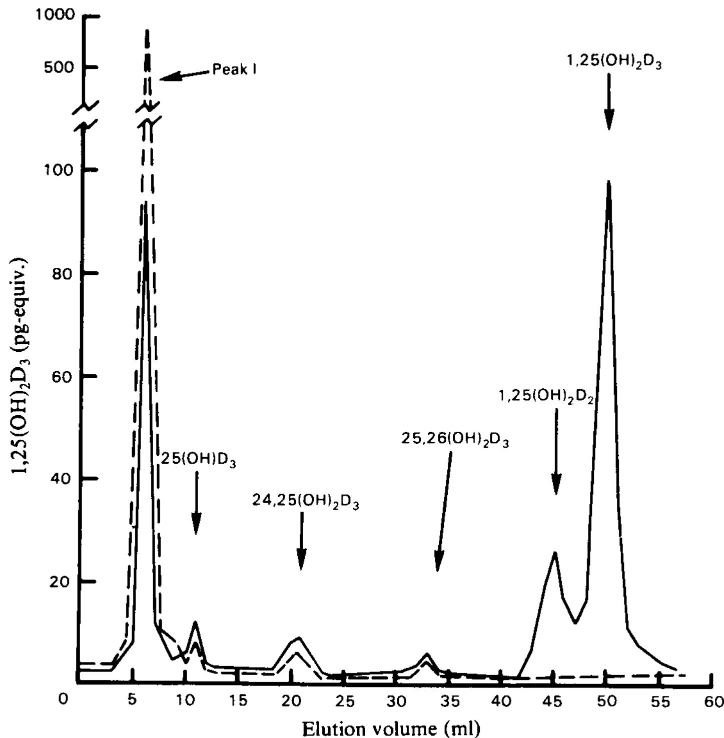


Fig. 14. *H.p.l.c. on Zorbax-SIL, with detection by assay with chick intestinal cytosol binding protein, of the dihydroxyvitamin D-containing fraction from Sephadex LH-20 isolated from normal (——) and anephric (----) human plasma*

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.

	Plasma concentration (ng/ml)		
Metabolite	Normal	Normal with high exposure to sun	Anephric
Vitamin D_2	1.2 ± 1.4	1.0 ± 0.7	1.3 ± 0.6
Vitamin D_3	2.3 ± 1.6	26.1 ± 7.2	1.4 ± 0.6
Total vitamin D	3.5 ± 2.5	27.1 ± 7.9	2.7 ± 0.8
$25(OH)D_2$	3.9 ± 3.1	1.3 ± 0.4	18.7 ± 9.0
$25(OH)D_3$	27.6 ± 9.2	55.5 ± 3.8	17.7 ± 12.2
Total 25(OH)D	31.6 ± 9.3	56.8 ± 4.2	36.4 ± 16.5
$24,25(OH)_2D$	3.5 ± 1.4	4.3 ± 1.6	1.9 ± 1.3
$25,26(OH)_2D$	0.7 ± 0.5	0.5 ± 0.2	0.6 ± 0.3
$1,25(OH)_2D$	0.031 ± 0.009	—	n.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_3, $25(OH)D_3$, $24,25(OH)_2D_3$, $25,26(OH)_2D_3$ and $1,25(OH)_2D_3$ 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 D_2 in these samples was 0.5–4.6 ng/ml, and vitamin D_3 was 0.7–5.7 ng/ml. Total 25(OH)D in normal samples ranged from 20.6 to 45.7 ng/ml ($n = 19$). $25(OH)D_2$ and $25(OH)D_3$ 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 D_2 concentrations were 1.5 and 0.5 ng/ml, and vitamin D_3 was 31.2 and 21.0 ng/ml. $25(OH)D_2$ was 1.6 and 1.0 ng/ml, and $25(OH)D_3$ 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 D_2 and vitamin D_3 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 $25(OH)D_2$ and $25(OH)D_3$ 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

the inherent variability and sensitivity to interfering compounds associated with competitive binding assays. Furthermore this method allows the individual measurement of vitamins D_2 and D_3 and $25(OH)D_2$ and $25(OH)D_3$. Attempts to analyse normal plasma samples for vitamin D on the Zorbax-ODS 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 $24,25(OH)_2D$ and $25,26(OH)_2D$ 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 $24,25(OH)_2D$ in rat and chick plasma shows the need for an additional reversed-phase h.p.l.c. system. Thus extensive purification of $24,25(OH)_2D$ and $25,26(OH)_2D$ isolated from plasma by h.p.l.c. is necessary before meaningful competitive binding analysis is possible.

The $1,25(OH)_2D$ 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 poly(ethylene 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 lifeguards (Haddad & Chyu, 1971).

Our mean value for the normal plasma concentration of $24,25(OH)_2D$ 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 $24,25(OH)_2D$ 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 $24,25(OH)_2D$ 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 $24,25(OH)_2D$ in anephric-human plasma suggests that an extrarenal 25(OH)D 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 $25,26(OH)_2D$ do not change from our normal values upon nephrectomy or exposure to sunlight. Although $25,26(OH)_2D$ 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 $1,25(OH)_2D$ is in good agreement with that previously reported (Eisman *et al.*, 1976; Brumbaugh *et al.*, 1974). The lack of $1,25(OH)_2D$ 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.* (1977*a*,*b*) for human plasma. The former measures total 25(OH)D, $24,25(OH)_2D$ and $1,25(OH)_2D$ by competitive protein-binding assays with normal values in good agreement with ours, but it does not measure vitamin D and $25,26(OH)_2D$ 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.* (1977*b*) measures total vitamin D, 25(OH)D and $24,25(OH)_2D$ by h.p.l.c., and $1,25(OH)_2D$ by competitive protein-binding assay, but its reliability seems suspect. Their normal value for vitamin D

(Lambert *et al.*, 1977*b*) is much higher than that reported by us or Jones (1978) and lower still than in their original abstract (Lambert *et al.*, 1977*a*). Also, their original normal value for 24,25$(OH)_2D$ (Lambert *et al.*, 1977*a*) was much higher than reported by us or others. Yet their final value for 24,25$(OH)_2D$ (Lambert *et al.*, 1977*b*) 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$(OH)_2D$ and is unable to measure vitamins D_2 and D_3 or 25$(OH)D_2$ and 25$(OH)D_3$ individually.

In summary, we report a multiple assay procedure that can measure, with sensitivity and accuracy, vitamin D_2 and vitamin D_3, 25$(OH)D_2$ and 25$(OH)D_3$, 24,25$(OH)_2D$, 25,26$(OH)_2D$ and 1,25$(OH)_2D$ in a single 3–5ml sample of plasma. It is possible to streamline the procedure and measure metabolites of particular interest, e.g. 25(OH)D, 24,25$(OH)_2D$ and 1,25-$(OH)_2D$. 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, it was 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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