Analysis for 1,25-Dihydroxyvitamin D in Human Plasma, after a Liquid-Chromatographic Purification Procedure, with a Modified Competitive Protein-Binding Assay

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1,25-Dihydroxyvitamin D in plasma is measured by competitive protein-binding assay after isolation from plasma. The present method is improved, as compared with those hitherto described, with regard to receptor preparation, isolation of vitamin D metabolites from plasma, and procedure for the competitive protein binding. Receptor preparation from healthy rather than rachitic chicks, together with a fast isolation procedure, results in a high yield of active receptor protein: 12 animals provide receptor for about 4000 incubations. No loss of binding activity was observed during one year. 1,25-Dihydroxyvitamin D is isolated from plasma by a single extraction and a one-step chromatographic purification. Analytical recovery for the entire procedure averaged 78.1% (SD 4.7%). Other vitamin D metabolites (25-hydroxyvitamin D and 24,25-dihydroxyvitamin D) can also be prepared with this procedure. The main features of the modified binding assay are the use of a stabilized cytosol receptor and dextran-coated charcoal instead of polyethylene glycol. The smallest detectable amount in the binding assay is 1-2 pg (2.4-4.8 pmol). Intra- and interassay CVs are 7.9% and 4.8%, respectively. 1,25-Dihydroxyvitamin D concentrations in plasma of 20 healthy subjects averaged 51.7 (SD 10.8) ng/L [124 (SD 26) pmol/L]. Three anephric patients showed values of 3, 6, and 7 ng/L (7, 14, and 17 pmol/L).

Additional Keyphrases: binding protein source, reference intervals, values for anephric patients, measurement of active forms of vitamin D, receptor from duodenum from healthy chicks

Vitamin D must be hydroxylated before its biological activity can be expressed. This takes place in the liver at C-25, after which there is further conversion to biologically active metabolites by hydroxylation at C-1 and C-24, mainly in the kidney. The renally produced metabolite, 1,25(OH)₂D₃, is the most active metabolite known in regulating calcium and phosphorus metabolism as well as in bone resorption (1). Its measurement in human blood will be of great value in the search for possible disorders of vitamin D metabolism in patients with calculous disease (2), decreased renal function (3), and (or) disorders of bone metabolism (4). A prerequisite to the analysis for vitamin D metabolites in clinical investigation is the availability of a relative simple and fast procedure for their measurement.

Previous techniques used for 1,25(OH)₂D assay are bioassay (5), radioimmunoassay (6), mass-fragmentography (7), and radioimmunoassay (8-10). Bioassay and mass-fragmentography are not very suited for routine analysis of 1,25(OH)₂D in plasma, because these methods are either time consuming or require too-large volumes of plasma. Both radioimmunoassay and radioimmunoassay can be adapted for the analysis of many samples. The advantage of the radioreceptor assay is that the receptor is much easier to obtain than an antiserum for use in the radioimmunoassay.

In this report we describe a method for measurement of 1,25(OH)₂D based on the procedure of Eisman et al. (6), with a simple isolation procedure and a modified competitive protein-binding procedure. Moreover, we describe a procedure for preparing receptor protein in high yield and with a high binding capacity. These modifications result in a relatively convenient and fast assay with high precision and a low detection limit. Also, this procedure will be suited for use in isolation of other important vitamin D metabolites in the same run.

Materials

Vitamin D Metabolites

Crystallized 25(OH)D₃ (Phillips Duphar, Weesp, The Netherlands); 24R,25(OH)₂D₃, and 1α,25(OH)₂D₃ (Hoffmann-LaRoche, Basle, Switzerland) were dissolved in absolute ethanol and stored at -20 °C. 25-Hydroxy[26(27)-methyl-³H]cholecalciferol (spec. act. 8 kCi/mol), 24R,25-dihydroxy[23,24(1)-³H]cholecalciferol (spec. act. 68 kCi/mol), and 1α,25-dihydroxy[23,24(1)-³H]cholecalciferol (spec. act. 82 kCi/mol) were purchased from the Radiochemical Centre, Amersham, U.K., and stored in absolute ethanol at -20 °C. 1,25(OH)₂[³H]D₃ used to monitor recovery during plasma purification, was purified by liquid chromatography on a Nucleosil 10–NO₂ column, developed with n-hexane/isopropanol/water (90/10/0.35 by vol) at a flow rate of 2 mL/min.

Reagents

All solvents for extraction and "high-performance" liquid chromatography (HPLC) were of analytical grade and were used without further purification.

Dextran (Grade C) was obtained from BDH Chemicals, Poole, U.K. Activated charcoal (analytical grade) was from

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1 Nonstandard abbreviations used: 1,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; 1,25(OH)₂D, 1α,25-dihydroxyvitamin D (including both D₂ and D₃ derivatives); 25(OH)D₃, tritium-labeled 25-hydroxyvitamin D₃ as specified in Materials; 24,25(OH)₂D₃, tritium-labeled 24R,25-dihydroxyvitamin D₃ as specified in Materials; CPB, competitive protein binding; HPLC, "high-performance" liquid chromatography.

Received Sept. 22, 1980; accepted Dec. 24, 1980.
Merck Nederland, Amsterdam, The Netherlands. Nucleosil 10-NO₂ for HPLC columns was obtained from Machery-Nagel, Duren, F. R. G. Conical polyethylene tubes (13 × 55 mm) for competitive protein binding were from Baker Chemicals, Deventer, The Netherlands. Gelatin came from Brocades, ACF, Maarsen, The Netherlands.

Radioactivity in aqueous solutions was counted in “Lumagel” scintillation fluid (Baker Chemicals, Deventer, The Netherlands) with 36% counting efficiency.

Radioactivity in ethanol solutions was counted in “Permafluor III” (Packard Instrument Belenux S.A., Bruxelles, Belgium) diluted 10-fold (counting efficiency, about 50%).

**Apparatus**

HPLC was performed with two Model 110A pumps (Altex Scientific, Berkeley, CA, 94710) controlled by an Altex 420 microprocessor and provided with a 1203 UV III detector (254 nm; Laboratory Data Control, Riviera Beach, FL, 33404) and an automatic sample processor (Model WISP 710A; Waters Associates, Milford, MA, 01767). Fractions were collected with a Redirac 2112 collector (LKB, Bromma, Sweden). In train with the HPLC column, a three-way valve (Model 7030A, fitted with a Model 7163 solenoid valve; Rhodyne, Inc., Berkeley, CA, 94710) was mounted to direct the flow to the fraction collector or to the ultraviolet detector. Both the fraction collector and the three-way valve were controlled by the Altex 420 microprocessor via home-made interfaces.

Columns for HPLC were packed with a Model 70.00 packing apparatus (Knauer, Berlin, F.R.G.).

Radioactivity was counted in a Model 1215 Rackbeta liquid scintillation counter provided with automatic quench correction (LKB/Wallac OY, Turku, Finland).

**Animals**

Hybro chickens were raised at the “Spelderholt Institute for Poultry Research,” Beekbergen, The Netherlands. The animals were adequately supplemented with calcium (1.1 g/kg of feed), phosphorus (0.83 g/kg of feed), and vitamin D (1800 int. units/kg of feed).

At four weeks of age the chickens were killed for receptor preparation.

**Methods**

**Preparation of Cytosol Receptor**

Batches of 12 chickens were killed by decapitation. The duodenal loops were removed and rinsed with 5 mL of phosphate buffer (50 mmol/L potassium phosphate, 50 mmol/L potassium chloride, 1 mmol/L dithiothreitol, pH 7.4). The mucosa was scraped from the serosa and washed three times with five volumes of phosphate buffer. The mucosa was then homogenized in one volume of buffer with three strokes of a Potter–Elvejem Teflon/glass homogenizer. The whole procedure was carried out at 4 °C within 45 min. After centrifugation in a 10 × 10 mL titanium head at 65 000 rpm (MSE 75 ultracentrifuge, 2 °C; total running time 35 min, max. g value 300 000), the supernates, including the lipid layers, were pooled, divided into 2-mL aliquots, and subsequently frozen in liquid nitrogen. These aliquots were lyophilized overnight and stored under the original freeze-drying vacuum at −40 °C. The protein content of each batch was measured according to Lowry et al. (11).

**Binding Properties of the Cytosol Receptor**

Binding capacity of each batch of receptor protein was determined by incubating increasing amounts of 1,25(OH)₂[³H]D₃ with a constant amount of receptor with and without 5 ng of unlabeled 1,25(OH)₂D₃.

The effect of the presence of gelatin in the incubation solution on the saturation of the receptor was separately studied. The influence of 0.6 and 1.2 g of gelatin per liter in the incubation buffer (see CPB section below) was examined. Temperature dependence of binding was examined by measuring binding of 1,25(OH)₂[³H]D₃ to cytosal receptor at 20, 25, 30, and 37 °C at various intervals.

The effect of different preincubation intervals of receptor with 1,25(OH)₂[³H]D₃ was tested in two experiments. In the first, 1,25(OH)₂[³H]D₃ was added to a freshly prepared solution of cytosal receptor and subsequently incubated at 0 °C for 10, 15, 30, and 45 min. After these preincubations the mixture was transferred to incubation tubes containing 40 pg of unlabeled 1,25(OH)₂D₃. The final composition of the incubation mixture was the same as is described below for the CPB procedure for plasma samples. The incubation tubes were maintained at 20 °C in a water bath. The resulting binding of 1,25(OH)₂[³H]D₃ to the receptor was examined after various times at 20 °C. In a second experiment we tested the effect of short preincubations (1–17.5 min), using the same procedure. Binding at 20 °C in the presence of 40 pg of 1,25(OH)₂D₃ was measured after 75 min.

**Extraction of Plasma**

To 5 mL of plasma was added 6000 dpm of 1,25(OH)₂[³H]-D₃ in 50 µL of ethanol. After equilibration at room temperature for 30 min, the plasma was extracted with 25 mL of n-hexane/isopropanol/n-butanol (93/3/4 by vol). The mixture was vortex-mixed during 3 min and the phases were separated by centrifugation. The aqueous phase was frozen in liquid nitrogen. The organic phase was then decanted into another extraction tube, where it was taken to dryness in a stream of nitrogen.

**Purification of the Plasma Extract**

The residue of the plasma extract was dissolved in 210 µL of n-hexane/isopropanol (9/1 by vol), transferred to low-volume inserts of the automatic HPLC sample processor, and injected onto a Nucleosil 10-NO₂ column (12 × 0.46 cm) fitted with a Nucleosil 10-NO₂ precolumn (4 × 0.46 cm).

1,25(OH)₂D was separated from other vitamin D metabolites and unknown interfering compounds by gradient elution with two solvents (solvent A and B). Solvent A consisted of a mixture of n-hexane/isopropanol/water (80/20/0.7 by vol). Solvent B was n-hexane. Starting with 100% solvent B at a flow rate of 4 mL/min, lipid contaminants were removed from the column. The vitamin D metabolites were then eluted by gradually increasing the proportion of solvent A to 50%. Concurrently, the flow rate was reduced to 2 mL/min. Remaining interfering compounds were removed with 100% solvent A at a flow rate of 4 mL/min. A detailed illustration of the program is given in Figure 1.

Because the first injections of plasma extracts on a new column cause a considerable decrease in the retention times of the vitamin D metabolites, accompanied by poorer resolution, every new plasma was primed by injection of 10 extracts of 5-mL aliquots of rejected plasma.

After each 10 plasma samples the retention times of the vitamin D metabolites were verified by injecting a mixture of 25(OH)₂D₃, 24,25(OH)₃[³H]D₃, and 1,25(OH)₂[³H]D₃ dissolved in n-hexane/isopropanol (9/1 by vol). The fraction containing 1,25(OH)₂D was collected, evaporated to dryness under nitrogen, and the residue was redisolved in 215 µL of absolute ethanol. Recovery determination and CPB were both carried out in duplicate with 50-µL aliquots.

Separation of 1,25(OH)₂D from other plasma constituents that displace 1,25(OH)₂[³H]D₃ from the intestinal receptor was verified by monitoring the displacement activity in the fractions of a 5-mL aliquot of the plasma pool after HPLC. Fractions were collected at 1-min (0–6 min), 0.5-min (6–29
double the binding capacity of the preparation. In this way, 12 four-week-old, healthy Hybro chicks provided about 1.7 g of lyophilized receptor protein, sufficient for about 4000 incubations. When stored lyophilized at –40 °C under the original freeze-drying vacuum, no loss of binding potency was observed during one year.

**Binding Properties of the Cytosol Receptor**

A saturation curve of the receptor protein showed no complete saturation of specific binding, even at high concentrations of 1,25(OH)2[3H]D3. In the presence of 50 pg of 1,25(OH)2[3H]D3, 0.4 mg of receptor protein bound 40 to 50% of the labeled hormone in the described incubation mixture. This resulted in a specific binding that could be accurately measured without the need for excessive counting times.

Addition of 0.6 and 1.2 g of gelatin per liter to the incubation buffer did not change the amount of 1,25(OH)2[3H]D3 necessary to saturate the receptor. Specific binding, however, increased 15 and 29%, respectively, as compared with incubations without gelatin. For practical reasons the incubation buffer was prepared with 1 g of gelatin per liter.

Binding of 1,25(OH)2[3H]D3 to the cytosol receptor increased with decreasing temperature. However, equilibration time also increased with decreasing temperature. These properties accorded with previous results for the receptor prepared from rachitic chickens (6). To obtain a high binding without the need for a long incubation, we chose an incubation at 20 °C for 75 min.

As can be seen in Figure 2, prolonged preincubation of cytosol with receptor resulted in a decrease in specific binding. The specificity of the binding decreased with increasing time of preincubation, indicating a decrease in affinity of the receptor.

**Results**

**Receptor Preparation**

We compared receptor prepared from duodena of four-week-old rachitic chickens with that from nonrachitic chickens. The use of nonrachitic animals provided at least twice as much receptor protein as rachitic chickens because the nonrachitic chickens grew much faster. Moreover, several of the rachitic chickens died before they were four weeks old.

Shortening the total ultracentrifuge running time to 35 min
tosol with receptor at 0°C caused substantial binding of 1,25(OH)$_2$[3H]D$_3$ to the receptor, which increased with preincubation time.

The differences in binding that existed after the preincubation still partly persisted after various incubation times at 20°C in the presence of 40 pg of unlabeled 1,25(OH)$_2$D$_3$. An incubation time of 40 min at 20°C seems to be sufficient to obtain nearly equilibrium conditions. To be completely sure of equilibrium conditions, irrespective of varying preincubation times, we used an incubation time of 75 min at 20°C. Under these circumstances short preincubation times (1–17.5 min) were re-examined. The final binding appeared to be constant when preincubation time was not longer than 5 min. Preincubating more than 5 min resulted in an increase of the final binding, indicating partly irreversible binding of 1,25(OH)$_2$[3H]D$_3$ during the preincubation. To prevent differences in binding within a series of assay tubes, we limited the preincubation time to less than 5 min.

Extraction

The one-step extraction procedure with the new solvent resulted in a high extraction efficiency. The recovery of 1,25(OH)$_2$[3H]D$_3$ added to 5 mL plasma was 89.7% (SD 1.3%) (n = 10).

Under the same circumstances, 25-hydroxyvitamin D$_3$ and 24,25-dihydroxyvitamin D$_3$ were recovered from plasma in a high yield (94% and 89%, respectively).

Purification of the Plasma Extract

The Nucleosil 10-NO$_2$ packing material, used in preparing the HPLC columns, showed relatively short retention times for the vitamin D metabolites studied. In addition, this material showed good compatibility with repeated gradient elution of plasma extracts. More than 100 plasma extracts could be purified with one column.

The quality of the separation procedure was determined by fractionating the complete eluate from the HPLC after injection of a plasma extract. The fractions tested for displacement activity in the 1,25(OH)$_2$D$_3$ assay showed four peaks (Figure 3). The first and second peak coeluted with 25(OH)-[3H]D$_3$ and 1,25(OH)$_2$[3H]D$_3$, respectively; the third peak probably consisted of an impurity introduced by the purification procedure, because it was also present in the HPLC profile of a water extract. The last peak was caused by a rather polar compound present in plasma extracts that displaces 1,25(OH)$_2$[3H]D$_3$ from its intestinal cytoisol receptor. The compound could be removed from the column with 100% solvent A. Under the conditions we used, the whole HPLC run took place within 55 min. Re-equilibration of the column with solvent B took another 10 min. The isolation procedure described yielded well-purified fractions with 1,25(OH)$_2$D$_3$. Water blanks carried through the whole procedure contained no displacement activity in the 1,25(OH)$_2$D$_3$ assay (less than 2 pg per tube).

The one-step purification resulted in very reproducible and high recoveries. Overall recovery after HPLC for 1,25(OH)$_2$-[3H]D$_3$ was 78.1% (SD 4.7%).

Competitive Protein Binding

High intra-assay reproducibility of the assay could be attained by observing the following details:

1. The ethanol content of the incubation mixture should be exactly the same for every incubation tube. For that purpose evaporation of the ethanol in which the samples and standards were dissolved was minimized by adding 100 μL of phosphate buffer to the tubes before samples and standards were added.

2. Receptor and 1,25(OH)$_2$[3H]D$_3$ were combined before addition to the assay tubes. This resulted in a more stable receptor protein and in a very constant amount of receptor and 1,25(OH)$_2$[3H]D$_3$, in exactly the same ratio, for each tube.

3. Separation of bound from free 1,25(OH)$_2$D$_3$ was very conveniently carried out with dextran-coated charcoal; this resulted in better reproducibility of standard curves than does the polyethylene glycol method.

The procedure developed appeared to be convenient and reliable, with sensitive standard curves (Figure 4a). When the reciprocals of specific binding of the standards were plotted against the amount of unlabeled 1,25(OH)$_2$D$_3$, straight lines resulted, which means that the curves correspond with theoretical isotope-dilution curves (Figure 4b). The detection limit of the assay (when expressed as two standard deviations of the binding without unlabeled 1,25(OH)$_2$D$_3$) was 1–2 pg per tube.

Precision of the Assay

The intra- and interassay CVs were determined by analyzing a pool of plasma from 20 healthy subjects eight times
on six different days (Table 1). Intra-assay CV averaged 7.0 (SD 5.2)%. Interassay CV, derived from the mean of eight measurements on six days, was 4.8%. The overall CV was 9.0%. Addition of 34.4 ng/L and 57.1 ng/L of 1,25(OH)2D3 to plasma from the pool, which had an endogenous 1,25(OH)2D3 of 53.7 ng/L, resulted in a mean recovery of the added metabolite of 97 (SD 12)% and 103 (SD 13%), respectively (Table 2).

Concentrations in Plasma

Individual values for plasma from the subjects contributing to the plasma pool averaged 51.7 (SD 10.8) ng/L (n = 20; range 32.0–78.5 ng/L). Plasma 1,25(OH)2D concentrations measured 3, 6, and 7 ng/L in three anephric patients.

Discussion

Receptor Preparation

Hitherto, various protein preparations have been used for the competitive protein-binding assay of 1,25(OH)2D3: cytosol receptors from chicken duodenum (6, 12–14) and antibodies raised against 1,25(OH)2D3 (8–10). With the use of receptor protein, problems were encountered from the slight stability (6, 12, 15, 16) and from the need for rachitic chickens used as a source of the duodenal receptor protein. A disadvantage of the antibody was the long time necessary to raise sufficient titers in a suitable animal.

Because of these time limitations we chose to use the cytosol receptor and had to cope with the difficulties mentioned. The modified procedure resulted in a high yield of stable receptor, which makes the use of the cytosol receptor very attractive again.

Binding Properties of the Cytosol Receptor

Binding capacities of the receptor preparations reported hitherto vary strongly (6, 17, 18), probably because of the instability of the receptor and the very crude preparations used. The impurity of the protein preparation may also account for the fact that the saturation curve did not show complete saturation of the receptor with increasing 1,25(OH)2D3 concentrations. The increase of specific binding caused by gelatin is not fully understood. Probably gelatin prevents adsorption of the 1,25(OH)2D/receptor complex to charcoal.

McCain et al. (15) and Kream et al. (16) reported stabilization of the solubilized receptor by adding 1,25(OH)2D3 to the receptor at temperatures below 4 °C. We tried this and found that binding was almost completely reversible when preincubation was limited to less than 5 min. Even better results could be obtained by preincubating at -3 °C. The use of receptor stabilized with 1,25(OH)2[3H]D3 offered the opportunity to improve the reproducibility of the CPB assay.

Extraction

Generally 1,25(OH)2D3 is extracted by a multi-step procedure, sometimes combined with an additional clean-up of the extracts. The developed extraction had the advantage that the examined vitamin D metabolites were extracted in a high yield by a one-step procedure.

Purification of the Plasma Extract

Almost all reports on the assay of 1,25(OH)2D in plasma or serum describe the preparufication of plasma extracts on Sephadex LH-20 before HPLC. The improved HPLC procedure enabled us to omit the preparufication with Sephadex LH-20.

A special feature of our purification procedure was the detection in human plasma of a component more polar than 1,25(OH)2D that effectively displaced 1,25(OH)2[3H]D3 from its intestinal cytosol receptor. Remarkable was the large amount of displacement activity in the polar range of the chromatogram, at least four times as much as in the 1,25(OH)2D region with our procedure. Columns generally used for preparation probably retain the observed polar compound(s), thus precluding its detection in the subsequent CPB assay. The nature and possible biological function of this (these) compound(s) are not clear, but will be subject to further investigation.

Separation from 24,25-dihydroxyvitamin D and 25,26-dihydroxyvitamin D was not critical, because they did not interfere in our CPB assay as far as it concerned their physiological concentrations (Figure 3). Nevertheless, our HPLC procedure provided a good separation of all vitamin D metabolites studied. This will be discussed in forthcoming reports.

Retention times of the vitamin D metabolites were constant enough that the HPLC procedure could be mechanized, which offered the opportunity to carry out sample purification overnight.

Competitive Protein Binding

Good reproducibility of extraction and purification enabled the addition of a constant amount of 1,25(OH)2[3H]D to the standard solutions, to compensate for the remaining spike in the plasma samples. The use of dextran-coated charcoal instead of hydroxyapatite (17) or polyethylene glycol (6) had the advantage of fewer practical complications and better precision. Short preincubation of receptor and 1,25(OH)2[3H]D3 eliminated the systematic intra-assay variation caused by instability of the receptor protein. Improved precision of the assay allowed a limitation to duplicate measurements of standards and samples, which increased the number of samples that could be assayed with a given amount of receptor protein.

Plasma Samples

Concentrations of 1,25(OH)2D reported in plasma previously (Table 3) were generally lower than the concentrations...
we found in healthy subjects. Possible explanations for the divergent values that have been measured might be geographical, nutritional, or racial differences in the investigated populations or the use of different purification and assay procedures, including systematic errors owing to extremely diluted standard solutions. Moreover, the small size of our study population also might account for the differences observed.

The 1,25(OH)2D concentrations measured in the three anephric subjects were very low but significantly different from zero. Until now, 1,25(OH)2D in anephric subjects was reported to be undetectable (8, 12, 14, 19–22). The detection limit of these earlier assays, however, was 4–10 ng/L.

Gray et al. (23) reported the presence of a compound in the plasma of anephric subjects treated with dihydrotachysterol that interfered with the 1,25(OH)2D measurement. The three subjects we examined had no dihydrotachysterol therapy and had not received blood transfusions for at least four weeks. Whether the measured concentration of 1,25(OH)2D represented extrarenal production, intake of exogenous 1,25(OH)2D in some way, or an artefact remains to be established.

We conclude that our assay procedure has high precision and a low detection limit. The purification procedure is potentially suited for the analysis of other vitamin D metabolites and can be automated. The rather large number of plasma or serum samples that can be processed this way makes it an attractive assay for clinical application.

We thank the Spelderholt Institute for Poultry Research at Beekbergen for their willingness to raise rachitic and healthy chickens for our investigation. The Radio Nucliden Centrum of the Vrije Universiteit is kindly acknowledged for its hospitality.

This work was supported by a grant from the Nier Stichting Nederland.

References
1. DeLuca, H. F., Recent advances in our understanding of the vitamin D endocrine system. J. Steroid Biochem. 11, 35–52 (1979).

