A Radioimmunoassay for 1,25-Dihydroxycholecalciferol

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We describe a radioimmunoassay for 1,25-dihydroxycholecalciferol in human serum. We raised antisera in rabbits to 1,25-dihydroxycholecalciferol-3-hemisuccinate coupled to bovine serum albumin, and obtained sensitive, high-titer antibodies. These antibodies had a high affinity for 1,25-dihydroxycholecalciferol and cross-reacted mainly with 25-hydroxycholecalciferol and 24,25-dihydroxycholecalciferol. Addition of 1 mL of normal rabbit serum per liter reduced this interference to 4 and 9%, respectively. However, these interfering steroids are present in large excess, so extensive purification of 1,25-dihydroxycholecalciferol from serum is necessary. The steroid was extracted with ethyl acetate/cyclohexane, purified on Sephadex LH-20, and then chromatographed on a column of silicic acid. The radioimmunoassay is sensitive to 5 pg/tube (3 ng/L of serum). The between-assay CV was 14%. The mean concentration of 1,25-dihydroxycholecalciferol in the serum of 54 healthy adults was 38 (SD 12) ng/L, with no sex-related difference. The assay was further validated by the finding of low or undetectable concentrations in patients with chronic renal failure and of increased concentrations in the serum of patients with primary hyperparathyroidism. In comparison with previously described methods, the major advantage of the present assay is the use of stable gamma-globulins, which are available in large amounts, as binding protein.

Additional Keyphrases: assessing vitamin D status • rickets • reference values • vitamin D and its metabolites • liquid chromatography • age-related effects

Cholecalciferol is metabolized in the liver to 25-hydroxycholecalciferol; further hydroxylation can then occur at either position 24 or 26, probably representing biological inactivation, or at position 1, resulting in 1,25-dihydroxycholecalciferol, a biologically highly active compound (1).

Many assays have been described for measuring 25-hydroxycholecalciferol, and its concentration in plasma reflects the status of the organism with regard to its access to nutritional or skin-produced cholecalciferol (2–9). However, the key enzyme of vitamin metabolism is the renal 25-hydroxycholecalciferol-1-hydroxylase (EC 1.14.13.13) (1) and measurement of 1,25-dihydroxycholecalciferol is therefore necessary for the evaluation of vitamin D metabolism in various circumstances and diseases. For this measurement, in most such assays the natural receptor from chick intestine is used as binding protein (7–12), but this receptor protein is not dependably stable and requires a regular supply of rachitic chicks.

A sensitive bioassay (13) and an isotope dilution–mass fragmentographic assay for 1,25-dihydroxycholecalciferol (14) have recently been described, but, owing to the technical difficulties and/or instrumentation involved, these are more reference methods than routine assays.

Most other steroid hormones are now measured by radioimmunoassay, because the initially described receptor assays were too cumbersome. We therefore tried to raise antibodies to, and to develop a radioimmunoassay for, 1,25-dihydroxycholecalciferol.

Materials and Methods

Materials

Crystalline cholecalciferol was obtained from Philips-Duphar, Amsterdam, The Netherlands; 25-hydroxycholecalciferol was a gift from Roussel-Uclaf, Paris, France; 24,25-dihydroxycholecalciferol and 1,25-dihydroxycholecalciferol were gifts from Hoffmann-La Roche, Basle, Switzerland. 1,25-Dihydroxy[23,24(n)-3H]cholecalciferol (spec. acty. 110 kCi/mol) was purchased from the Radiochemical Centre, Amersham, U.K., and purified before use by high-pressure liquid chromatography. Sephadex LH-20 and Dextran T70 were obtained from Pharmacia Fine Chemicals, Upsala, Sweden. Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO 63178. Charcoal (Norit A) came from ICN Pharmaceuticals, Cleveland, OH 44128, and complete Freund’s adjuvant from Difco Laboratories, Detroit, MI 48232. Rhesus ampuoles were obtained from Forma Vitrum, St. Gallen, Switzerland. All solvents and reagents came from Merck, Darmstadt, F.R.G., and were of analytical grade.

The high-pressure liquid chromatography was performed with a Waters System (Solvent Delivery System, Model 600A, Intelligent Sample Processor, Model 710A, and an Absorbance Detector, Model 440; all from Waters Associates, Milford, MA 01757). We used a recorder (Rec 61 Servographe; Radiometer, Copenhagen, Denmark). The three-way valve (LKB valve 11320) and the automatic fraction collector (Ultrarac II) came from LKB-Producer AB, Bromma, Sweden. The column used for high-pressure liquid chromatography was a Zorbax SIL column (0.62 × 25 cm) from Dupont Instruments (DuPont de Nemours, ‘s Hertogenbosch, The Netherlands) as described previously (6). Radioactivity was measured with a β-scintillation counter (Tri-Carb 2450; Packard Instrument Co., Downers Grove, IL 60515), with “picofluor” (Packard Instrument Co.) as scintillator.

Methods

1α, 25-Dihydroxycholecalciferol-3-hemisuccinate (Figure 1, 5) was prepared from 1α,25-dihydroxy-7-dehydrocholesterol (Figure 1, 1), which in turn was obtained by deacetylation of the corresponding triacetate (15, 16). Treatment of 1 with 2,2,2-trichloroethylsuccinyl chloride at low temperature and in the presence of pyridine gave selectively 2. Photolysis (15–17) of 2, separation of the corresponding precholecalciferol 3 from the photolysis mixture by high-performance liquid chromatography, and thermal isomerization gave the cholecalciferol 4. The desired 1α, 25-dihydroxycholecalciferol-3-hemisuccinate (5) was then obtained by treatment of 4 with zinc dust in the presence of potassium dihydrogen phosphate (18). Details of the preparation follow.

Mono-2,2,2-trichloroethyl succinate. A mixture of 5 g (50.0
mmol) of succinic anhydride and 6 mL (62.5 mmol) of trichloroethanol (Aldrich Chemical Co., Milwaukee, WI 53233) in 50 mL of dry pyridine was kept at 100 °C for 15 h. This gave 5.9 g of a pale-yellow solid, which could be crystallized from n-hexane–ether to give 5.2 g of pure mono-2,2,2-trichloroethyl succinate, m.p. 73–74 °C. IR (KBr) 2685, 2580, 1753, 1716 cm⁻¹. NMR (CDCl₃) δ 10.50 (bs, 1H), 4.76 (s, 2H), 2.77 (s, 4H) ppm.

2,2,2-Trichloroethylsuccinyl chloride. A solution of 5.0 g (20.0 mmol) of mono-2,2,2-trichloroethyl succinate in 50 mL of absolute ether was refluxed for 10 h with 2.5 mL (34.3 mmol) of thionyl chloride. Solvent and excess reagent were then removed under reduced pressure, and the residue was distilled to give 4.8 g of a colorless liquid, b.p. 75–76 °C (0.2 mm Hg). NMR (CDCl₃) δ 4.77 (s, 2H), 3.07 (AA’BB’ system, Δν = 28 Hz, 4H).

1α,25-Dihydroxy-7-dehydrocholesterol-3-(2',2',2'-trichloroethyloxycarboxylate) (Figure 1, 2). A solution of 500 mg (1.2 mmol) of 1α,25-dihydroxy-7-dehydrocholesterol in a mixture of 30 mL of absolute tetrahydrofuran and 3 mL of anhydrous pyridine was treated dropwise at -5 °C with a solution of 400 mg (1.5 mmol) of 2,2,2-trichloroethylsuccinyl chloride in 2 mL of absolute tetrahydrofuran. After 1 h at -5 °C, the crude product was purified by chromatography to give, after crystallization from n-hexane–ethyl acetate, 448 mg of product, m.p. 93–95 °C; [α]D⁰ = -14.73 (c = 0.5, EtOH). Analysis: Calculated for C₂₅H₄₄O₄Cl₂: C, 61.16; H, 7.62; Cl, 16.41. Found: C, 61.09; H, 7.86; Cl, 16.33. UV (EtOH) λmax (e) 251 (4100), 262 (7600), 272 (10 650), 282 (11 400), 294 (6700) nm. IR (KBr) 1758, 1733, 1650, 1600 cm⁻¹. NMR (CDCl₃) δ 5.73 (bd, J = 6 Hz, 1H), 5.38 (m, 1H), 4.76 (s, 2H), 3.80 (bs, 1H), 1.22 (s, 6H), 0.97 (d, 3H), 0.96 (s, 3H), 0.69 (s, 3H) ppm. MS (70 eV) m/e (rel. abundance) 398 (92), 380 (100).

1α,25-Dihydroxyprocholecalciferol-3-(2',2',2'-trichloroethyloxycarboxylate)(Figure 1, 3). A solution of 500 mg of 1α,25-dihydroxy-7-dehydrocholesterol-3-(2',2',2'-trichloroethyloxycarboxylate) (2) in 200 mL of a 4:1 mixture of spectroscopic grade n-hexane and tetrahydrofuran was irradiated at 0 °C with a medium-pressure mercury lamp (Hanovia, 450 W) through quartz glass, until approximately 50% of the starting material was converted. After the solvents were evaporated, the components of the photolysis mixture were separated by use of a Waters Associates high-performance liquid chromatograph, Model 244, and a 245 cm x 1 cm Porasil A column, eluted with a mixture of n-hexane–ethyl acetate (1:5/1 by vol) to give 216 mg of recovered starting material and 162 mg of 1α,25-dihydroxyprocholecalciferol-3-(2',2',2'-trichloroethyloxycarboxylate) (3) as a thick, colorless oil. NMR (CDCl₃) δ 5.87 (bs, 2H), 5.53 (bs, 1H), 5.10 (bm, 1H), 4.77 (s, 2H), 4.2 (bm, 1H), 1.20 (s, 6H), 0.97 (bd, J = 5 Hz, 3H), 0.70 (s, 3H).

1α,25-Dihydroxycholcalciferol-3-(2',2',2'-trichloroethyloxycarboxylate) (Figure 1, 4). A solution of 400 mg of 1α,25-dihydroxycholcalciferol-3-(2',2',2'-trichloroethyloxycarboxylate) in 20 mL of dioxane was refluxed under an argon atmosphere for 30 min. After the solvent was evaporated, the components of the reaction mixture were separated by high-performance liquid chromatography (same conditions as above) to give 50 mg of starting material and 235 mg of product 4, as a white solid. [α]D⁰ = -32.11 (e = 0.3, EtOH). Analysis: Calculated for C₂₅H₄₄O₄Cl₂: C, 61.16; H, 7.62. Found: C, 61.18; H, 7.68. UV (EtOH) λmax (e) 210 (16 000), 283 (14 550) nm. IR (KBr) 3540–5360, 1758, 1735 cm⁻¹. NMR (CDCl₃) δ 3.64 (d, J = 12 Hz, 1H), 5.60 (d, J = 12 Hz, 1H), 5.38 (bs, 1H), 5.25 (bm, 1H), 4.73 (s, 2H), 4.40 (bm, 1H), 1.21 (s, 6H), 0.93 (bd, J = 5 Hz, 3H), 0.54 (s, 3H) ppm. MS (70 eV) m/e (rel. intensity) 616 (9), 614 (10), 366 (100).

1α,25-Dihydroxycholecalciferol-3-hemisuccinate (Figure 1, 5). A solution of 400 mg of 1α,25-dihydroxycholecalciferol-3-(2',2',2'-trichloroethyloxycarboxylate) (4) in 50 mL of tetrahydrofuran and 20 mL of water was stirred at 0 °C under argon with 10 g of zinc dust and 10 mL of an aqueous potassium dihydrogen phosphate solution (1 mol/L). After 3 h, the reaction mixture was filtered and, after evaporation of the tetrahydrofuran present, the residue was dissolved in ethyl acetate. The crude product was purified with a Waters Associates high-performance liquid chromatograph, Model 244, and a 120 x 1 cm Bondapac-Phenyl column, eluted with a mixture of methanol/water (3/1 by vol) to give 265 mg of pure 5 as a white foam. UV (EtOH) λmax (e) 211 (14 300), 264 (14 700) nm. IR (KBr) 3480–3330, 1735, 1715 cm⁻¹. NMR (CDCl₃) δ 6.32 (d, J = 12 Hz, 1H), 6.00 (d, J = 12 Hz, 1H), 5.33 (bs, 1H), 5.20 (bm, 1H), 5.00 (bs, 1H), 4.40 (bm, 1H), 1.22 (s, 6H), 0.92 (bd, J = 5 Hz, 3H), 0.54 (s, 3H) ppm. MS (70 eV) m/e (rel. intensity) 498 (11), 380 (100).

Production of Antisera
Preparation of the immunogen. We prepared a conjugate of bovine serum albumin with 1,25-dihydroxycholecalciferol according to the procedure previously described for other steroids (19). Five milligrams of 1,25-dihydroxycholecalciferol-3-hemisuccinate was solubilized in 0.1 mL of redistilled dioxane; 3 μL of tri-n-butylamine and 1.5 μL of isobutylchlorocarbonate were added and the mixture was cooled at 4 °C, then added to 1 mL of bovine serum albumin (34 mg, diluted in dextrose/water, 1/1 by vol, previously adjusted to pH 9.2 with sodium hydroxide, 1 mol/L solution). The pH was kept between 8.5 and 9 by adding minute amounts of the sodium hydroxide solution. After magnetic mixing for 4 h at 4 °C, the solution was applied on the top of a 0.9 x 20 cm Sephadex G-50 (fine) column and eluted with a sodium phosphate buffer (0.1 mol/L, pH 7.2). Two ultraviolet-absorbing peaks were seen. The first one corresponded to the protein–steroid conjugate and was stored in small aliquots at - 20 °C. The second corresponded to the unreacted 1,25-dihydroxycholecalciferol-3-hemisuccinate. Eighty percent of the steroid was incorporated into bovine serum albumin, as calculated from the difference in absorbance at 265 nm between normal bovine serum albumin and the protein–steroid conjugate. This represents a 15/1 molar ratio of steroid to bovine serum albumin.

Immunization. Twelve female rabbits (three months old) were immunized by multiple intradermal injections of various amounts of immunogen emulsified in 1 mL of complete Freund's adjuvant. At monthly intervals thereafter, the same mixture was given subcutaneously. Ten days later, blood was
taken from an ear vein and the serum was stored at -20 °C until used. Three rabbits who had received more than 0.5 mg of the conjugate died within two months, from hypercalcemia (serum calcium >150 mg/L). Four other rabbits were killed after five months because their serum was of low titer and low affinity for 1,25-dihydroxycholecalciferol. Two of them had received a monthly dose of 250 μg of immunogen; the other two, 125 μg. The best antisera was obtained in rabbit H after 10 monthly injections of 250 μg of immunogen, and we used this antisera in the present study.

Assay Methodology

**Extraction of 1,25-dihydroxycholecalciferol from plasma.** Five milliliters of serum or plasma, pre-incubated for 10 min with 10 μL of ethanol containing 8 pg of 1,25-dihydroxy[PH]-cholecalciferol is extracted with 5 mL of ethyl acetate/cyclohexane (equal volumes), vigorously shaken for 1 min and centrifuged (3000 rpm, 5 min). Four milliliters of the supernate are removed and replaced by 5 mL of the same solvent mixture, again shaken for 1 min, and centrifuged. Five milliliters of the supernate is removed and combined with the solvent mixture, which was evaporated at 40 °C under reduced pressure (we used a Buchler Vortex Evaporator).

**Sephadex LH-20 chromatography.** The plasma extract is taken up in two 0.2-mL portions of solvent (chloroform, n-heptane, ethanol, water, 49/49/2/saturation, by vol) and applied on the top of a 0.5 × 7.5 cm column of LH-20 and eluted with the same solvent mixture. The first 8 mL is collected and evaporated as the initial plasma extract.

**Liquid chromatography.** The dried extract is taken up in two 1-mL portions of solvent (n-heptane/isopropanol, 9/1, by vol) and filtered through a Millipore filter (cat. no. LSWP 013.00, 5-μm pore size), dried again in the evaporator, and the residue dissolved in 120 μL of the same mixture; 100 μL is transferred to a glass limited-volume insert of the Waters Intelligent Sample Processor (WISP) system. The sample (85 μL) is then automatically injected by the WISP system into a 0.62 × 25 cm Zorbax SIL column at a constant flow of 4 mL and a pressure of 60 to 70 MPa. The outlet of the column is connected to a three-way valve and then directed either to a waste collector or to an automatic fraction collector (Figure 2). Nine fractions are collected in such a way that fraction 8 contains all the 1,25-dihydroxycholecalciferol in 8 mL (eluting about 15 to 17 min after fraction). Figure 2 is a schematic representation of the automatic liquid-chromatographic procedure.

The fraction containing 1,25-dihydroxycholecalciferol is evaporated as before and redissolved in 1 mL of ethanol; 300 μL is transferred to a glass counting vial and evaporated; 10 mL of scintillator is added, and the radioactivity in the sample is counted. Six hundred microliters is transferred to a rhesus ampoule and evaporated for use in the radioimmunoassay.

**Radioimmunoassay.** The assay tubes contain either 1,25-dihydroxycholecalciferol purified from plasma as described above or various amounts of the crystalline steroid (0, 6.25, 12.5, 25, 50, 100, 250, or 1000 pg) initially added in 600 μL of ethanol and evaporated. Then 300 μL of antisera, diluted 40 000-fold in sodium phosphate buffer (0.1 mol/L, pH 7.2) containing bovine serum albumin (1 g/L) and normal rabbit serum (1 mL/L), is added. This mixture is vortexed, then incubated for 48 h at 4 °C. Then 1,25-dihydroxy-[PH]cholecalciferol (20 pg) is added in 10 μL of ethanol, mixed, and again incubated for 2 h at 4 °C. One milliliter of cold dextran-coated charcoal (Norit A, 120 mesh, 1.6 mg and Dextran 1770, 0.16 mg) is added, and after 30 min the tubes are centrifuged (4 °C, 3000 rpm, 10 min). The supernate is transferred to a scintillation vial, 10 mL of scintillation mixture is added, and the radioactivity in the sample is counted for 10 min.

**Results**

**Antiserum Production**

Rabbits immunized with 1,25-dihydroxycholecalciferol-3-hemisuccinate, coupled to bovine serum albumin, developed antiserum against this new immunogen. Indeed, there are several reasons to believe that 1,25-dihydroxycholecalciferol was bound to the γ-globulins of these antisera and not to the natural vitamin D-binding α-globulin: labeled 1,25-dihydroxycholecalciferol incubated with these antisera sedimented in the 7S region on sucrose gradient ultracentrifugation and
precipitated in the γ-globulin fraction on fractionation with ammonium sulfate. Moreover, the steroid specificity of these antisera differed markedly from that of the vitamin D-binding protein (20, 21). Figure 3 shows the titer and affinity for 1,25-dihydroxycholecalciferol of six of these antisera.

Extraction and Purification of Plasma 1,25-Dihydroxycholecalciferol

Extraction of 1,25-dihydroxycholecalciferol from plasma with an equal volume of an equilibrium mixture of ethyl acetate and cyclohexane was found to be efficient; 93% (SD 1%, n = 5) of the labeled steroid could be recovered. Only 73% of labeled 25-hydroxycholecalciferol could be extracted by this procedure. The analytical recovery of radioactive 1,25-dihydroxycholecalciferol on the Sephadex LH-20 columns was 76% (SD 2%, n = 5). The overall analytical recovery after plasma extraction, chromatography on Sephadex LH-20, and liquid chromatography, monitored systematically by adding labeled 1,25-dihydroxycholecalciferol, was 54% (SD 4%) for 50 consecutive plasma samples.

Precision, Sensitivity, and Recovery

The between- and within-assay CV’s, estimated by the repeated measurement of a pool of human serum containing 42 pg of 1,25-dihydroxycholecalciferol per milliliter, were 14% (n = 25) and 11% (n = 5), respectively.

The sensitivity of the system, defined as the point two standard deviations below the B⁰ (40% of the total radioactivity), was 5 pg. This corresponds to a sensitivity of 3 to 4 ng/L when 5 mL of serum is extracted and processed as described. We measured 1,25-dihydroxycholecalciferol in four samples after extraction from 5 or 2.5 mL of serum. The amount of 1,25-dihydroxycholecalciferol measured in the 2.5 mL extract was 47% (SD 1%) of the amount found in the 5-mL extract.

Analytical recovery was estimated at two levels: when 50 or 100 pg of 1,25-dihydroxycholecalciferol was added to 5 mL of plasma, 52% (SD 10%, n = 4) and 100% (SD 18%, n = 7) of the added amount was recovered, respectively.

Specificity

The antisera against 1,25-dihydroxycholecalciferol-3-hemisuccinate was not specific for 1,25-dihydroxycholecalciferol. In the absence of added normal rabbit serum the most cross-reactive steroids were 25-hydroxycholecalciferol (33%) and 24,25-dihydroxycholecalciferol (10%). However, addition of 1 mL of normal rabbit serum per liter decreased the cross-reactivity of these metabolites to 5 and 4%, respectively (Figure 4). The other vitamin D metabolites (1α-hydroxycholecalciferol, 25-hydroxyergocalciferol, and cholecalciferol) cross react much less than 1% in the assay. The possible influence of large amounts of several vitamin D metabolites on the measurement of 1,25-dihydroxycholecalciferol was negligible; addition of 500 ng of cholecalciferol, 500 ng of 25-hydroxycholecalciferol, or 100 ng of 24,25-dihydroxycholecalciferol to a pool of serum before the extraction did not influence the measurement of 1,25-dihydroxycholecalciferol. Extraction of 5 mL of water instead of serum resulted invariably in zero values for 1,25-dihydroxycholecalciferol.

Clinical Results

Normal values were obtained by use of samples from 54 apparently healthy blood donors and laboratory assistants (23 men and 31 women). Their mean age was 38 years (range, 19 to 66). The mean concentration of 1,25-dihydroxycholecalciferol was 38 (SD 12) ng/L. No sex-related difference was observed (Figure 5). In some elderly subjects (older than 75 years), very low concentrations of 1,25-dihydroxycholecalciferol were detected, not explicable by the presence of chronic renal failure or other diseases. In patients with chronic renal failure being treated with chronic hemodialysis, concentrations of 1,25-dihydroxycholecalciferol were low [8.2 (SD 4) ng/L, n = 12] and in five anephric patients the 1,25-dihydroxycholecalciferol concentration was <10 ng/L. Patients with surgically confirmed primary hyperparathyroidism showed increased concentrations of 1,25-dihydroxycholecalciferol (Figure 5).

Discussion

Because 1,25-dihydroxycholecalciferol is important in calcium homeostasis, its measurement has many potential investigational and clinical applications. In the most widely used assays, however, a chick intestinal receptor protein is used, which requires specific conditions for its conservation (12, 22) and necessitates a regular supply and handling of rachitic chicks (7–12). The production of antibodies against vitamin D metabolites, although theoretically possible in view

![Fig. 3](image-url)  
Fig. 3. Rabbit antibodies against 1,25-dihydroxycholecalciferol.

Rabbits were immunized with 1,25-dihydroxycholecalciferol-3-hemisuccinate, coupled to bovine serum albumin, and emulsified in complete Freund’s adjuvant. The antisera were tested after four monthly injections of 250 pg of immunogen in rabbits L, Q, R, S, and T and after 10 injections of the same amount of immunogen in rabbit H. The final dilution of the antisera is given in the figure. The incubation was performed as described in Methods, radioimmunoassay.

![Fig. 4](image-url)  
Fig. 4. Cross-reactivity of anti-1,25-dihydroxycholecalciferol antisera (rabbit H) with other vitamin D metabolites.

The antisera was diluted 40 000-fold in sodium phosphate buffer containing 1 g of bovine serum albumin per liter, with (upper panel) or without (lower panel) normal rabbit serum, 1 mL/L. The percentage cross-reaction is calculated at 50% of the B⁰ (40% of total radioactivity).

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of the experience with other steroids, has been hampered by the lack of suitable immunogens. 1,25-Dihydroxycholecalciferol-25-hemisuccinate coupled to bovine serum albumin reportedly is immunogenic (23, 24). We used a similar immunogen, differing only in the position of the hemisuccinate at the first hydroxyl group of the steroid, but our prolonged immunization resulted in antibodies with a higher titer and better affinity for 1,25-dihydroxycholecalciferol. The antibody, however, reacts with several vitamin D metabolites and even seems to recognize the 25-hydroxyl group better than the 1-hydroxyl group (Figure 5). The addition of a small amount of normal rabbit serum (1 mL/L) markedly enhances the specificity of the radioimmunoassay for 1,25-dihydroxycholecalciferol. This can be explained by the presence of the natural vitamin D-binding protein, which has a much higher affinity for 25-hydroxycholecalciferol and 24,25-dihydroxycholecalciferol than for the most active steroid (20, 21). The specificity of our assay still necessitates extensive purification of the steroid because the competing substances are present in the serum in 100- to 1000-fold excess (2–9).

Although the assays in which intestinal receptor is used are a little more specific than our radioimmunoassay, a similar purification of 1,25-dihydroxycholecalciferol is required to remove nonspecific interfering substances (7–12). The cross reactivity of our assay for 1,25-dihydroxyergocalciferol could not be tested, but the antisera discriminates well between 25-hydroxycholecalciferol and 25-hydroxyergocalciferol, so that the ergocalciferol metabolites might not be completely detected in our assay. This would then imply that our antibody assay underestimates the concentration of biologically active vitamin D metabolites in vitamin D2-treated subjects.

Our assay procedure follows the general scheme previously used by others (7–12). Extraction of the steroid with ethyl acetate/cyclohexane is at least as efficient as, but much simpler than, previously used techniques (7–14). The dimensions of the Sephadex LH-20 column were kept to a minimum, and this step was only included to decrease the amount of lipid that would be injected into the chromatograph. The use of an automatic sample injector coupled to an automatic sample collector permits handling of many samples without the constant attendance of a technician.

The sensitivity of the assay was optimized by using a small incubation volume and non-equilibrium conditions. A similar sensitivity can be obtained by the recent adaptations (8, 9) of the receptor assay in which tracers of high specific activity are used.

Results obtained with our radioimmunoassay correspond well to those obtained previously with other methods (7–12). Indeed, our normal mean value of 38 (SD 12) ng/L does not differ markedly from those of Hughes et al. (33 ± 6, ref. 7), Elsman et al. (29 ± 9, ref. 11), and others using similar techniques. For reasons that are unexplained, slightly lower values were found for a bioassay (13) and slightly higher ones for an isotope dilution–mass fragmentography assay (14). Both of these techniques were only applied to a few samples. Low or undetectable concentrations of 1,25-dihydroxycholecalciferol in serum were found in patients with nonfunctional or no kidneys (Figure 5), as could be expected for a hormone produced exclusively in the kidney (1). Increased concentrations were observed in serum of patients with primary hyperparathyroidism, probably related to the stimulatory effect of excess parathyrin and low phosphorus concentrations (1). In some elderly subjects, unexpectedly low concentrations of 1,25-dihydroxycholecalciferol were found, but it remains to be explored whether this represents a normal phenomenon of aging or a real disease.

The present report thus confirms that a radioimmunoassay for 1,25-dihydroxycholecalciferol is feasible but requires extensive preliminary purification of the steroid, because serum contains a large excess of related metabolites that compete with the antisera. The radioimmunoassay has the advantage of a stable gamma-globulin, which can be produced in large amounts, instead of the more labile natural receptor protein from rachitic chicks. The sensitivity of the present assay is comparable to that of previously reported methods and gives much the same results as they for normal and pathological human sera. Clemens et al. (25) recently described a radioimmunoassay for 1,25-dihydroxycholecalciferol with use of similar general procedures, but the antibodies were raised against 1,25-dihydroxycholecalciferol-25-hemisuccinate. Their assay is slightly less sensitive than ours and the cross reaction with other vitamin D metabolites was higher, because no normal rabbit vitamin D-binding protein was added. The intrinsic cross reactivity of their and our antibodies are, however, quite similar. The use of other immunogens, exposing both the A ring and the side chain of the steroid may result in more specific antisera, reducing the technical difficulties involved in all the currently available methods.

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