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Note

High-performance liquid chromatographic determination of 24,25-dihydroxyvitamin D_3 in serum

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The second most abundant vitamin D metabolite in serum is 24,25-dihydroxyvitamin D (see ref. 1). Several procedures have been described in the literature for quantifying 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] in serum [1]. Most of these employ high-performance liquid chromatography (HPLC) for initial purification and competitive protein binding (CPB) as the final step in the analytical procedure. CPB is used because of its sensitivity and because relatively low concentrations of 24,25$(OH)_2$D are found in serum (1—5 ng/ml) [1]. For determination of vitamin D and its hydroxylated metabolites in serum, we have recently reported a multiple assay procedure using HPLC and CPB [2]. Because the CPB technique is sensitive to interfering compounds, we have now modified the procedure so that it allows quantification of 24,25-$(OH)_2$D using direct UV detection. Two forms of 24,25$(OH)_2$D may occur in serum, one derived from vitamin D_3 (cholecalciferol) and the other from vitamin D_2 (ergocalciferol). In this procedure they can be measured separately.

EXPERIMENTAL

Chemicals and instruments

Crystalline 25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 were generous gifts from F. Hoffmann-La Roche (Basel, Switzerland). Radioactive 24,25$(OH)_2$-[23,24(n)-^{3}H] D_3 (82 Ci/mmol) and 25,26$(OH)_2$-[23,24(n)-^{3}H] D_3 (gift) were obtained from The Radiochemical Centre (Amersham, Great Britain). All solvents and analytical grade chemicals were supplied by E. Merck (Darmstadt, G.F.R.). Hexane and propan-2-ol were dried for HPLC by molecular sieving and filtered under vacuum. 2,5-Diphenyloxazole (PPO) and *p*-bis-2(5-phenyl-oxazolyl)-benzene (POPOP) were

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purchased from NEN Chemicals (Frankfurt am Main, G.F.R.). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

The HPLC system consisted of an Altex high-performance liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.) equipped with a Model 110A pump, a pressure filter, a Model 153 UV detector with 8-μl flow-through cell, and a Rheodyne Model 7125 20-μl-loop injector. A stainless steel column (20 cm × 4.0 mm I.D.) prepacked with 7-μm spherical microparticulate silica (Nucleosil 50-7) and equipped with a precolumn (5 cm × 4.0 mm I.D.) packed with Polygosil 60-30 precolumn packing was obtained from Macherey-Nagel (Düren, G.F.R.). A stainless steel column (20 cm × 4.0 mm I.D.) prepacked with 7-μm microparticulate octadodecyl silica (Nucleosil 7-C_{18}) was also supplied by Macherey-Nagel and was equipped with a precolumn (3 cm × 4.0 mm I.D.) packed with Bondapak C_{18}/Corasil (Waters Assoc., Milford, MA, U.S.A.). A Model Ultrobeta 1210 liquid scintillation counter (LKB Wallac, Turku, Finland) was used at 15°C for scintillation counting. ^{3}H-labeled fractions were counted for recovery estimations with about 40% counting efficiency in a toluene scintillant containing 4 g of PPO and 50 mg of POPOP per liter of toluene.

Serum extraction

Serum samples were collected from students and healthy adult laboratory workers in September 1982. To each aliquot of serum (2—5 ml) 4500 dpm of 24,25(OH)$_2$[^{3}H]D$_3$ was added as an internal standard for calculating the final metabolite recovery. After vortex mixing, the sample was allowed to equilibrate for 30 min and it was then extracted with chloroform—methanol using a modification of the method of Bligh and Dyer [3], as described previously [2]. Briefly, 3.75 volumes of chloroform—methanol (1:2, v/v) were added, and after shaking the mixture was allowed to stand for 30 min. The phases were separated by adding 1.25 volumes of chloroform. The lower chloroform layer was collected and the upper aqueous layer was re-extracted twice with an additional 1.25 volumes of chloroform. The combined chloroform layers were then washed with an equal volume of saturated aqueous sodium chloride solution and evaporated on a rotary evaporator at reduced pressure.

Sephadex LH-20 and HPLC purification of 24,25(OH)$_2$D

The serum extract was then chromatographed at 20°C on a column (10 × 1 cm) containing 2.5 g of Sephadex LH-20 in a solvent system of hexane—chloroform—methanol (9:1:1, v/v). The extract was applied in 0.5 ml of the column solvent and rinsed with an additional 0.5 ml of solvent. The 15—25 ml fraction containing 24,25(OH)$_2$D and a number of other dihydroxylated vitamin D metabolites was collected and dried under nitrogen. HPLC on a Nucleosil 50-7 column equilibrated in propan-2-ol—hexane (1:9, v/v) and eluted at a constant flow-rate of 1.0 ml/min was used for further purification of 24,25(OH)$_2$D. Absorbance of the eluate was monitored continuously at 254 nm. Standards of nonradioactive 25(OH)D$_3$, 24,25-(OH)$_2$D$_3$, and 1,25-(OH)$_2$D$_3$ were injected to determine their elution positions. The 24,25(OH)$_2$-[^{3}H]D$_3$ eluted identically with nonradioactive 24,25(OH)$_2$D$_3$. The 24,25-

$(OH)_2D$ fraction (5—8 min) was collected and dried under nitrogen.

HPLC quantitation of 24,25$(OH)_2D_3$

The dried extract was redissolved in 25 μl of the eluent, and in the final step serum 24,25$(OH)_2D_3$ was purified by reversed-phase HPLC on a Nucleosil 7-C_{18} column equilibrated in water—methanol (1:9, v/v) at a constant flow-rate of 0.7 ml/min. Absorbance of the eluate was monitored continuously at 254 nm. The 25$(OH)D_3$, 24,25$(OH)_2D_3$, and 1,25$(OH)_2D_3$ were injected to determine their elution positions. The 24,25$(OH)_2[^3H]D_3$ eluted identically with the nonradioactive standard. The 24,25$(OH)_2D$ fraction (7—10 min) was collected into a counting vial, evaporated, and counted in the toluene scintillant along with the initial portion of 24,25$(OH)_2[^3H]D_3$. The height of the 24,25$(OH)_2D_3$ peak was divided by the percentage recovery to yield a corrected peak height, which was related to peak heights of 24,25$(OH)_2D_3$ standards to calculate the amount of 24,25$(OH)_2D_3$ in the original sample. Dividing by the sample volume gave the concentration in ng/ml.

RESULTS

A typical standard curve obtained after injection of 1—32 ng of 24,25-$(OH)_2D_3$ into a Nucleosil 7-C_{18} column and elution as described above was linear (r = 0.999). When the injection of 24,25$(OH)_2D_3$ was repeated, the

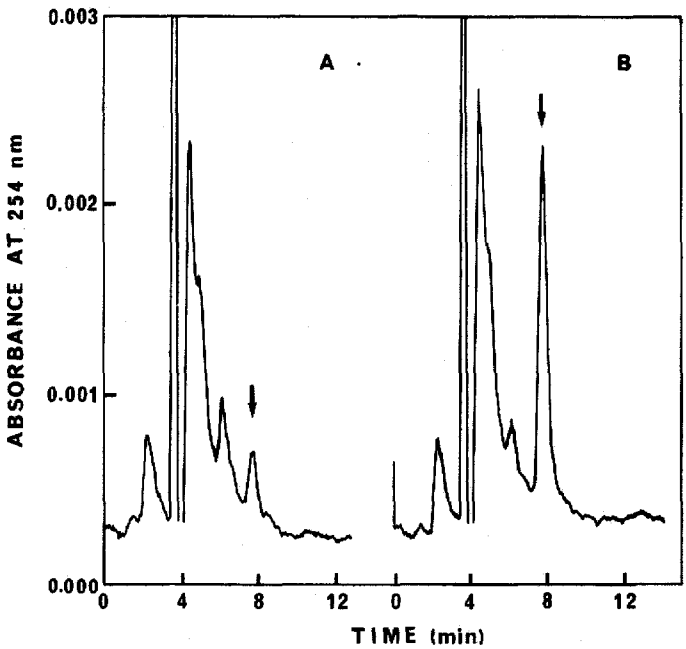

Fig. 1. (A) UV-absorption profile of extract of human serum containing 1.7 ng/ml 24,25-$(OH)_2D_3$. The UV detector was set at 0.005 absorbance units full scale. A reversed-phase column was employed with water—methanol (1:9, v/v) as eluent. The flow-rate was 0.7 ml/min. The 24,25$(OH)_2D_3$ peak elutes about 8 min after injection (arrow). (B) (UV-absorption profile of a similar serum sample to which 8.2 ng/ml 24,25$(OH)_2D_3$ had been added before extraction (100.1% recovery). Conditions for chromatography were as in A.

response was highly reproducible (16.4 ng corresponded to 9.3 ± 0.1 cm S.D., n = 7, coefficient of variation 1.3%). One nanogram of $24,25(OH)_2D_3$ was readily detected using UV detection set at 0.005 absorbance units full scale.

The UV-absorption profile observed after injection and elution of a serum extract is shown in Fig. 1. The $24,25(OH)_2D_3$ was eluted at about 8 min. When standard $24,25(OH)_2D_3$ was added to the serum before extraction, more than 95% of the added amount was measured in the final UV-absorption profile when the peak height was corrected for ^{3}H-recovery. Addition of $25(OH)D_3$ or $1,25(OH)_2D_3$ did not alter the determination of $24,25(OH)_2D_3$. The recovery of $24,25(OH)_2[^3H]D_3$ was 41.7 ± 7.0% (S.D., n = 10) with a range of 31.8—50.7%. $25,26(OH)_2[^3H]D_3$ was well resolved from $24,25(OH)_2[^3H]D_3$ (not shown). Intra-assay variability was 5.5% (10.0 ng/ml, n = 6) and inter-assay variability 9.3% (10.0 ng/ml, n = 6). In normal adults (age 20—24 years) the $24,25(OH)_2D_3$ concentration was 2.3 ± 0.9 ng/ml (5.5 ± 2.2 nmol/l, mean ± S.D., n = 10) with a range of 0.9—4.2 ng/ml. This was 9.1% of the concentration of $25(OH)D_3$ in the same samples (25.2 ± 6.5 ng/ml). Analysis of serum from rats treated previously with large amounts of vitamin D_2 indicated that $24,25(OH)_2D_2$ is eluted about 1 min later than $24,25(OH)_2D_3$. However, the UV method is not sensitive enough to measure $24,25(OH)_2D_2$ (if present) in serum samples from normal adults.

DISCUSSION

The 25-hydroxylation of vitamin D_3 in the liver is only partially regulated by feedback, whereas further hydroxylations of $25(OH)D_3$ to $24,25(OH)_2D_3$ or $1,25(OH)_2D_3$ in the kidneys are strictly regulated by the concentrations of serum calcium, phosphate and parathyroid hormone [4]. The $24,25(OH)_2D_3$ may play a role in bone mineralization [5]. In various physiological and pathological states and during treatment with vitamin D or its metabolites, disturbances may occur in the metabolic system of vitamin D, which requires that reliable assay techniques are available. The present study describes a reproducible and sensitive technique using UV quantitation for estimating $24,25(OH)_2D_3$ in serum. This technique avoids the variability and interference of unknown UV-absorbing and binding compounds associated with the CPB method [1, 6]. The assay is easily combined with currently used HPLC and CPB techniques for the assay of other vitamin D metabolites in serum [1].

The procedure described above enables assay of serum $24,25(OH)_2D_3$ using UV detection with a coefficient of variation of 9%. All major metabolites of vitamin D are separated from $24,25(OH)_2D$ during the purification and final quantitation steps. If present in high amounts, $24,25(OH)_2D_2$ can be quantitated separately from $24,25(OH)_2D_3$, but with physiological levels the UV method is not sensitive enough to measure $24,25(OH)_2D_2$ (if present). The assay is sufficiently sensitive for use in some clinical studies. With 5 ml of serum, 1.0 ng/ml $24,25(OH)_2D_3$ can be detected (normal range 1—5 ng/ml) based on an average recovery of 42%. The mean concentration of $24,25(OH)_2D_3$ found in serum in this study is in agreement with values obtained with techniques where HPLC has been used for purification [6—11]. A recently published spectrophotometric assay for plasma $24,25(OH)_2D_3$ is similar to

the present method, except that a different extraction procedure and two sequential straight-phase HPLC columns were used [12]. The advantages of final quantitation on a reversed-phase column are that less UV-absorbing material is eluted at the elution position of $24,25(OH)_2D_3$ and that the elution time is shortened with increased peak sharpening.

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