A Modified Radioimmunoassay for 1,25-Dihydroxycholecalciferol

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A radioimmunoassay for 1,25-dihydroxycholecalciferol which did not cross react with 1,25-dihydroxyergocalciferol is described. IgG fractions were prepared from the serum of rabbits that had been immunized with 1,25-dihydroxycholecalciferol-3-hemisuccinate coupled to bovine albumin. Radioligand binding by the IgG fractions was time-, temperature-, and pH-dependent. The IgG fractions had a high affinity for 1,25-dihydroxycholecalciferol but cross reacted with 25-hydroxycholecalciferol and 24,25-dihydroxycholecalciferol. Vitamin D3 metabolites did not cross react in the assay when amounts up to 9 ng per tube were tested. The determination of 1,25-dihydroxycholecalciferol in human serum required an organic extraction and chromatographic isolation of the metabolite. Radioligand binding was influenced by the presence and concentration of the proteins in the phosphate buffer. The mean concentration of 1,25-dihydroxycholecalciferol in serum from normal adults was 56 (SEM 5.7) ng/L. 1,25-Dihydroxycholecalciferol was not detectable in serum from a nephrectomized subject and the concentration in serum was lower than normal in hypoparathyroid patients. Ingestion of 1,25-dihydroxycholecalciferol by nephrectomized or hypoparathyroid patients restored the concentration of 1,25-dihydroxycholecalciferol in serum to the normal range. The stability of the IgG fraction, the relatively short incubation interval, and the ability to measure 1,25-dihydroxycholecalciferol without interference from 1,25-dihydroxyergocalciferol are unique aspects of this radioimmunoassay.

Additional Keyphrases: reference intervals • "high-pressure" liquid chromatography • values for nephrectomized or hypoparathyroid patients • vitamins D

1α,25-Dihydroxycholecalciferol (1,25(OH)2D3) is the most active metabolite of cholecalciferol (vitamin D3) (1). The development and application of protein-binding assays to measure the concentration of 1,25(OH)2D3 in biological fluids has provided important information about this metabolite (2-5). The binding proteins in these assays were obtained from the cytosolic fraction of chick intestinal mucosa (2-5). The procurement and storage of these proteins requires special attention and technique (5). The need for an easily obtained and stable binding protein motivated several groups to develop radioimmunoassays for 1,25(OH)2D3 (6-8). This report describes the development of a radioimmunoassay in which antibodies reacting with 1,25(OH)2D3 but not with 1,25-dihydroxyergocalciferol are used, in contrast to the intestinal cytosol proteins, which react with both metabolites.

Materials and Methods

Materials

The 3-hemisuccinate derivative of 1,25(OH)2D3 and the synthetic vitamin D3 metabolites were donated by M. Uskokovic, Ph.D., Hoffmann-La Roche, Nutley, NJ. The vitamin D3 metabolites were synthesized and purified in the laboratory of one of us (G.J.). [26, 27-3H]-1,25(OH)2D3 with a specific activity of 160 kCi/mol and the Aquasol solution were obtained from New England Nuclear, Boston, MA. The epimer, 1β,25(OH)2D3, was generously donated by Sally A. Holick, Ph.D., Endocrine Unit, Massachusetts General Hospital, Boston, MA. Bovine albumin (Fraction V), dextran (industrial grade), and swine skin gelatin (type II) were purchased from Sigma Chemical Co., St. Louis, MO. Freund's adjuvant was supplied by Miles Lab., Inc., Elkhart, IN. Acid-washed charcoal (Norit A) was obtained from Nutritional Biochem. Corp., Cleveland, OH. Goat anti-rabbit immunoglobulin (IgG) was purchased from Nichols Institute Diagnostics, San Pedro, CA. Sephadex LH-20 resin (25-100 μm particle size) was supplied by Pharmacia Fine Chemicals, Piscataway, NJ. The organic solvents of high-pressure liquid chromatography (HPLC) grade were bought from Fisher Scientific Co., Fairlawn, NJ. An HPLC system (Laboratory Data Control, Riviera Beach, FL) equipped with a microsilica column (Zorbax-Sil, 25 cm × 4.6 mm; Dupont, Inc.) was used for the chromatographic separation and identification of 1,25(OH)2D3. The liquid-scintillation system was a Beckman LS3000, which performed at 38% efficiency.

Procedures

Partially purified IgG was prepared from the sera of immunized rabbits. Ammonium sulfate (250 g/L of serum) was added and the mixture was left at ambient temperature for 20 h. After centrifugation at 3000 × g the pellet was washed with a 1.75 mol/L solution of (NH4)2SO4. One hour later, centrifugation was repeated and the pellet was washed twice in the same fashion. The final pellet was dialyzed vs. distilled water for 48 h. The fluid within the dialysis sac was tested for the presence of (NH4)2SO4 by adding BaCl2; if no turbidity was seen the fluid was centrifuged at 800 × g and the supernate was removed and diluted with phosphate buffer (pH 7.4, 0.1 mol/L) to the original volume of the serum. The IgG fraction was frozen and stored at −20 °C until further use. To test the purity of the IgG fractions, chromatography and immuno-diffusion were performed on several IgG preparations. The IgG fraction prepared from the rabbit serum was chromatographed on diethylaminoethyl cellulose (DE-52) with use of
a phosphate buffer (pH 7.0, 10 mmol/L). The IgG fraction was dialyzed against this buffer for 2 h before being applied to the 2 × 4 cm column. Absorbance of the eluting fractions was monitored at 280 nm with an Isco UA-5 Absorbance Monitor. The Ouchterlony immunodiffusion test was performed in the laboratory of Don Gabriel, M.D., Dept. of Medicine, University of North Carolina School of Medicine.

Serum or plasma from human volunteers was stored at −20 °C. After thawing, [3H]-1,25(OH)2D3 (1000 cpm) was added to 2-mL aliquots. The purity of the isotope was confirmed by HPLC before use. Thirty minutes after isotope addition, 12 mL of methanol/chloroform (2/1 by vol) was added and mixed. Ten minutes later the mixture was centrifuged (150 × g). The supernate was decanted into a disposable tube containing 4 mL of phosphate buffer (pH 10.5, 0.1 mol/L) and 4 mL of chloroform. After gentle agitation this mixture was centrifuged at 150 × g, and the upper layer was aspirated and discarded. The lower layer was evaporated to 1–2 mL, then dried under a stream of nitrogen. The residue was dissolved in 750 μL of chloroform/hexane (65/35 by vol), applied to a 20 × 0.5 cm Sephadex LH-20 column, and eluted with chloroform/hexane (65/35 by vol). The eluates were collected as two pools, the first 7.5 mL being the 25(OH)D3 portion and the next 20 mL being the 1,25(OH)2D3 portion. The second pool was evaporated to 1–2 mL and dried under nitrogen. The residue was dissolved in 100 μL of hexane/isopropanol (90/10 by vol) and injected into the HPLC system. The mobile phase of the HPLC system was hexane/isopropanol (90/10 by vol), at a flow rate of 2 mL/min. The volumes co-eluting with synthetic 1,25(OH)2D3 were collected, dried under nitrogen, and dissolved in 100 μL of 95% ethanol. Three 20-μL aliquots were used in the radioimmunoassay and a fourth aliquot of 35 μL was used for the determination of [3H]-1,25(OH)2D3 recovery. The percentage recovery was calculated by correcting the radioactivity for background, dividing the corrected value by the total radioactivity (cpm) added, and multiplying this fraction by 100. The concentration of 1,25(OH)2D3 in serum was determined by calculating the average cpm of the triplicates, correcting the average value for the solvent blank, converting the corrected value from cpm to pmol from the standard curve, multiplying this figure by the dilution factor for the sample, dividing by the percentage recovery and by the volume of serum, and then converting the units from pmol/mL to pg/mL (ng/L).

Results

Antisera Production

Antisera directed against 1,25(OH)2D3 were produced in New Zealand White rabbits by immunization with 1,25(OH)2D3/3-hemisuccinate conjugated to bovine albumin according to the procedure of Vaitukaitis et al. (9). The 3-hemisuccinate derivative was linked to bovine albumin by a mixed-anhydride reaction (10). Two animals designated as controls were immunized with bovine albumin suspended in Freund’s adjuvant. Blood samples were obtained thereafter at four-week intervals. IgG fractions prepared from the rabbit serum produced a single peak on elution from the column of diethylaminoethyl cellulose and a single sharp band on the Ouchterlony plates.

Binding Characteristics

A dilution of the IgG fraction that bound 35–45% of the radioligand, [3H]-1,25(OH)2D3, was selected for the assay. Binding of the radioligand was dependent on time, temperature and buffer pH (Figures 1 and 2) and was maximal at 4 °C. At this temperature, saturation of the binding was observed after 2 h of incubation (Figure 1). Binding was maximal at a buffer pH of 7.4 and 4 °C (Figure 2).

![Fig. 1. Effect of temperature and time on the binding of [26,27-3H]-1,25(OH)2D3 to rabbit IgG no. 933 (625-fold dilution)](image)

Radioimmunoassay System

The initial assay reactants included 20 μL of the radioligand (2500 cpm), 30 μL of synthetic 1,25(OH)2D3, 200 μL of the IgG fraction diluted 625- to 1000-fold, and 300 μL of phosphate buffer (pH 7.4, 0.1 mol/L, and containing gelatin in a concentration of 0.1 g/L). These reactants were placed in borosilicate-coated glass tubes at the ambient temperatures, mixed, and incubated at 4 °C overnight. Dextran-coated charcoal (250 μL, charcoal/dextran 10/1 by wt) was added to separate bound and free radioactivity, mixed, and the tubes were centrifuged (1000 × g, 4 °C, 20 min). The supernates were aspirated and the radioactivity was counted in Aquasol solution. The results were expressed as the mean cpm vs. the 1,25(OH)2D3 concentration per tube.

Figure 3 depicts a typical displacement curve. Displacement of the bound radioligand increased as the 1,25(OH)2D3 concentration per tube was increased up to 1.25 pmol. The least detectable concentration in the assay depicted in Figure 3 was 0.025 pmol per tube, or 10 pg per tube. The range of 1,25(OH)2D3 concentrations encompassed by the displacement curve was from 0.025 to 0.5 pmol per tube, a 20-fold increase in 1,25(OH)2D3 concentration. A Scatchard plot revealed the linear relationship between the bound/free ratio and the 1,25(OH)2D3 concentration per tube (Figure 4).
affinity constant of the IgG fraction, calculated from five displacement curves, was $4 \times 10^9$ L/mol.

The controls in the radioimmunoassay included: (a) a solvent blank, to monitor potential interference from substances in the solvents; (b) a zero standard, to determine maximal binding by the IgG fraction; (c) a non-immune IgG fraction, to monitor nonspecific binding in the absence of antibodies; and (d) an excess of 1,25(OH)$_2$D$_3$ (9 ng per tube), to measure binding in the presence of excess standard.

Specificity of Binding

The amount of radioligand displaced by added 25(OH)D$_3$, 24,25(OH)$_2$D$_3$, and 1,24,25(OH)$_3$D$_3$ varied considerably (Figure 5), the relative order of displacement being 1,25(OH)$_2$D$_3 > 25$(OH)D$_3 > 24,25$(OH)$_2$D$_3 > 1,24,25$(OH)$_3$D$_3$. A useful criterion for comparison of specificity is the metabolite concentration associated with 50% displacement of the bound radioligand. Using this criterion, we determined that 50 pg of 1,25(OH)$_2$D$_3$, 128 pg of 25(OH)D$_3$, and 919 pg of 24,25(OH)$_2$D$_3$ displaced 50% of the bound radioligand. In contrast, added vitamin D$_2$ metabolites did not displace the radioligand (Figure 6), nor did addition of 25-, 24,25-, and 1,25-dihydroxyergocalciferol in amounts up to 9 ng per tube. An analog of 1a,25(OH)$_2$D$_3$ lacking biological activity, 1b,25(OH)$_2$D$_3$ (11), displaced the radioligand exactly the same as the 1a epimer. Binding specificity was studied further by testing the displacing effect of several steroids normally found in human serum. Hydrocortisone, 7260 ng per tube, displaced the same amount of radioligand as 5 pg of 1,25(OH)$_2$D$_3$. Progesterone, 6900 ng per tube, produced the same radioligand displacement as did 31 pg of 1,25(OH)$_2$D$_3$. Cholesterol, 7560 ng per tube, was equivalent in its radioligand displacement to 66 pg of 1,25(OH)$_2$D$_3$.

Separation Technique

We compared results by the specific second-antibody separation with those by the nonspecific dextran-coated charcoal separation. The second-antibody technique involved addition of goat anti-rabbit IgG, diluted 25-fold with phosphate buffer, pH 7.4, to the tubes after the incubation steps. One hour later, non-immune rabbit IgG (25 μL, 20-fold diluted in phosphate buffer, pH 7.4) was added and after an additional hour the mixture was centrifuged (1000 x g, 20 min, 4°C). The pellets were resuspended and the radioactivity measured. Table 1 shows the effect of increments in the second-antibody volume on the proportion of bound and free radioactivity. Volumes of second antibody up to 15 μL precipitated increasing amounts of bound radioactivity. Further increments in the second antibody decreased the bound radioactivity. The bound/free ratio associated with 15 μL of the second antibody was 0.404; the ratio associated with the dextran-charcoal was 0.85 (Table 1). Figure 7 depicts two displacement curves from assays that were identical except for the separation techniques, charcoal being used in one and second antibody in the other. As shown in Figure 7, the displacement curve associated...
Table 1. Comparison of Separation of Bound and Free Radioactivity: Second Antibody (I) vs Dextran-Coated Charcoal (II) *

<table>
<thead>
<tr>
<th>Volume, μL</th>
<th>cpm as [3H]-1,25(OH)2D3</th>
<th>B/F ratio</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>Bound (B)</td>
<td>Free (F)</td>
</tr>
<tr>
<td>10</td>
<td>506</td>
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</tr>
<tr>
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<td>515</td>
<td>1273</td>
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<td>475</td>
<td>1372</td>
</tr>
<tr>
<td>25</td>
<td>422</td>
<td>1569</td>
</tr>
</tbody>
</table>

* Rabbit IgG no. 934 (1:1000), 2-h incubation at 4 °C and separation. Rabbit nonimmune IgG was added 1 h after second antibody, to produce a pellet upon centrifugation.

Buffer Proteins

The gelatin concentration in the phosphate buffer influenced the radioligand binding by the IgG fractions. We studied four concentrations of gelatin (0.06, 0.1, 0.5, and 1.0 g/L) to determine their effect on binding and displacement of the radioligand. When the gelatin concentration was 1.0 g/L, binding was 15% greater and displacement of bound radioligand was less than that observed with a gelatin concentration of 0.1 g/L. When the concentration of 1.25(OH)2D3 added to the tubes was increased to 0.625 pmol per tube, the displacement of the radioligand was nearly identical in all four concentrations of gelatin.

Reportedly, addition of normal rabbit serum to the phosphate buffer in small amounts (1 mL/L) enhances the specificity and sensitivity of the assay (8), but we found that addition of rabbit serum to the phosphate buffer did not improve either sensitivity or specificity at incubation intervals of 4 or 48 h. In fact, the presence of rabbit serum decreased the total binding of the radioligand and the sensitivity of the assay.

Preliminary results suggested to us that serum extracts were associated with higher amounts of bound radioactivity than plasma extracts when the assay involved the second-antibody separation, but further experience showed no significant difference.

1,25(OH)2D3 Isolation from Serum

The mean (n = 20) recovery of [3H]-1,25(OH)2D3 after organic extraction and step-wise chromatography was 68% (SEM 3%). When 5 × 106 cpm of [3H]-25(OH)D3 was added to a serum sample before extraction and chromatography, <0.01% of the total radioactivity was accounted for in the 1,25(OH)2D3 fraction after HPLC. Figure 8 shows chromatographic separation of the dihydroxylated metabolites of vitamin D3, including 25,26(OH)2D3, under the conditions described elsewhere in this report.

Clinical Application

The mean concentration of 1,25(OH)2D3 in serum from 16 women, ages 20–40 years, was 57.8 (SEM 8.8) ng/L. These results were obtained from several assays in which charcoal was used in the separation. When the second-antibody separation was used, the mean concentration of 1,25(OH)2D3 in serum from six men and four women, ages 20–40 years, was 56.9 (SEM 5.9) ng/L. Because there is no statistically significant difference between these two sets of results, we combined them, giving a mean concentration in serum from 26 men and women, ages 20–40 years, of 57.3 (SEM 5.7) ng/L.

Using the second-antibody separation, we radioimmunoassayed 1,25(OH)2D3 in serum of patients with diseases known to alter vitamin D metabolism (7). 1,25(OH)2D3 was not detectable in serum from a nephrectomized patient. Another nephrectomized patient, who was ingesting 1,25(OH)2D3 daily, had a value of 44 ng/L. Two untreated hypoparathyroid patients had 1,25(OH)2D3 values of 10 and 18 ng/L. Two other hypoparathyroid patients, who were ingesting 0.5 μg of 1,25(OH)2D3 per day, had serum 1,25(OH)2D3 concentrations of 50 and 59 ng/L. The mean within-assay CV was 10%; the mean between-assay CV was 13%.

Discussion

The concentration of 1,25(OH)2D3 in biological fluids has been measured by investigators using the protein-binding assays for over six years (2, 3). Persistent problems with
availability and stability of the intestinal mucosal receptor have sustained an interest in the development and refinement of radioimmunoassays for 1,25(OH)2D3. The first report of a sensitive radioimmunoassay for 1,25(OH)2D3 was that of Clemens and his associates (6). Subsequently two other groups have reported the development of similar radioimmunoassays (7, 8). In the most recently reported radioimmunoassay (8) the 3-hemisuccinate derivative of 1,25(OH)2D3 coupled to bovine albumin was used as the immunogen, the same compound we used in the present radioimmunoassay. Unlike the assay system described by the three other groups, in the present radioimmunoassay we used IgG fractions from rabbit serum to bind the radioligand, [3H]-1,25(OH)2D3. Synthetic 1,25(OH)2D3 displaced in a linear fashion the radioligand bound to these IgG fractions. The antibodies possessed a high affinity for 1,25(OH)2D3 but cross reacted readily with 25(OH)D3 and 24,25(OH)2D3, necessitating the chromatographic separation of 1,25(OH)2D3 from these other metabolites. The vitamin D3 metabolites did not cross react in amounts up to 9 ng per tube. A biologically inactive dihydroxylated metabolite, 1,25(OH)2D3α, displaced the radioligand in the same fashion as 1α,25(OH)2D3, showing that the stereochemical position of the 1-hydroxyl group did not determine the immunoreactivity.

Initially when this radioimmunoassay was applied to human serum, the mean concentration of 1,25(OH)2D3 in serum from 16 women, ages 20–40 years, was found to be 57.8 (SEM 8.8) ng/L, a mean value higher than results reported by most other groups (2–8). This caused us to consider whether the value was falsely increased, artificially, or actually represented a biological fact. We examined several assay variables that conceivably might have contributed to spurious increases in 1,25(OH)2D3 concentration, including the technique of separating bound and free radioactivity, the presence of proteins other than the rabbit antibodies, and potential interference by vitamin D3 metabolites other than 1,25(OH)2D3 present in the serum extract. The effect on the assay of added non-immune rabbit serum to the phosphate buffer was also tested because of the above-mentioned report (8).

The dextran-coated charcoal, a nonspecific separation, seemed to overestimate the bound radioactivity as compared to the results produced by a specific separation technique, the second-antibody method. Despite this apparent overestimate, the mean 1,25(OH)2D3 concentrations in normal human serum were nearly identical when assayed by use of either charcoal or second-antibody separation. Evidently the standards and the unknown samples were altered to a comparable degree by the charcoal separation, thereby shifting the displacement curve to the right without increasing the unknowns that fell on the curve. When the gelatin concentration in the phosphate buffer was 0.5 or 1.0 g/L, the total binding of the radioligand was increased and the displacement of bound radioligand by synthetic 1,25(OH)2D3 was decreased. At a gelatin concentration of 0.1 g/L or less this non-IgG protein did not influence binding or displacement. The separation of the vitamin D3 metabolites by the "high-pressure" liquid-chromatographic system was acceptable in terms of sufficient time intervals at the baseline level between the elution volumes as shown in Figure 8. Furthermore, when [3H]-25(OH)D3 was added to a serum sample before extraction, less than 0.01% of the radioactivity was recovered in the 1,25(OH)2D3 fraction after liquid chromatography. Thus, 2 mL of serum containing 50 pg of 25(OH)D3 would contribute only 5 pg to the apparent 1,25(OH)2D3. Non-immune rabbit serum added to the buffer actually diminished the sensitivity of the radioimmunoassay. As a result of these studies we substituted the second-antibody separation for the separation with charcoal in the radioimmunoassay. When we assayed additional human sera, using the second-antibody separation, the mean concentration of 1,25(OH)2D3 in the serum from 10 normal men and women, ages 20–40 years, was 56.9 (SEM 4.5) ng/L, a value nearly identical to the mean of 57.8 (SEM 8.8) ng/L associated with the charcoal separation.

To further validate this radioimmuno assay we tested sera from patients with diseases known to alter vitamin D metabolism, with the results described above. These findings provide additional evidence that the radioimmunoassay measured 1,25(OH)2D3 rather than another metabolite of vitamin D3 or a non-vitamin substance.

This radioimmunoassay appeared to measure 1,25(OH)2D3 in human serum sensitively and reproducibly. Possibly, the concentrations of 1,25(OH)2D3 in serum from adults 20 to 40 years old are higher than values at other points in the life cycle. Measurement of the concentration of 1,25(OH)2D3 in serum from this age group has not been reported, with one exception. Kumar et al. (12), using a competitive protein-binding assay, reported the mean plasma concentration of 1,25(OH)2D3 in young adult women to be 52.8 (SEM 4.9) ng/L. This report lends credence to the argument that the results reported herein reflect the actual concentration of 1,25(OH)2D3 in the serum of young adults.

Values for 1,25(OH)2D3 in human sera as measured by this radioimmunoassay are of particular interest because the active metabolite of vitamin D3, 1,25(OH)2D3, did not cross react in this assay. Human serum may contain 1,25(OH)2D3 if vitamin D3 is present in the diet. The previously published assays apparently detected both vitamin D3 and D2 metabolites, so the values for 1,25(OH)2D3 measured by this assay should be less than the combined values. The fact that the present results are equal to or slightly higher than previously published values indicates differences in assay techniques and potential biological differences, differences that can be resolved by the exchange of antisera and serum samples among investigators for the sake of standardizing the 1,25(OH)2D3 assays. Once a standard assay has been established, methodological differences among laboratories and the biological factors influencing the concentration of 1,25(OH)2D3 in serum can be readily identified.

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References


