Improved Radioimmunoassay for Vitamin D and Its Use in Assessing Vitamin D Status

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We describe a faster, more-sensitive radioimmunoassay for vitamin D in plasma. Antibodies were generated in rabbits immunized with a new vitamin D analog, the 25,24,25,26,27-pentanor-C(22)-carboxylic acid of vitamin D, coupled directly with bovine serum albumin. After several months, Rivanol-treated sera from the rabbits contained high-titer antibodies, as determined by their abilities to bind 25-hydroxy[3H]cholecalciferol. The antibody, used at a 1:15,000 final dilution, cross-reacted equally with all cholecalciferol and ergocalciferol metabolites tested except 1,25-dihydroxycholecalciferol metabolites and the parent calciferols. 25-Hydroxycholecalciferol and similar forms were efficiently extracted from plasma or serum with acetonitrile. We separated bound from free 25-hydroxy-[3H]cholecalciferol by using a second antibody: goat antiserum to rabbit serum. The detection limit of the assay was 3.0 pg of 25-hydroxycholecalciferol and its equivalents per tube; thus only 1 µL of plasma is needed per assay tube. Results compared well with those from a liquid-chromatographic procedure involving specific ultraviolet detection of 25-hydroxycholecalciferol in plasma.

Additional Keyphrases: 25-hydroxyvitamin D status · vitamin D and its metabolites · reference values · liquid chromatography compared · cholecalciferol

Calciferol (vitamin D) metabolites are mostly 25-hydroxycholecalciferols [25-(OH)D₃], which are therefore considered to be the primary indicator of vitamin D status (1). These compounds can exist in two forms: cholecalciferol (vitamin D₃), which is derived from synthesis in the epidermis, and ergocalciferol (vitamin D₂), which is derived solely from plant sources (2, 3). Thus the overall vitamin D status of the organism depends on endogenous (sun exposure) and exogenous (dietary intake) sources (4, 5), and it is important to measure both forms.

Various assays have been developed for measuring 25-(OH)D in plasma. Most are competitive protein-binding assays, involving the vitamin D-binding protein from various animal species (6–8), and most require extracting the plasma with organic solvent followed by chromatographic purification before assay. Some nonchromatographic assays for determining vitamin D status have been described (7–9), but their validity has been questioned (10). Recently, two radioimmunoassays (RIAs) for vitamin D status have been described. One (11) requires preliminary chromatography and neither measures 25-hydroxyergocalciferol [25-(OH)D₂] (11, 12). Thus, their usefulness in assessing total vitamin D status in cases where vitamin D₂ is used widely as a dietary supplement is limited.

We have synthesized a compound, 23,24,25,26,27-pentanor-C(22)-carboxylic acid vitamin D [vitamin D-C(22)-acid], that lacks the calciferol side-chain. When coupled to bovine serum albumin, it thus can induce antibodies that cross react equally with vitamin D₃ and D₂ metabolites. We describe here our RIA directed against this new vitamin D analog and evaluate its utility in assessing the overall vitamin D status of human subjects.

Materials and Methods

Materials

Reagents. Crystalline cholecalciferol (vitamin D₃) and ergocalciferol (vitamin D₂) were obtained from Sigma Chemical Co., St. Louis, MO. Crystalline 25-hydroxycholecalciferol [25-(OH)D₃], 24,25-dihydroxycholecalciferol [24,25-(OH)₂D₃], 23,25-dihydroxycholecalciferol [23,25-(OH)₂D₃], 25S,26-dihydroxycholecalciferol [25S,26-(OH)₂D₃], 1,25-dihydroxyergocalciferol [1,25-(OH)₂D₃], and 1,25-dihydroxycholecalciferol [1,25-(OH)₂D₃] were from Hoffmann-La Roche Inc., Nutley, NJ. Biozthetic 25-hydroxyergocalciferol [25-(OH)D₂], 24,25-dihydroxyergocalciferol [24,25-(OH)₂D₂], and 23,25-dihydroxyergocalciferol [23,25-(OH)₂D₂] were gifts from Drs. T. A. Reinhardt and R. L. Horst, Ames, IA. 25-Hydroxy[26,27,3H]cholecalciferol (spec. acty. 90 kCi/mol) was synthesized as described elsewhere (13). Swine-skin gelatin, 6,9-diamino-2-ethoxyacridine lactate (Rivanol), and Freund's complete and incomplete adjuvants were from Sigma Chemical Co. Goat antirabbit second antibody precipitating complex was from Immuno Nuclear Corp., Stillwater, MN. "HPLC" grade acetonitrile was from Fisher Chemical Co., Pittsburgh, PA.
Unless otherwise noted, all other reagents were reagent grade.

Clinical samples. Plasma samples for this study were obtained from 50 apparently normal individuals, five infants known to have low 25-(OH)D concentrations, and 10 patients from southern Florida who were undergoing chronic hemodialysis.

Methods

Concentrations of vitamin D and its metabolites were determined by ultraviolet spectroscopy, with molar absorptivity $\varepsilon_{\text{mol}}$ of 18 300 mol$^{-1}$cm$^{-1}$ for vitamin D$_3$ and its metabolites and $\varepsilon_{\text{mol}}$ of 19 400 mol$^{-1}$cm$^{-1}$ for vitamin D$_2$ and its metabolites.

Preparation of antiserum. We immunized female rabbits by multiple intradermal injections of immunogen, 250 $\mu$g per injection, in 1.5 mL of complete Freund's adjuvant initially, with subsequent injections in incomplete adjuvant at monthly intervals. Ten days after each injection, we sampled blood from an ear vein and stored the serum at $-20^\circ$C. Before use in the radioimmunoassay we treated the serum from the immunized rabbits with Rivanol to remove $\alpha$-globulins (14), incubating 100 $\mu$L of immune serum overnight at 4°C with 500 $\mu$L of a 11 mmol/L solution of Rivanol. After centrifugation (2000 $\times$ g, 4°C, 5 min), the supernate was stored at $-20^\circ$C until used in the RIA. The highest-titer antiserum was obtained from rabbit 2 after six months of injection and was used in the present study.

Preparation of methyl-22,23-bisnor-5,7-cholesten-3-ol. We dissolved 1 g of 22,23-bisnor-5,7-cholesten-3-acetate (Steraloids, Wilton, NH) in 50 mL of diethyl ether, and to this a solution of diazomethane in ether until a stable yellow color was maintained. The solvent and excess diazomethane were evaporated under nitrogen, leaving 1.1 g of the methyl ester. We heated a mixture of the methyl ester (0.5 g, 1.25 mmol), sodium bicarbonate (0.7 g, 3.3 mmol), and 1,3-dibromo-5,5-dimethylhydantoin (0.2 g, 0.71 mmol) in 9 mL of hexane and 5 mL of benzene for 20 min at 90°C. Under nitrogen, then cooled and filtered the mixture. After the filtrate was washed with hexane, it was evaporated under reduced pressure. We dissolved the residue in 1.3 mL of 2,4,6-collidine and 14 mL of xylene and refluxed the mixture for 1.5 h under nitrogen. The reaction mixture was then cooled, diluted with 20 mL of benzene, and washed successively with 20 mL each of 1 mL of HCl, 50 mmol/L sodium bicarbonate, and water. The solvents were evaporated from the organic phase under reduced pressure. After dissolving the residue in 18 mL of dioxane, we added 70 $\mu$L of p-toluenesulfonic acid, and heated the solution at 70°C for 1 h. We cooled the reaction mixture, then added 18 mL each of water and diethyl ether. After washing the ether phase with 18 mL each of 50 mmol/L sodium bicarbonate, water, and saturated aqueous sodium chloride, we evaporated the organic phase under reduced pressure, then dissolved the residue in 6 mL of diethyl ether and 5 mL of 0.5 mol/L potassium hydroxide in methanol, and let the mixture stand at ambient temperature for 2.5 h. We then added 6 mL of water and ether, washed the ether phase repeatedly with 6 mL of water, and removed the solvents under reduced pressure. The product was purified by eluting it through a 0.9 × 20 cm silica gel column with ethyl acetate/hexane (1/3, by vol). Crystallization from hexane gave 347 mg of product (69% yield). On thin-layer chromatography with ethyl acetate/hexane (1/3, by vol) the product $R_f$ was 0.24; in ethanol, the product absorbed at 293, 282, 272, and 263 (shoulder) nm.

Preparation of C(22)-acid of vitamin D. A solution of the 5,7-diene (1.0 g) in 950 mL of diethyl ether was stirred under argon at 0°C in a photochemical reaction assembly (Ace Glass, Vineland, NJ 08360; cat. no. 7840-185) fitted with a Vycor filter (Ace Glass, absorption sleeve, cat. no. 7835-40). The solution was irradiated for 5 min with a 450-W, medium-pressure, quartz mercury-vapor lamp, then the solvent was evaporated under reduced pressure. We applied the residue to a $20 \times 20 \times 1$ cm silica gel thin-layer chromatographic plate and purified the previtamin by developing twice with ethyl acetate/hexane (3/7, by vol).

We refluxed the purified previtamin in ethanol for 2.5 h at 50°C under nitrogen. The solvent was removed under reduced pressure to yield 232 mg (23%) of the methylated C(22)-acid of vitamin D. By thin-layer chromatography with ethyl acetate/hexane (1/3, by vol) as the developing solvent system the $R_f$ of the product was 0.4. Its showed an absorbance maximum in ethanol at 267 nm and the characteristic absorption minimum at 229 nm ($\lambda_{\text{max}}$ = 2.2). By electron ionization mass spectroscopy peaks (and relative intensity) were at m/z 356 (M$^+$, 15), 340 (M$^+$-H$_2$O, 8), 325 (M$^+$-H$_2$O-CH$_3$, 12), 299 (M$^+$-COOCH$_3$, 271 (M$^+$-side chain, 5), 253 (M$^+$-H$_2$O-side chain, 16), 136 (A ring plus C(6) and C(7), 60), and 118 (m/z 136-H$_2$O, 100).

We heated the methyl ester (185 mg) at 50°C in 4.5 mL of a 250 g/L solution of potassium hydroxide in methanol for 5 h. After cooling the reaction mixture we adjusted the pH to 3 with 6 mol/L HCl, then added 4.5 mL each of diethyl ether and water. The phases were separated and the solvent was evaporated from the organic phase. We purified the residue by thin-layer chromatography, developing with 2-propanol/hexane (1/8, by vol), obtaining 133 mg (71%) of the free acid with an $R_f$ of 0.29. The compound showed an absorbance maximum in ethanol at 267 nm, with a characteristic absorbance minimum at 229 nm ($\lambda_{\text{max}}$ = 1.9). The compound was negative for chloride ion in the chemical ionization mass spectrum m/z 379 (M$^+$--SCN$^-$).

Preparation of the BSA conjugate of vitamin D-C(22)-acid. We added 36 mg of the C(22)-vitamin D acid to 150 $\mu$L of dioxane, then added 25 $\mu$L of tri-n-butylamine and 14 $\mu$L of isobutylchloroformate. After cooling the solution to 4°C, we added 1.8 mL of bovine serum albumin solution (130 mg, 2 $\mu$mol/L) in an equivalent mixture of water and dioxane. The pH of the reaction mixture was maintained at 8.5 by adding sodium hydroxide. The reaction mixture was stirred for 40 h, then applied to a 0.9 × 20 cm column of Sephadex G-50. The conjugate was eluted with 25 mL of 0.1 mol/L sodium phosphate buffer (pH 7.2). The ratio of vitamin to albumin was calculated as 22/1, as determined by the difference in absorbance between bovine serum albumin and the conjugate at 265 nm. The yield of conjugate was 51.2 mg.

Extraction of 25-hydroxycholecalciferol from plasma. We extracted 25-(OH)$_2$D$_3$ and related metabolites from human plasma as follows. We placed 1.2 mL of acetonitrile in 12 × 75 mm borosilicate glass tubes, added 25 $\mu$L of plasma sample by dropping it through the acetonitrile, and capped the tube. After vortex-mixing for 15 s, we let the sample stand for 15 min, then centrifuged (2000 × g, 4°C, 5 min), and transferred 25 $\mu$L of the supernate to a 12 × 75 mm borosilicate glass tube.

Radioimmunoassay. Prepare assay tubes containing 25-(OH)$_2$D$_3$ extracted from plasma as described above or standards of authentic 25-(OH)$_3$D$_3$ (0, 5, 10, 20, 40, 60, 80, 150, 200 pg—or 25 000 pg to estimate nonspecific binding) in ethanol. Evaporate the solvent from all sample and standard tubes, under nitrogen. To each tube add 25-[$^3$H](OH)$_2$D$_3$, 5000 counts/min in 50 $\mu$L of absolute ethanol, and gently vortex-mix. Then add to each tube 500 $\mu$L of antisemur dilute 15 000-fold in sodium phosphate buffer (50 mmol/L, pH 7.4) containing 0.4 g of swine-skin gelatin.
per liter. Vortex-mix the contents of the tubes and incubate them for 2 h at 4°C. Then add 500 μL of the second-antibody precipitating complex to each tube, vortex-mix, incubate at 4°C for 15 min, and centrifuge (4°C, 2000 x g, 15 min). Discard the supernate, and redissolve the pellet from each tube in 400 μL of 50 mmol/L NaOH, transfer the sample to a scintillation vial containing 5 mL of scintillation fluid, and count the radioactivity in each vial. Our liquid-scintillation system (Beckman LS-8000) performed at 40% efficiency.

Direct ultraviolet detection of 25-(OH)D in plasma after "high-performance" liquid chromatography or competitive protein-binding assay. Total 25-(OH)D [25-(OH)D2 and 25-(OH)D3] was determined in plasma by direct quantification of ultraviolet absorbance after liquid-chromatographic purification by a previously described method (15). For the samples from infants we used a competitive protein binding assay (8) to determine total 25-(OH)D in serum.

Calculations. We calculated the 25-(OH)D-equivalent value (picograms per tube) by a logit/log plot of the data (see Figure 1 below) (16). The results of this RIA are expressed in terms of 25-(OH)D-equivalents because the assay detects not only 25-(OH)D but also the various other metabolites of vitamin D contained in plasma (see Table 1 below).

Results

Antibody production. Rabbits immunized with vitamin D-C(22)-acid coupled to bovine serum albumin developed antibodies against the antigen that bound 25-[3H](OH)D3 when incubated with diluted Rivanol-treated immune serum and could be selectively precipitated with a goat antirabbit second antibody. Nonimmune Rivanol-treated rabbit serum failed to bind 25-[3H](OH)D3.

Extraction of 25-(OH)D3 from plasma. Extraction of 25-(OH)D3 from plasma with acetonitrile was quantitative, 100.7% (SD 2.5%; n = 6), as determined by the analytical recovery of 25-[3H](OH)D3 that had been pre-incubated with the plasma. But for this recovery to be quantitative, one must add the plasma to the acetonitrile, not vice versa. When this order of addition is reversed, one recovers only 53.3% (SD 3.5%; n = 6) of the 25-[3H](OH)D3.

Specificity of radioimmunoassay. Table 1 summarizes the cross reactivity of vitamin D and several of its metabolites with the antibody generated against the vitamin D-C(22)-acid. Several vitamin D metabolites could equally well displace 25-[3H](OH)D3 from the antibody. However, vitamins D2, D3, 1-25-(OH)D2, and 1,25-(OH)D3 were less effective. Cholesterol demonstrated no ability to displace 25-[3H](OH)D3 from the antibody. The steroid specificity of the antiserum differed from that of the vitamin D-binding protein (17).

Sensitivity, analytical recovery, and precision. The sensitivity of the RIA, defined as 2 SD from the mean for data on the zero sample, was 3 pg per tube (Figure 1), corresponding to a detection limit of 3 μg of 25-(OH)D equivalents per liter of plasma. Bound tracer was displaced 50% at about 60 pg of 25-(OH)D3 per tube. In the absence of 25-(OH)D3 standard, 30% of the total 25-[3H](OH)D3 was bound to the antibody.

We estimated the analytical recovery of 25-(OH)D3 in the assay at various concentrations of 25-(OH)D3 added to a vitamin D-deficient plasma sample (Table 2). An average of 108% (SD = 18.8%, n = 4) of the added 25-(OH)D3 was accounted for. Intra- and interassay CVs for the immunoassay were <13%.

Comparison of RIA with independent assay method. Results obtained by the present method for the 50 healthy subjects were compared with those by a conventional assay (15) with which 25-(OH)D was measured in plasma by direct quantification of ultraviolet absorbance after liquid chromatography (Figure 2). Assessment of vitamin D status as determined by the two assay methods was quite similar, 26.0 (SD 11.0) μg of 25-(OH)D3-equivalents per liter of plasma for the RIA vs 25.5 (SD 11.8) μg of 25-(OH)D3 per liter of plasma for direct quantification.

The corresponding values for 10 renal-disease patients

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**Table 1. Cross Reactivity of Some Compounds with 15 000-fold Diluted Antiserum to 23,24,25,26,27-Pentanor-C(22)-carboxylic Acid Vitamin D**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reactivity, %</th>
</tr>
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<tbody>
<tr>
<td>Vitamin D2</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td>10</td>
</tr>
<tr>
<td>25-(OH)D2</td>
<td>100</td>
</tr>
<tr>
<td>25-(OH)D3</td>
<td>100</td>
</tr>
<tr>
<td>24,25-(OH)D2</td>
<td>100</td>
</tr>
<tr>
<td>24,25-(OH)D3</td>
<td>100</td>
</tr>
<tr>
<td>25,26-(OH)D2</td>
<td>100</td>
</tr>
<tr>
<td>25,26-(OH)D3</td>
<td>100</td>
</tr>
<tr>
<td>23,25-(OH)D2</td>
<td>100</td>
</tr>
<tr>
<td>1,25-(OH)D2</td>
<td>5</td>
</tr>
<tr>
<td>1,25-(OH)D3</td>
<td>5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Ability to displace 50% of the 25-[3H](OH)D3 from the antiserum (from rabbit 2) diluted 15 000-fold.

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**Table 2. Analytical Recovery of 25-(OH)D3 from Vitamin D-Deficient Serum**

<table>
<thead>
<tr>
<th>Added 25-(OH)D3 μg/L</th>
<th>Measured</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.0</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>7.3</td>
<td>4.3</td>
</tr>
<tr>
<td>10</td>
<td>16.8</td>
<td>13.8</td>
</tr>
<tr>
<td>20</td>
<td>24.2</td>
<td>21.2</td>
</tr>
<tr>
<td>40</td>
<td>43.7</td>
<td>40.7</td>
</tr>
</tbody>
</table>

* Determined in duplicate by radioimmunoassay as described in text.
who were undergoing chronic hemodialysis were respectively 32.2 (SD 16.3) and 31.7 (SD 19.2) µg per liter of plasma. Those for five infants’ serum samples were 3.1 (SD 1.9) and 3.0 (SD 1.8) µg per liter, respectively.

Discussion

Although many methods have been developed for determination of vitamin D status in humans (6–12), a rapid nonchromatographic assay is still needed, such as the present one, an RIA based on use of a new vitamin D analog, vitamin D-C(22)-acid. Coupling this compound to bovine serum albumin allowed us to generate antibodies that cross reacted equally with most vitamin D₂ and D₃ metabolites (Table 1). The structures for vitamins D₂ and D₃ differ only with respect to their side chains: vitamin D₂ contains a double bond between carbons 22 and 23 and has a C(28) methyl group attached to carbon 24. Because our analog retained the intact structure of vitamin D only up to carbon 22, the structural differences between vitamins D₂ and D₃ were not involved in the antibody recognition, and antibodies directed against this analog could not discriminate with respect to side-chain metabolism of vitamin D. The antibodies should, however, be specific for the open B-ring cis-triene structure containing a 3β-hydroxyl group that is inherent in all vitamin D compounds—and indeed the antibodies generated against this analog generally displayed these characteristics.

As Table 1 demonstrates, all side-chain metabolites tested, except 1,25-(OH)₂D₂ and 1,25-(OH)₂D₃, interacted equivalently with the antibody. The inability of 1,25-(OH)₂D₂ and 1,25-(OH)₂D₃ to interact effectively with the antibody can be attributed to the 1α-hydroxyl group on these compounds. Surprisingly, the parent compounds, vitamins D₂ and D₃, interacted less well with the antibody, but we believe this may be related to their solubility. In the aqueous RIA incubation solution, the parent compounds are much less soluble than 25-(³H)OH-D₂ and this greatly impedes their interaction with the antibody. This same problem has been encountered when competitive protein binding assay was used to measure the parent compounds in plasma (16). The structural requirement for the cis-triene system as a prerequisite for binding to the antibody is demonstrated by the inability of cholesterol to interact with the antibody (Table 1).

The assay system used here to determine vitamin D status is simple, sensitive, and rapid. However, it is important to remove α-globulins from the immune serum with Rivanol before it is used in the RIA. This step eliminates vitamin D-binding protein from the immune serum, thus eliminating the possibility of its interference by competing with the antibody for binding of the vitamin D compounds in the assay solution.

Because the extraction procedure is quantitative, determining the individual recovery of each sample is unnecessary. The sensitivity of the RIA, 3 µg of 25-(OH)D-equivalent per liter, is such that this assay can be used with plasma in the vitamin D deficiency range (19). Use of only 1 µL of plasma extract per sample tube also eliminates the nonspecific lipid interferences that are observed in other nonchromatographic vitamin D assays (9, 10).

We used 25-(OH)D₃ and 25-(³H)(OH)D₃ as standard and tracer, respectively, for this RIA because they are widely available and also because nutritional vitamin D status has been defined in terms of the amount of circulating 25-(OH)D (1). Although many vitamin D metabolites other than 25-(OH)D are present in the circulation, they contribute by only a small percentage (6–7%) to the overall assessment of nutritional vitamin D status as compared with 25-(OH)D (20). The parent compounds, vitamins D₂ and D₃, are unreliable indicators of overall vitamin D status and usually are present in the circulation in very low concentrations (20, 21).

On comparing results with those by other methods for determining the 25-(OH)D content of plasma, we found good agreement with respect to 25-(OH)D status in a population of normal subjects (Figure 2), and in renal-disease patients’ plasma and cord blood. The amounts determined by RIA also agree well with those reported in previous studies (1, 6, 11, 12, 20). Thus we conclude that our RIA is valid for assessing nutritional vitamin D status, and we believe it to be an important clinical tool.

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References