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Determination of Vitamin D Status by Radioimmunoassay with an 125I-Labeled Tracer

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We report here the first radioimmunoassay for a vitamin D metabolite utilizing a radioiodinated tracer. Antibodies were generated in a goat immunized with the vitamin D analog 23,24,25,26,27-pentanor-C(22)-carboxylic acid of vitamin D, coupled directly with bovine serum albumin. The 125I-labeled tracer was prepared by reacting a 3-amino-propyl derivative of vitamin D-C(22)-amide with Bolton–Hunter reagent. The primary antisera, used at a 15,000-fold dilution, cross-reacted equally with all cholecalciferol and ergocalciferol metabolites tested except 1,25-dihydroxycholecalciferol metabolites and the parent calciferol; the antisera did not cross-react with dihydrocholesterol. Calibrators were prepared in vitamin D-stripped human serum. 25-Hydroxycholecalciferol was quantitatively extracted from serum or plasma (50 μL) with acetonitrile. The assay consists of a 90-min incubation at room temperature with primary antisera, followed by a 20-min incubation with a second antisera and separation of bound from free fractions by centrifugation. The detection limit of the assay was 2.8 μg/L for 25-hydroxycholecalciferol. Results with the present assay compared well with those from a liquid-chromatographic procedure involving specific ultraviolet detection of 25-hydroxycholecalciferol in plasma.

Indexing Terms: 25-hydroxyvitamin D · vitamin D metabolites

It is well established that vitamin D nutritional status is a function of circulating 25-hydroxycholecalciferols

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[25-(OH)D] (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 individual depends on endogenous (sun exposure) and exogenous (dietary intake) sources (4, 5), and measurement of both forms is important.

Many assays have been developed for assessing circulating 25-(OH)D. The majority of these assays are competitive protein-binding assays, involving the vitamin D-binding protein from various animal species (6–10), and most require extracting the serum with organic solvent followed by chromatographic purification before assay. Nonchromatographic assays for determining vitamin D status have been described (7–9), although their validity has been questioned (10). Several high-performance liquid chromatographic assays for 25-(OH)D have been described; although accurate, they are labor intensive and require costly equipment and large sample volumes (11–13).

We had previously described (14) a radioimmunoassay (RIA) based on the same antigen used in the present study. Although this former assay was satisfactory, it was based on the use of [³H]25-(OH)D₃ as a tracer; an RIA based on the use of a radioiodinated tracer would be more desirable. Thus, we have modified our first assay by incorporating the use of vitamin D-C(22)-amide labeled with ¹²⁵I via Bolton–Hunter reagent. We describe here our modified RIA based on this novel tracer and evaluate its utility in assessing the overall vitamin D status of human subjects.

Materials and Methods

Reagents

Crystalline cholecalciferol (vitamin D₃), ergocalciferol (vitamin D₂), and dihydrotachysterol (DHT) were obtained from Sigma Chemical Co., St. Louis, MO. Crystalline 25-hydroxycholecalciferol [25-(OH)D₃], 24,25-dihydroxycholecalciferol, 25S,26-dihydroxycholecalciferol, 1,25-dihydroxycholecalciferol [1,25-(OH)₂D₃], and 1,25-dihydroxyergocalciferol [1,25-(OH)₂D₂] were obtained from Hoffmann-La Roche Inc., Nutley, NJ. Biosynthetic 25-hydroxyergocalciferol [25-(OH)D₃], 25(OH)D₃ 26-23-lactone, 24,25-dihydroxyergocalciferol, and 25,26-dihydroxyergocalciferol were gifts from R. L. Horst, Ames, IA. Bolton–Hunter reagent (2000 kCi/mol) was obtained from New England Nuclear, Boston, MA. Goat anti-25-(OH)D primary antibody and donkey anti-goat secondary antibody precipitating complex were both obtained from Incstar Corp., Stillwater, MN. HPLC-grade acetonitrile was from Fisher Chemical Co., Pittsburgh, PA. Unless otherwise noted, all other reagents were reagent grade.

Clinical Samples

Serum or plasma samples for this study were obtained in the fall from 36 apparently normal individuals, ages 20–40 years. Twelve serum samples were obtained from children with extrahepatic biliary atresia, ages 6 months–2 years; these children received 2500–5000 IU of vitamin D₃ daily. Serum samples were also obtained from six hypoparathyroid and two pseudohypoparathyroid children, ages 7–15 years, who were taking various pharmacological doses of vitamin D₂ or 25-(OH)D₃ for the maintenance of serum calcium. The procedures followed were in accordance with the ethical standards of this institute.

Methods

Concentrations of vitamin D and its metabolites were determined by ultraviolet spectroscopy, with molar absorptivity (ε₉₀₄) of 18 300 L mol⁻¹ cm⁻¹ for vitamin D₃ and its metabolites and ε₉₀₄ of 19 400 L mol⁻¹ cm⁻¹ for vitamin D₂ and its metabolites.

Preparation of radioiodinated vitamin D. The 23,24,25,26,27-pentanor-C(22)-carboxylic acid of vitamin D was synthesized as previously described (14). A solution of the C(22)-acid of vitamin D (13 mg) and 1,1-carbonyldiimidazole (10 mg) in dimethylformamide (0.2 mL) was allowed to react at 4 °C for 2 h. 1,3-Diaminopropane (0.33 mL) was then added and the resulting solution was allowed to react at 4 °C for 16 h and then at 25 °C for 3 h. The product, 45 nmol of a 3-aminopropyl derivative of vitamin D-C(22)-amide, was combined with Bolton–Hunter reagent (5 mCi) in 50 μL of dioxane. The solution was allowed to react at ambient temperature for 15 min and then at 4 °C for 16 h. The resulting side-chain radioiodinated vitamin D analog was isolated and purified by silica gel chromatography. The ¹²⁵I-labeled vitamin D derivative appeared to be stable for the useful life of the isotope when stored in an equivolume solution of ethanol and 0.01 mol/L phosphate buffer at ambient temperature.

Preparation of assay calibrators. Human serum was "stripped" free of 25-(OH)D by treatment with activated charcoal. Absence of 25-(OH)D in the stripped sera was confirmed by direct ultraviolet detection of 25-(OH)D in serum after high-performance liquid chromatography as previously described (13). Subsequently, crystalline 25-(OH)D₃ dissolved in absolute ethanol was added to the stripped sera to yield calibrators at concentrations of 0, 5, 10, 30, 70, and 150 μg/L. The calibrators were then stored at 4 °C.

Radioimmunoassay. Calibrators and sample sera were extracted with acetonitrile as previously described (14). We prepared assay tubes containing 25 μL of 25-(OH)D extracted from calibrators and samples. To each tube we added ¹²⁵I-labeled vitamin D derivative (50 000 counts/min in 50 μL of the ethanol/phosphate buffer, pH 7.4). We then added to each tube 1 mL of primary antiserum, vortex-mixed, and incubated the
tubes for 90 min at room temperature. After this, we added 0.5 mL of the second-antisem precipitating complex to each tube, vortex-mixed, incubated the samples at room temperature for 20 min, and centrifuged the contents (room temperature, 2000 × g, 20 min). We discarded the supernate and counted the radioactivity in the tubes in a γ-radiation well-type counting system.

Direct ultraviolet detection of 25-(OH)D in plasma or serum after high-performance liquid chromatography. Total 25-(OH)D was determined in plasma or serum by direct quantification of ultraviolet absorbance after liquid-chromatographic purification by a previously described method (13).

Calculations. 25-(OH)D-equivalent values were calculated directly by the γ-radiation counting system with use of a smooth-spline method. 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 in plasma (see Table 1).

Results

Specificity of radioimmunoassay. Table 1 depicts the cross-reactivity of vitamin D and several of its metabolites with the goat antiserum generated against the 23,24,25,26,27-pentanor-C(22)-carboxylic acid calciferol. Several vitamin D metabolites could equally displace 125I-labeled vitamin D from the antibody. Exceptions to this were vitamins D2 and D3, DHT, 1,25-(OH)2D2, and 1,25-(OH)2D3, which were far less effective in displacement. The steroid specificity of this antiserum is similar to one we reported earlier (14) but different from that of the vitamin D-binding protein (15).

Detection limit, analytical recovery, precision, and assay parallelism. The detection limit of the RIA, defined as 3 SD from the mean for data on the zero sample, was 2.8 μg/L. Bound tracer was displaced 50% by 25-(OH)D3 at ~40 μg/L.

We estimated the analytical recovery of 25-(OH)D3 in the assay with four separate human serum samples at two different amounts of added vitamin D3. An average of 97.3% (SD = 10.4%, range 85–115%, n = 8) of the added 25-(OH)D3 was accounted for.

Assay precision (CV), both within- and between-assay variation, was determined at three different points on the standard curve. At all points the assay variation was nearly identical (Table 2). Within-assay variation was ~6%; between-assay variation was ~15%.

Assay parallelism was assessed in four different serum samples by serial dilution of the original plasma in vitamin D-free serum. As shown in Table 3, sample dilution did result in assay linearity.

Comparison of RIA with independent assay method. Results obtained by the present method for 63 human subjects were compared with those by a conventional assay (13), in which 25-(OH)D is measured in serum by direct quantification of ultraviolet absorbance after liquid chromatography. Assessment of vitamin D status as determined by the two assay methods was quite similar (Figure 1).

Assay utility in the assessment of vitamin D status. The respective values for 36 normal subjects, 12 biliary atresia patients, and 8 vitamin D therapy patients were 25.7 (SD 15.8), 6.3 (SD 2.0), and 145 (SD 43.6) μg of 25-(OH)D per liter of serum (Table 4).

Discussion

During the past 20 years many methods have been developed for determining vitamin D status in humans (6–14). These techniques have involved competitive protein-binding assay (6–10), direct ultraviolet detection after high-performance liquid chromatography (11–13), and RIA (14). One major deficiency in these proce-
Fig. 1. 25-(OH)D3-equivalent values obtained by the present method (y) and by direct ultraviolet quantification of 25-(OH)D after liquid chromatography (x).

Table 4. Concentration of 25-(OH)D in Various Physiological States

<table>
<thead>
<tr>
<th>Subject type</th>
<th>n</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>36</td>
<td>25.7</td>
<td>9.9-41.5</td>
</tr>
<tr>
<td>Biliary atresia</td>
<td>12</td>
<td>6.3</td>
<td>4.3-8.3</td>
</tr>
<tr>
<td>Vitamin D therapy</td>
<td>8</td>
<td>145</td>
<td>92-202</td>
</tr>
</tbody>
</table>

* Determined in duplicate by the radioimmunoassay described in the text.
* Samples from subjects in Minnesota in October.
* Samples from subjects with hypoparathyroidism or pseudohypoparathyroidism receiving pharmacological doses of vitamin D2.

In conclusion, we have developed the first RIA for a vitamin D metabolite based on the use of a radioiodinated tracer. We believe that this assay provides a significant advance with respect to ease of use and speed when compared with tritium-based assays of a similar type.

References
How and How Long to Store Urine Samples before Albumin Radioimmunoassay: A Practical Response

Ottavio Giampietro, Giuseppe Penno, Aldo Clerico, Lorella Cruschelli, and Mauro Cecere

We used three study protocols to check the dependence of albumin stability, measured by an RIA, on different temperatures, durations, and materials (i.e., assay tubes) of urine storage. Albumin values obtained for samples stored in three types of assay tubes (glass, polyethylene, and polystyrene) throughout the 2 months of the first (prospective) protocol were superimposable. The 24-h storage of six urine samples at room temperature or at 4 °C, as well as 72-h storage at 4 °C, did not affect the albumin measurement by RIA. After 2 months of storage of these same six urine samples at −20 °C, there was still no albumin decrease. A significant albumin decrease occurred (a mean of ∼5% per year, throughout the range of albumin concentrations tested) when samples stored at −20 °C were reassayed by RIA after >2 years (second protocol, retrospective). Finally, 3 of 21 (14.3%) urine pools stored at −20 °C for various periods (4–21 months) showed a significant albumin loss after storage; the time of storage or its impact on the decrease of these 3 pools differed from each other (third protocol, retrospective). Short- and medium-term (2–6 months) freezing of urine samples at −20 °C does not significantly affect the stability of immunoassay albumin. For longer preservation periods, storage of urine samples at −70 °C may be preferable.

Measurement of urinary albumin excretion in the range of 20–200 mg/day, so-called microalbuminuria, is used to evaluate kidney involvement in people with diabetes mellitus (1, 2). Indeed, diabetic renal disease may be reversible by tight metabolic control, if detected early (1). Accordingly, screening to determine urinary albumin excretion has become a frequent part of diabetes care (1, 2).

Contradictory evidence (2–9) has been reported about the effect of specimen storage conditions on the results of these assays. Albumin in urine has been stated to be stable at room temperature for several hours and for a few weeks at 4 °C without added preservative, which implies that urine samples can be stored for a relatively short time (from hours to some days) at 4 °C until assay (2). However, epidemiological and (or) multicenter studies require that several samples be collected, stored for long periods (from several months to some years), and eventually shipped to the laboratory before the assay. Hence, storage conditions such as room temperature or 4 °C are not suitable; instead, freezing the urine samples seems more appropriate. Some authors claim that storage of samples at −20 °C for determination of urinary albumin excretion by laser immunonephelometry (5) or RIA (7) yields falsely low results, so that freezing urine samples is not suitable. Others (3, 8), like us (4, 6, 9), are of the opinion that freezing urine samples at −20 °C (or −70 °C) does not in itself affect the albumin assay, and thus recommend storing specimens at −20 °C (or −70 °C) if the assay cannot be performed within a few days (2–3 days at most).

To determine the best way to stabilize the albumin in urine until assay, we stored under different conditions and for different periods a large number of urine samples and pools treated in various ways.