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ISOLATION AND QUANTITATION OF ENDOGENOUS VITAMIN D AND ITS PHYSIOLOGICALLY IMPORTANT METABOLITES IN HUMAN PLASMA BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

We have developed methodology for the precise measurement of vitamin D, 25-OHD, 24,25-$(OH)_2$D and 1α,25-$(OH)_2$D in relatively small quantities (3–5 ml) of normal human plasma. It involves initial extraction with methanol–methylene chloride and separation of phases by centrifugation in polypropylene tubes, followed by discontinuous flow gradient chromatography on LH-20. These procedures accomplish the removal of interfering lipophilic substances and excellent resolution of vitamin D metabolites with good recovery (94%). Further separation of metabolites from interfering U.V. absorbing lipophilic substances is achieved by HPLC using isocratic conditions in normal and reverse phase systems. The U.V. absorbing peak areas eluting from HPLC in the positions corresponding to standard vitamin D, 25-(OH)D and 24,25-$(OH)_2$D are reproducibly quantitated by automated integration. 1α,25-$(OH)_2$D Is measured by ligand binding assay using intestinal cytosol because plasma concentrations of this metabolite are too low for optical detection. Values in normal human plasma obtained in late summer are: vitamin D = 15.2 ± 1.8 ng/ml; 25-OHD = 28.6 ± 2.1 ng/ml; 24,25-$(OH)_2$D = 2.1 ± 0.5 ng/ml; 1α,25-$(OH)_2$D = 0.035 ± 0.003 ng/ml (±S.D.). The methodology described or modification thereof should provide a formidable tool in the investigation of the regulation of vitamin D metabolism *in vivo* in both physiologic and pathologic states.

INTRODUCTION

Separate ligand binding assays have been reported for 25-OHD [1–6], 24,25$(OH)_2$D [9, 10] and 1.25$(OH)_2$D [7, 8] and they have been applied to measurements of these vitamin D metabolites in biological fluids. The single ligand binding assay for vitamin D itself which has been reported detects vitamin D_3 3–4 times better than vitamin D_2 [12, 13]. Therefore, it is not possible to obtain accurate measurements of total vitamin D (D_2 plus D_3) in serum or plasma at the present time [12, 14, 15].

Recent technological advances with high pressure liquid chromatography (HPLC) have permitted the qualitative detection of vitamin D and its known metabolites in serum after appropriate purification of extracts [16–18]. It has also been used for the purification of 1,25$(OH)_2$D prior to ligand binding assay [19].

The objective of the present investigation was to develop a quantitative approach to the measurement of total vitamin D and all its known natural metabolites using HPLC and optical detection. Two major technical problems were encountered: (1) the contamination of purified vitamin D and metabolite fractions with lipophilic substances which produced optical background noise during HPLC, and (2) the inability to detect the small quantities of circulating vitamin D and metabolites using available optical systems.

In this paper, we describe solutions to these problems which have permitted us to quantitate total vitamin D, 25-OHD and 24,25$(OH)_2$D using HPLC and optical detection of these compounds with sufficient sensitivity to measure them in small (3 ml) quantities of normal human plasma. Plasma concentrations of 1,25$(OH)_2$D are also reported, but these measurements required larger quantities of plasma (5 ml) and ligand binding assay after HPLC chromatography because of the extremely small quantities (pg) of circulating metabolites.

The following trivial names and abbreviations are used in this paper: When no subscript is present after D, both D_2 (ergocalciferol) and D_3 (cholecalciferol) are implied. D = ergocalciferol and cholecalciferol (combined); D_2 = ergocalciferol; D_3 = cholecalciferol; 25-OHD = 25-hydroxyergocalciferol and 25-hydroxycholecalciferol; 25-OHD_3 = 25-hydroxycholecalciferol; 24,25-$(OH)_2$D = 24R,25-dihydroxyergocalciferol and 24R,25-dihydroxycholecalciferol; 24,25$(OH)_2D_3$ = 24R,25-dihydroxycholecalciferol; 1α,25-$(OH)_2$D = 1α,25-dihydroxyergocalciferol and 1α,25-dihydroxycholecalciferol; 1α,25-$(OH)_2D_3$ = 1α,25-dihydroxycholecalciferol; 1α-OHD_3 = 1α-hydroxycholecalciferol; HPLC = high pressure liquid chromatography.

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MATERIALS AND METHODS

Solvents. All solvents were of spectroanalyzed grade and were either redistilled or filtered through 1 μ pore size. Millipore fluoropore filters (Millipore Corp., Bedford, MA) prior to use. The solvents used were the following: Methanol*, methylene chloride*, chloroform*, *n*-hexane†, and *iso*-propanol†.

Biological samples. Human plasma was obtained from 10 to 20 healthy volunteers (ages 20–50 years) during the late summer or winter. Vitamin D deficient chick plasma was obtained from White Leghorn pullets raised from 1 day post-hatching on a modified vitamin-D-deficient diet [20] for 3–4 weeks. All biological samples not immediately used were stored under N_2 at $-20°$.

Sterols. The following reference compounds were obtained commercially in crystalline form: vitamin D_2 and D_3 from Grand Island Biological Co., Grand Island, NY; 25-OHD_2 was kindly supplied by Dr. J. Babcock of the UpJohn Co., Kalamazoo, MI; 1α-OHD_3, 25-OHD_3, 24,25-$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ were a generous gift from Dr. M. Uskokovic of Hoffman–LaRoche Inc., Nutley, NJ. The following radioactive sterols were obtained commercially: [26,27-^{3}H]-25OHD_3 (9.3 Ci/mmol) from New England Nuclear, Boston, MA; [1,2-^{3}H] vitamin D_3 (12.3 Ci/mmol) from Amersham–Searle Corp., Arlington Heights, IL; [26,27-^{3}H]-24,25-$(OH)_2D_3$ (9.3 Ci/mmol and [26,27-^{3}H]-1α,25-$(OH)_2D_3$ (9.3 Ci/mmol) were biosynthesized *in vitro* with high yield from [26,27-^{3}H]-25OHD_3 utilizing modifications [20] of existing techniques [21–25]. All sterols were quantitated by micro-U.V. spectrophotometry after purification by HPLC. Prior to use, all sterols were stored under N_2 in deoxygenated absolute ethanol in sealed ampules at $-20°$.

Extraction. All glassware involved in extraction and chromatography was siliconized. Siliconization was accomplished by immersing the glassware in a 1% silicone solution (Siliclad, Becton Dickinson and Co., Parsippany, NJ) for 5 s and then heating at 100° for 10–15 min. Five ml of plasma was placed in a 30 ml polypropylene centrifuge tube in an ice bath and a radioactive internal standard of each metabolite (10,500 d.p.m.) was added to monitor recovery through the extraction and chromatographic procedures. The sample was extracted with 3 vol. of cold methanol–methylene chloride (2:1, v/v) per vol. of plasma and vortexed for 2 min. One vol. of cold methylene chloride was then added, vortexed for 2 min and centrifuged at 23,300 *g* for 10 min at 0–5° in a Sorvall HB-4 rotor. The lower organic phase was siphoned off via a Teflon tube into a polypropylene tube and the aqueous phase was reextracted with 2 vol. of cold methylene chloride. All organic phases were combined and evaporated to dryness under nitrogen.

Gel chromatography. The residue from these organic solvent extracts were solubilized in 100 μl of *n*-hexane–chloroform–methanol (9:1:1, by vol.) [8] and applied to a Sephadex LH-20 column (0.9 × 15 cm; Pharmacia Fine Chemicals) equilibrated with the same solvent system. The polypropylene tubes were washed twice with 100 μl of solvent and these washes were used to wash the sample into the column bed. Vitamin D and its metabolites were eluted from the column with the non-linear "flow gradient". The "flow gradient" consisted of the following incremental changes in flow rate: 0.5 ml/min for the first 10 ml of eluant (for vitamin D); 1.3 ml/min for the next 15 ml (for 25-OHD); and 1.5 ml/min for the remainder of the gel chromatography elution (for 24,25-$(OH)_2D$ and 1α,25-$(OH)_2D$).

High pressure liquid chromatography (HPLC) of vitamin D and its metabolites. Two basic systems were developed: one involving a solvent gradient which was used to assess the purity of the endogenous vitamin D and its metabolites eluting from the LH-20 chromatography; the other involved isocratic solvent conditions which were used in the quantitation of these metabolites. The vitamin D, 25OHD, 24,25-$(OH)_2D$ and 1α,25-$(OH)_2D$ peaks from the Sephadex LH-20 gel chromatography were evaporated under nitrogen and dissolved in 50 μl of redistilled absolute ethanol. They were separately applied to a Waters Model 202 High Pressure Liquid Chromatograph (Waters Associates, Milford, MA). A reverse phase system operated at room temperature was used for quantitating the compounds. It consisted of two 0.6 × 30 cm μ Bondapak C_{18} columns (Waters Associates) in tandem. The following separate isocratic solvent systems were used: for vitamin D = CH_3OH–H_2O (90:10, v/v) at 1.8 ml/min and 3000 psi; for 25-OHD = CH_3OH–H_2O (87:13, v/v) at 1.4 ml/min and 2400 psi; for 24,25-$(OH)D_2$ = CH_3OH–H_2O (80:20, v/v) at 1.5 ml/min and 3200 psi. The 1α,25-$(OH)_2D$ peak from LH-20 chromatography was applied to a normal phase system which consisted of two 0.6 × 30 cm μ-Porasil columns (Waters Associates) in tandem and an isocratic solvent system of *n*-hexane–isopropanol (88:12, v/v) at 1.5 ml/min and 800 psi. For the solvent gradient, dual 6000 Model pumps with an automatic solvent programmer (Waters Associates) were used to generate the solvent gradient for the separation of the compounds on reverse phase as described above. This enables rapid monitoring of purity of these metabolites. The system was initiated with CH_3OH–H_2O (75:25, v/v) and terminated with CH_3OH–H_2O (95:5, v/v) over a 40 min period with a flow rate of 1.5 ml/min and 3500 psi.

Quantitation of endogenous vitamin D and its metabolites. Quantitation of endogenous vitamin D, 25-OHD and 24,25-$(OH)_2D$ in eluting from the HPLC was done by electronic integration of the peak areas of the U.V. absorbing material. A micro-ultra-

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violet differential spectrophotometer (Waters Associates) with a 100 μl cell was used at a wavelength setting of 254 nm. The spectrophotometer signal was recorded on a Hewlett–Packard 3380A recording integrator which registered retention times as well as calculated the relative peak area of each of the eluting U.V. absorbing components. Enhanced sensitivity was achieved using a log scale attenuation as the integrator.

Measurement of 1α,25-$(OH)_2$D in the fractionated HPLC peak containing these metabolites was done using a ligand binding assay modified [20] from that of Eisman *et al.* [8]. This method employs intestinal mucosal cytosol from rachitic chicks as the receptor system. A competitive protein binding standard curve was obtained by incubating cytosol binding protein and biosynthesized [26,27-^{3}H]-1α,25-$(OH)_2$D with increasing amounts of synthetic 1α,25-$(OH)_2D_3$. Polyethylene glycol was used to separate bound from free 1α,25-$(OH)_2$D.

Liquid scintillation counting. [^{3}H] Radioactivity of specific chromatography fractions was determined by the addition of fractions to 10 ml of liquid scintillator (32.0% Triton X-100, 3.0% PPO and 0.05% POPOP in toluene) and then counting on a Beckman LS-100 C liquid scintillation counter at 27–33% efficiency.

RESULTS

Figure 1 shows the structures of vitamin D_3 and its metabolites which are discussed in this paper with respect to quantitation in human plasma. Figure 2 outlines the method described in this paper on the quantitation of endogenous vitamin D and three of its physiologically important metabolites. The reason for each of the steps (i.e., extraction, LH-20 chromatography and HPLC) will be discussed below with respect to purification and recovery of each of the vitamin D metabolites. The Sephadex LH-20 gel chromatography profile showing the separation of vitamin D, 25-OHD, 24,25-$(OH)_2$D as well as 1α,25-$(OH)_2$D from a methanol–methylene chloride extract of 5 ml of human plasma is shown in Fig. 3. A significant proportion of the U.V. absorbance of lipophilic compounds from the plasma extracts were separated from the vitamin D peak by means of the slow flow rate (0.5 ml/min) and small elution fractions (0.5 ml). Rapid and effective resolution of the remaining vitamin D metabolites was achieved with an increase in flow rate beginning at 1.3 ml/min for 25-(OH)D followed by a 1.5 ml/min for 24,25-$(OH)_2$D and 1α,25-$(OH)_2$D.

Evidence for effective separation and purification of these endogenous metabolites by the above described LH-20 chromatography was shown by HPLC as described in the Methods Section and shown in Fig. 4. As discussed in the Methods Section the solvent gradient system was found to be superior for analyzing the purity of these metabolites. The more polar the metabolites the earlier they elute from the column in the reverse phase system. This system represents the best of many systems tested for separating mixtures of the various metabolites of vitamin D in

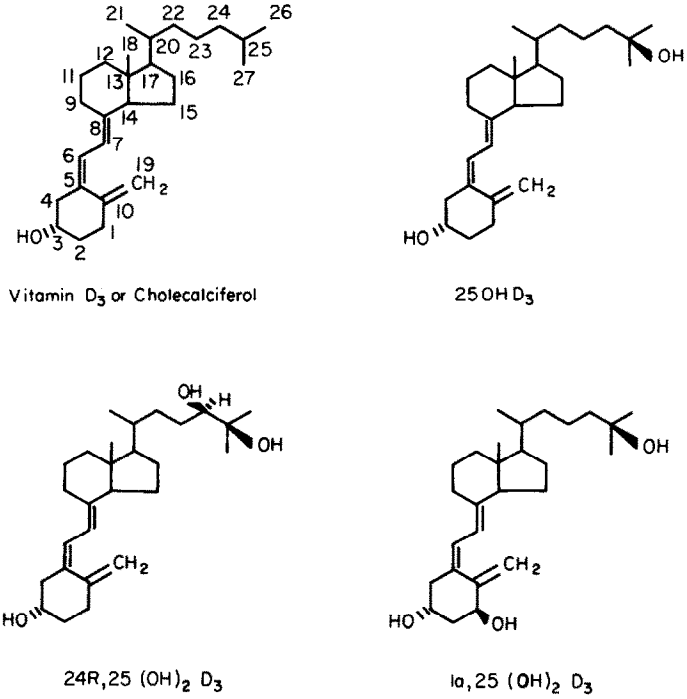

Fig. 1. Structure of vitamin D_3 and its metabolites. Vitamin D_2 or ergocalciferol and its metabolites have an additional double bond between C-22 and C-23.

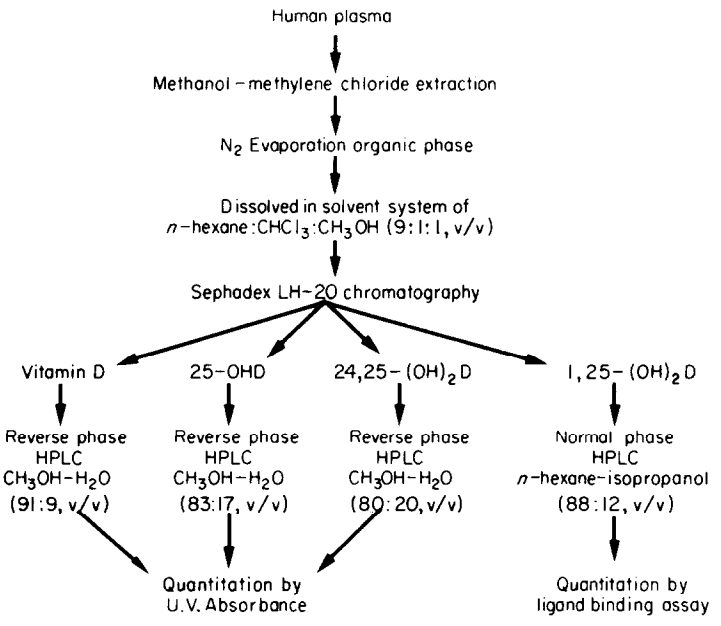

Fig. 2. Summary of the quantitation of vitamin D and three of its physiologically important metabolites.

a single pass. In some instances, the stereoisomers were resolved in this system (see Fig. 4). The retention times of the labeled standards correspond to those of the respective unlabeled standards. The labeled standards were added to and then extracted from human plasma and fractionated by Sephadex LH-20 chromatography as described above. Each of the eluted peaks of radioactivity was then applied to HPLC. The HPLC chromatographs showed that the LH-20 gel chromatography separates the vitamin D compounds with high resolution.

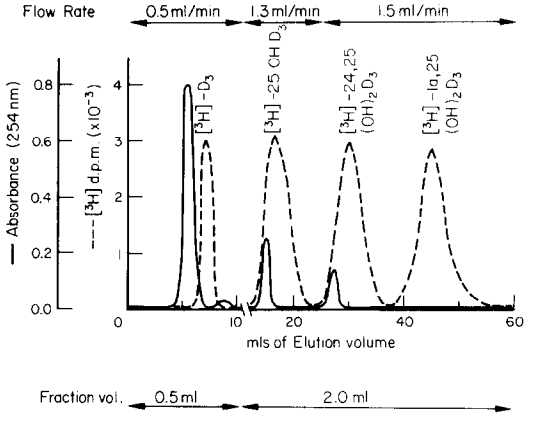

Fig. 3. Sephadex LH-20 gel chromatography profile of the organic solvent extract of normal human plasma containing [^{3}H] labeled vitamin D and metabolite markers. The [1,2-^{3}H]-D_3, [26,27-^{3}H]-25-OHD_3, [26,27-^{3}H]24,25-$(OH_2)D_3$ and [26,27-^{3}H]-1α,25-$(OH)_2D_3$ were added as labeled markers prior to extraction. Chromatography was carried out in a 0.9 × 15 cm column with a solvent system of *n*-hexane–$CHCl_3$–CH_3OH (9:1:1, v/v) and a nonlinear flow rate gradient as described in the Results and in the figure. The [^{3}H]-d.p.m. are plotted as a function of elution vol. for vitamin D, 25-OHD, 24,25-$(OH)_2D$ and 1α,25-$(OH)_2D$ and the absorption at 254 nm (——). The absorption measurements were performed using a 1 ml cuvette in a Beckman 2000 spectrometer which cannot detect vitamin D metabolites in normal plasma but only large quantities of other plasma, lipophilic, U.V. absorbing substances.

For quantitation of various metabolites, however, isocratic systems on reverse phase were found to be superior. Figure 5 represents the HPLC elution pattern of various vitamin D metabolites from human plasma using an isocratic system selected for its ability to separate vitamin D from other U.V. absorbing substances. Standard [1,2-^{3}H]-D_3 was added to the plasma to serve both as a reference for elution from the chromatographic column and to estimate the extraction yield of vitamin D. Figure 5(a) displays the elution pattern of the various standard unlabeled vitamin D metabolites using this isocratic system. The labeled [1,2-^{3}H]-D_3 was not applied directly to the HPLC as were the unlabeled standards but instead was added to plasma and extracted as described earlier. This labeled standard coelutes with the unlabeled standard vitamin D. The contaminating lipophilic substances in the vitamin D extract from the LH-20 chromatography only slightly altered the retention time of the standard vitamin D. Clearly, vitamin D was resolved from the other metabolites. Figure 5(b) represents the HPLC chromatogram of the U.V. absorbing lipophilic material from human plasma present in the fraction of the LH-20 chromatography eluant which coelutes with labeled vitamin D. The vitamin D standard coelutes with one peak of U.V. absorbing material. The plasma from both normal chicks as well as humans displayed this particular peak on HPLC. If this particular peak of U.V. absorbing material does represent vitamin D, then this peak should be reduced in size or absent when analyzing the plasma of vitamin D deficient chicks. As shown in Fig. 5(c), this is clearly the case. The

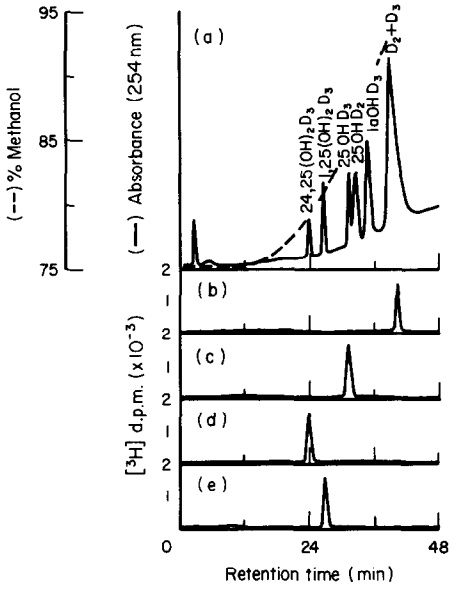

Fig. 4. Qualitative analysis of standard synthetic vitamin D and its metabolites by high pressure liquid chromatography. Panel 4(a) shows the HPLC elution pattern obtained after a mixture of vitamin D, its metabolites, and the analogue 1α,OHD_3 was applied to a reverse phase system using a concave gradient of changing solvent (see Methods section). The mixture contained 200 ng of vitamin D_2 and vitamin D_3, 150 ng of 25-OHD_2 and 25-OHD_3, 150 ng of 1α,OHD_3, 200 ng of 1α,25-$(OH)_2D_3$ and 150 ng of 24,25-$(OH)_2D_3$ and was injected in 100 μl of redistilled absolute ethanol. A 40 min concave gradient program on the Waters solvent programmer beginning at CH_3OH–H_2O (75:25, v/v) and ending with CH_3–H_2O (95:5, v/v) at a flow rate of 1.5 ml/min was used. Two 6 mm × 30 cm $C_{18}\mu$ Bondapak columns (Waters Associates) at room temperature were used in tandem with and the per cent methanol in the solvent (---) are plotted as a function of the elution time. Figs 4(b), 4(c), 4(d) and 4(e) represent the HPLC profiles of [^{3}H] vitamin D_3, [^{3}H]25-OHD_3 [^{3}H]24,25-$(OH)_2D_3$, and [^{3}H]1α,25-$(OH)_2D_3$ respectively. Each radioactive standard was added to plasma, extracted, and chromatographed on Sephadex LH-20 columns and the respective peaks applied to the HPLC. The same chromatographic conditions as described in Fig. 4(a) above were used in 4(b), 4(c), 4(d) and 4(e) except that 0.5 ml fractions of the eluant were collected, counted as described in Methods and the [^{3}H]-d.p.m. was plotted versus the elution time.

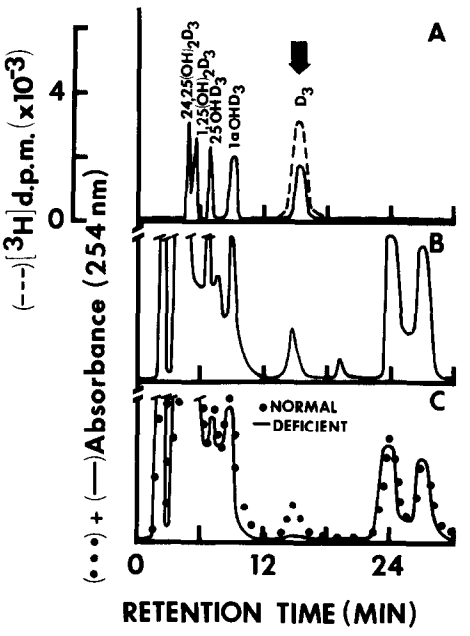

Fig. 5. Quantitation of endogenous vitamin D in normal plasma using high pressure liquid chromatography. Figure 5(a) shows the elution profile of U.V. absorbing material (——) after 200 ng each of the unlabeled standard metabolites of vitamin D are applied to the HPLC. The elution of the [1,2-^{3}H]-D_3 (---) which was added to and then extracted from plasma, chromatographed on LH-20, and applied to HPLC, is also shown. Figure 5(b) represents the elution profile of U.V. absorbing material, extracted from human plasma, which coelutes with the labeled vitamin D standard on the LH-20 chromatography (see Fig. 3). Figure 5(c) shows the elution profile of U.V. absorbing material extracted from plasma of normal (·····) and vitamin D deficient-(——) chicks which coelutes with labeled standard vitamin D on the LH-20 chromatography. All patterns are generated by a reverse phase, isocratic system of methanol–water (90:10, v/v) using two $C_{18}\mu$ Bondapak columns in tandem at room temperature, a 1.8 ml/min flow rate, and 3000 psi pressure. Radioactivity was assessed by collecting and counting the 0.5 ml fractions from the HPLC in the Triton X-100 toluene base fluor as described in the Methods.

plasma of vitamin D deficient chicks showed no U.V. absorbing peak with a retention time which matched the vitamin D standards while the plasma of normal humans and chicks contained this peak. The "vitamin D" peak shown in Fig. 5(b) constituted 5.8% of the total U.V. absorbing lipophilic material that eluted from the HPLC (as calculated by the integrator). It should be stated here that in all the isocratic systems described in this paper, the stereoisomers (D_2 and D_3) of vitamin D and 25-OHD coelute.

Similar results were obtained with modified chromatographic conditions using isocratic systems selected for their ability to separate and quantitate 25-(OH)D (Fig. 6) and 24,25-$(OH)_2$D (Fig. 7). Again, plasma samples from normal humans or chicks or from vitamin D deficient chicks were extracted with organic solvents, chromatographed on LH-20, and the peaks of interest applied to HPLC. Again, the labeled standards extracted from the plasma, coelute closely with the unlabeled standards applied directly to HPLC. In each instance, (1) the particular metabolites of interest eluted from the HPLC with retention times different from those of the bulk of U.V. absorbing material and from the remaining vitamin D metabolites; (2) the plasma from normal humans and chicks displayed a U.V. absorbing peak which coeluted with the respective standards, and (3) the plasma from vitamin D deficient chicks displayed no such peak. The 25-OHD and 24,25-$(OH)_2$D peaks shown in Fig. 6(b) and 7(b) constituted 8.4% and 0.8% respectively of the total U.V. absorbing lipophilic material that was eluted from the HPLC (as calculated by the integrator).

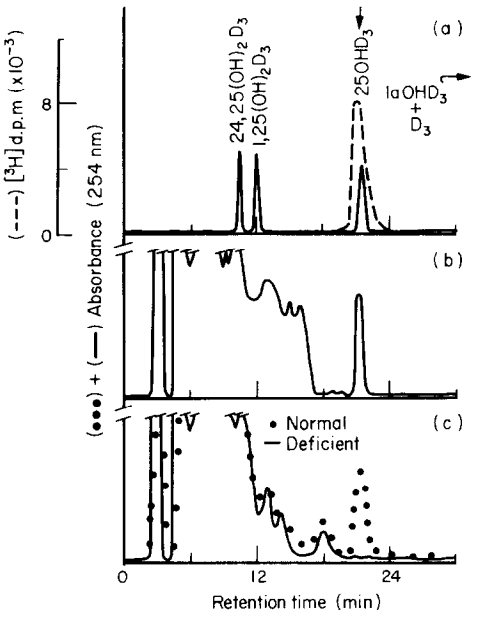

Fig. 6. Quantitation of 25-OHD from plasma using high pressure liquid chromatography. Figure 6(a) represents the elution profile of U.V. absorbing material (——) after 200 ng each of the unlabeled standard metabolites of vitamin D are applied to the HPLC. The elution of [26,27-^{3}H]-25OHD$_3$ (---) which was added to and then extracted from plasma, chromatographed on LH-20, and applied to HPLC, is also shown. Figure 6(b) represents the elution profile of U.V. absorbing material extracted from human plasma, which coelutes with the labeled 25-OHD standard on LH-20 chromatography (see Fig. 3). Figure 6(c) represents the elution profile of U.V. absorbing material, extracted from plasma of normal- (·····) and vitamin D deficient- (——) chicks, which coelutes with the 25-OHD standard on LH-20 chromatography. All patterns are generated by the same system as described in the legend of Fig. 5 except that the isocratic conditions of a methanol–water (87:13, v/v) solvent was used at 1.4 ml/min and 2400 psi. The radioactive material was assayed by collecting and counting the 0.5 ml fractions from the HPLC in a Triton X-100 toluene based fluor as described in Methods.

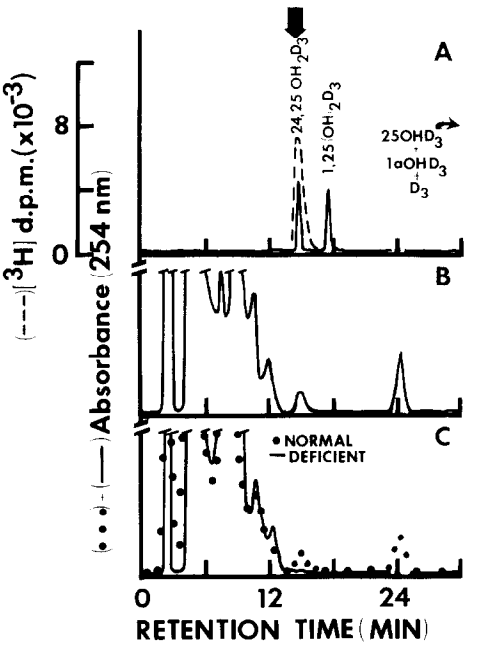

Fig. 7. Quantitation of endogenous 24,25(OH)$_2$D in normal plasma using high pressure liquid chromatography. Figure 7(a) shows the elution profile of U.V. absorbing material (——) after 200 ng each of the unlabeled standards of the vitamin D metabolites are applied to the HPLC. The elution of [26,27-^{3}H]-24,25-(OH)$_2$D$_3$ (——) which has been added to and then extracted from plasma, chromatographed on LH-20, and applied to HPLC, is also shown. Figure 7(b) represents the elution profile of U.V. absorbing materials extracted from human plasma, which coelutes with the labeled 24,25(OH)$_2$D$_3$ standard on LH-20 chromatography. Figure 7(c) represents the elution profile of U.V. absorbing material extracted from plasma of normal- (·····) and vitamin D deficient- (——) chicks which coelutes with the labeled 24,25(OH)$_2$D$_3$ standard on LH-20 chromatography. All patterns are generated by the same system as described in the legend of Fig. 5 except that the isocratic conditions of methanol–water (80:20, v/v) at 1.5 ml/min and 3200 psi were used. The radioactive material was assessed by collecting and counting the 0.5 ml fractions from the HPLC in the Triton X-100 toluene based fluor as described in the Methods Section.

In each of the isocratic systems described above, the quantitation of the vitamin D, 25-OHD and 24,25-(OH)$_2$D was achieved with a Hewlett–Packard recording integrator using a log scale attenuation to enhance the sensitivity of the low values of absorption (at 254 nm). Tests were then performed to assess the quantitative reproducibility of the HPLC/integrator analysis of the vitamin D and its metabolites. Increasing concentrations of the unlabeled standards were added to 5 ml samples of vitamin D deficient chick plasma, the plasma samples extracted, chromatographed on an LH-20 column and applied to the HPLC as described earlier. Figure 8 shows the calibration of the integrator values versus the ng of each standard injected. The mean and standard deviation of 10 replicate analysis at each level is shown. These linear plots demonstrate the reproducibility and accuracy of this method for quantitating endogenous vitamin D, 25-OHD and 24,25-(OH)$_2$D. Since our method uses 5 ml plasma samples, our assays of human/chick plasma for these vitamin D metabolites are in the regions of 10–150 ng on this scale.

Of the many conditions tested, a normal phase system, described in the Methods section, proved to be superior for isolating and quantitating 1α,25(OH)$_2$D. In this normal phase system, the 1α,25-(OH)$_2$D is separated from the contaminating U.V. absorbing material whereas the other metabolites of vitamin D are not. Figure 9 shows the chromatogram of the various vitamin D metabolites on the normal phase HPLC. This method represented a slight modification of that reported by Jones and DeLuca [17]. As shown in Fig. 9(a) the standard 1α,25-(OH)$_2$D elutes well after the elution of the contaminating lipophilic substances, and vitamin D, and the other metabolites. Figure 9(a) also shows the elution of [26,27-^{3}H]-1,25-(OH)$_2$D$_3$ which was added to the plasma, extracted, and isolated on the LH-20 gel

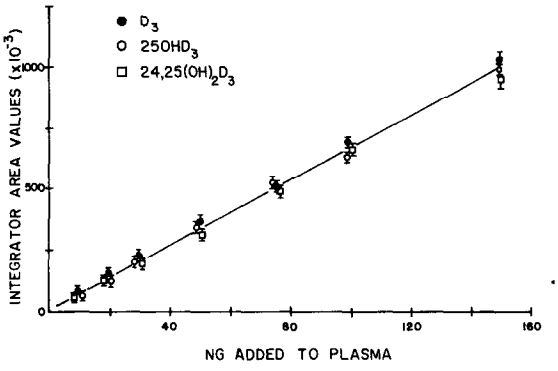

Fig. 8. Calibration curves for the quantitation of various standard vitamin D metabolites applied to HPLC. The quantitation of vitamin D (●), 25-OHD (○) and 24,25-$(OH)_2$D (■) are shown. The methods for quantitating these compounds are described in the legends of Figs 5, 6, and 7 respectively. The determination of peak area for each metabolite was determined by the integrator. These values are plotted against ng quantities of each of the unlabeled standard metabolites which was added to the vitamin D deficient chick plasma, extracted, and fractionated first by LH-20 chromatography and then by HPLC. The mean and standard deviation of recovered amounts are given.

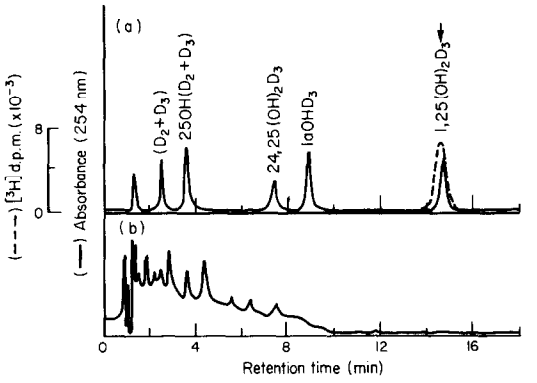

Fig. 9. Elution profile of 1α,25-$(OH)_2D_3$ and vitamin D metabolites on normal phase HPLC. Figure 9 represents the elution profile of the U.V. absorbing material (——) after 200 ng of each of the unlabeled standard vitamin D metabolites are applied to the HPLC. The elution of [26,27-^{3}H]1,25-$(OH)_2D_3$ (----) which has been added to and then extracted from plasma, chromatographed on LH-20, and applied to HPLC is also shown. Figure 9(b) represents the elution profile of U.V. absorbing material, extracted from normal human plasma, which coelutes with the labeled 1α,25$(OH)_2D_3$ standard on the LH-20 chromatography. A normal phase isocratic system of *n*-hexane–isopropanol (88:12, v/v) with two μ Porasil columns in series at room temperature are used with a flow rate of 1.5 ml/min and 1200 psi. The radioactive material was assessed by collecting and counting 0.5 ml fractions from the HPLC in the Triton X-100 toluene-base fluor as described in the Methods.

chromatography as discussed above. This radioactive standard coeluted with the unlabeled standard. The level of 1α,25-$(OH)_2D_3$ is known to be far below that of the other metabolites of vitamin D, and as shown in Fig. 9(b), we were unable to see any absorption peak displaying the retention time of standard 1α,25-$(OH)_2D_3$. Consequently, it was necessary to quantitate this metabolite using a modification of the ligand binding assay as described by Eisman *et al.* [8]. Thus, plasma extracts were chromatographed on LH-20 columns and the eluants eluting with the standard 1α,25-$(OH)_2$D collected and applied to HPLC. The regions of the HPLC eluant coeluting with standard 1α,25-$(OH)_2$D, were collected and quantitated by the binding assay.

The actual recovery of each of the metabolites after extraction from plasma, LH-20 chromatography and HPLC are shown in Table 1. Final recoveries of 82–88% of the original with ±2 to 3% S.D. were calculated using the radioactive metabolites as internal standards. The above described methods were then applied to human plasma to assess the concentrations of these endogenous metabolites and the variations of this analysis. Table 2 shows that the mean plasma levels in the late summer or winter for 10–20 normal individuals are: vitamin D = 15.2 ± 1.8 ng/ml; 25-OHD = 25.4 ± 2.1 ng/ml; 24,25-$(OH)_2$D = 2.1 ± 0·5 ng/ml; 1α,25-(OH)D = 0.035 ± 0.003 ng/ml (S.D.).

The technique described above and outlined in Fig. 2 represents one approach to the quantitative analysis of vitamin D and its physiologically important metabolites. The method appears to be reproducible (with minimum variation) if performed carefully. For good

Table 1. Determinations of the recoveries from human plasma of vitamin D and its metabolites*

Metabolite	[^{3}H]d.p.m. Added to plasma	Recovery following step 1 (extraction) and step 2 (gel chromatography)		Final recovery following step 3 (HPLC)	
		[^{3}H]d.p.m.	% ± S.D.	[^{3}H]d.p.m.	% ± S.D.
[1,2-^{3}H]-D_3	11,600	11,365	98% ± 1	10,672	92% ± 3
[26,27-^{3}H]-25OHD_3	10,350	9936	96% ± 2	9108	88% ± 2
[26,27-^{3}H]-24,25-$(OH)_2D_3$	10,045	9141	91% ± 2	8438	84% ± 2
[26,27-^{3}H]-1α,25-$(OH_2)D_3$	3500	3115	89% ± 2	2870	82% ± 3

* Radioactive internal standard for each metabolite was added to the 5 ml plasma sample followed by extraction (Step 1), Sephadex LH-20 gel chromatography (Step 2), HPLC (Step 3) and scintillation counting as described in the Methods. The mean and standard deviations of the recovered radioactivity from the samples from 10 normal individuals are given.

Table 2. Mean levels for vitamin D and its physiologically important metabolites in human plasma*

Metabolite	N†	ng/ml + S.D.
D	20 (M)	15.2 ± 1.8
25-OHD	10 (S)	25.4 ± 2.1
24,25$(OH)_2$D	20 (M)	2.1 ± 0.5
1α,25$(OH)_2$D	10 (S)	0.035 ± 0.003

* Plasma was obtained from 10–20 normal individuals. Quantitation of vitamin D, 25-OHD and 24,25-$(OH)_2$D were achieved using automatic integration of the peak areas of U.V. absorbing components eluting on HPLC, as described in Materials and Methods. 1α,25-$(OH)_2$D values were obtained in applying the HPLC peak to a ligand binding assay, as described elsewhere [8, 19, 20]. The means and standard deviations are given.

† Periods of sampling: S = September; M = March.

recoveries, the quantitative handling of the sample, use of nitrogen as opposed to air, use of siliconized glassware and polypropylene vessels, and quantitative injections into the HPLC are required.

DISCUSSION

Assessment of the physiology and pathophysiology of vitamin D metabolism is dependent, at least in part, on the ability to accurately determine the concentrations of vitamin D and its biologically important metabolites in biological fluids. Organic solvent extraction and LH-20 chromatography have been utilized for years in the analysis of various lipids. Recently these techniques have been combined with HPLC for analysis of 1α,25-$(OH)_2$D in plasma [19]. The method described in this paper and outlined in Fig. 2 permits one to measure with precision endogenous vitamin D, 25-OHD, 24,25-$(OH)_2$D as well as 1α,25-$(OH)_2$D in a single extract of human plasma for the first time. Each of the 3 basic steps in the quantitative analysis of endogenous vitamin D and its metabolites will be discussed below in terms of its selection over other conditions tested and its value in the ultimate quantitation. Besides the qualitative separation of one vitamin D metabolite from another, the primary purpose of these steps is to separate these compounds from U.V. absorbing lipophilic substances which interfere with the quantitative analysis.

Firstly, concerning the initial extraction from plasma, the accurate quantitation of vitamin D and its metabolites using existing extraction techniques has partly been hampered by excessive contaminations of U.V. absorbing lipophilic substances which interfere with the quantitation and/or reduced recovery of certain metabolites. With the use of methylene chloride to extract plasma [8], the amount of extracted lipophilic material is greatly reduced but unfortunately there is a significant decrease in the recovery of certain metabolites (e.g., 25-OHD). On the other hand, use of a methanol–chloroform extraction solvent [20] results in excellent recoveries of vitamin D and its metabolites, but is plagued by a substantial contamination with interfering lipophilic substances. The use of the methanol–methylene chloride (2:1, v/v) solvent [6] in our method and the separation of the phases by centrifugation in polypropylene tubes offers a compromise, providing both a significant reduction in these interfering substances and satisfactory recovery of the vitamin D metabolites.

Secondly, the Sephadex LH-20 chromatography, first applied for 1α,25-$(OH)_2$D quantitation in plasma by Eisman *et al.* [19], not only reduces the level of optically interfering substances but also, using the flow gradient modifications described in this paper, provides a major fractionation step for separating the various metabolites of vitamin D. A slow flow rate is initially used until the bulk of the U.V. absorbing lipophilic material elutes from the column. This is followed by an increased rate of flow to elute the metabolites of vitamin D. In our hands, these modifications enhance the resolution of the various vitamin D metabolites and the removal of the undesired lipophilic material.

The third step involves the first reported direct application of the HPLC for quantitating vitamin D and its metabolites in human plasma. Major advances have been made in the qualitative analysis and purification of these compounds with HPLC [16–18]. By selecting proper conditions for the HPLC and using samples already partially purified by the above described extraction and LH-20 chromatography, quantitation of each of the endogenous metabolites of vitamin D and vitamin D itself is now possible.

The presence of nonpolar, lipophilic substances in the sample represents a greater problem in normal phase chromatography because these substances have retention times similar to vitamin D and some of its metabolites. This U.V. absorbing material interferes with the optical quantitation. However, both the gradient (Fig. 4) and isocratic (Figs 5, 6 and 7) systems on reverse phase chromatography more effectively separate these lipophilic substances from the vitamin D and its metabolites and thus allow more sensitive optical quantitation. Although the gradient solvent system can resolve most of the known metabolites in one run, it was not found to be the best method for the quantitation of these compounds due to: (1) better removal of undesired U.V. absorbing lipophilic substances in the isocratic system; (2) the more stable base line in the isocratic system, and finally (3) the better resolving power of each of the described isocratic systems for each of the respective metabolites.

Lastly, the application of a sensitive ultraviolet absorbance spectro-photometer combined with electronic quantitation of the absorbance measurements as described in the Methods allows detection of nanogram concentrations. This quantitative approach combined with the excellent recoveries of the vitamin D metabolites examined, permits accurate determinations of the plasma levels of these compounds.

Because of the very low circulating levels of 1α,25-$(OH)_2$D (<50 pg/ml) [8, 15, 26], quantitations

by analysis of peak areas from the high pressure liquid chromatography was not feasible. However, as demonstrated recently, HPLC provides a means for highly effective purification necessary for measurement of this metabolite by competitive ligand binding assays. Using minor modifications of the method of Jones and DeLuca [17], the 1α,25-$(OH)_2$D peak from LH-20 gel chromatography was run on normal phase HPLC. With this normal phase system, the more polar 1α,25-$(OH)_2$D elutes well behind the lipophilic substances. The methods described in this paper consistently yield a high recovery of 1α,25-$(H)_2$D which is a very suitable preparation for the ligand binding assay.

The values for the concentrations of vitamin D, 25-OHD and 1α,25-$(OH)_2$D in human plasma reported in this paper generally agree with those previously reported by ligand binding assays [1, 2, 8, 26]. However, the circulating vitamin D concentrations, as determined by the less sensitive bioassay technique [11, 12, 14], and the 24,25-$(OH)_2$D concentrations recently reported by ligand binding assay [9, 10], are slightly different from our values. Aside from obvious differences in methodology and patient population, we can only speculate at the present time on the importance of such influencing factors as seasonal variation, dietary calcium and vitamin D intake. Certainly, a larger population, more carefully selected with the latter aspects in mind, is required for representative normal values. Our results strongly indicate that the metabolites of vitamin D represent the only U.V. absorbing compounds eluting from the HPLC at the designated periods. This is primarily based on the analysis of plasma vitamin D deficient chicks. There remains the possibility that in human plasma U.V. absorbing components other than the eluting vitamin D metabolites are present. If this is the case, our values for the levels of some or all of the vitamin D metabolites in human plasma would be high. We have used a variety of conditions for the HPLC to alter the elution periods of the various metabolites and have obtained similar quantitative results. Mass spectra analysis or ligand binding assays of the eluted peak material for each metabolite would be more definitive ways of answering this problem. Both of these approaches are presently under investigation.

In conclusion, the methods described in this paper, allow for the first time the quantitation of total vitamin D, 25-OHD, 24,25-$(OH)_2$D and 1α,25-$(OH)_2$D in an individual 5 ml sample of human plasma. The described methodology (outlined in Fig. 2) should provide a potentially useful tool in the identification of abnormalities of the critical conversion reactions of vitamin D and its biologically important metabolites in disease states.

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