A Simplified Assay for Dihydroxylated Vitamin D Metabolites in Human Serum: Application to Hyper- and Hypovitaminosis D

Rebecca S. Mason, Dianne Lissner, Harry S. Grunstein, and Solomon Posen

We describe a simplified assay for 24,25- and 1,25-dihydroxvitamin D in human serum. It involves two preparative steps, and normal chick intestine is used in preparing cytosol-binding protein. Our results for 24,25-dihydroxvitamin D indicate a reference interval of 2.9–16 nmol/L (1.2–6.7 μg/L), a mean of 6.7 nmol/L (2.8 μg/L), an intra-assay CV of 11%, and an interassay CV of 22%. For 1,25-dihydroxvitamin D, these data were 29–168 pmol/L (12–70 ng/L), 86 pmol/L (36 ng/L), 12%, and 22%, respectively. In hypoparathyroid patients with vitamin D intoxication, mean concentrations of 25-hydroxvitamin D and 24,25-dihydroxvitamin D in serum were significantly above normal; the 1,25-dihydroxvitamin D concentrations were significantly below normal. Patients with malabsorption and/or post-gastrectomy states had significantly subnormal values for both 25-hydroxvitamin D and 24,25-dihydroxvitamin D in serum, and there was a significantly negative correlation between each of these biochemical values and the severity of osteomalacia. We also discuss cost effectiveness of assaying vitamin D metabolites in human serum.

Additional Keyphrases: “high-performance” liquid chromatography · reference intervals · values in disease states · economics of laboratory operation · hypoparathyroidism · malabsorption · osteomalacia

Since 1974, when Brumbaugh et al. (1) first described a method for measuring 1,25-dihydroxvitamin D (1,25diOHD) in human plasma, several alternative methods and modifications have been presented (2). Eisman et al. (3, 4) published an assay in which three chromatographic steps were replaced by the use of Sephadex LH20 and “high-performance” liquid chromatography, and in which the binding protein was cytosol rather than a cytosol/chromatin preparation.

Assays for 24,25-dihydroxylated vitamin D metabolites have been described (5, 6). These involve chromatography on Sephadex LH20, followed by a competitive protein-binding assay similar to that used for 25-hydroxvitamin D (25-OHD).

Modified methods have appeared (7–9), together with methods based on bioassay (10), radioimmunoassay (11), and mass fragmentography (12).

In this paper we present two methodological simplifications: (a) a one-step extraction and purification preceding liquid chromatography, and (b) the use of intestinal cytosol from normal chicks. Some technical problems we encountered are described.

Materials and Methods

Materials

Non-radiolabeled 25-OHD3, 24,25diOHD3, and 1,25diOHD3 were donated by Roche Products Pty., Ltd., Dee Why, N.S.W., Australia. Tritiated 25-hydroxy-[23,24n-3H] cholecalciferol (90–110 kCi/mol) was obtained from the Radiochemical Centre, Amersham, U.K. Tritiated 1,25-dihydroxy-[23,24n-3H]-cholecalciferol (90–110 kCi/mol) was either obtained from the Radiochemical Centre or synthesized according to a modification (13) of the method of Boyle et al. (14). Tritiated 24,25diOHD3 was made according to a modification of the method of Holick et al. (15) or purchased (Radiochemical Centre). Tritiated chemicals were purified by liquid chromatography (see Methods) at least every two months.

The following buffers were used: buffer A, diethyl barbiturate/acetate buffer, pH 5.6; buffer B, Triton X-100, 5 g/L in buffer A; buffer C, tris(hydroxymethyl)aminomethane hydrochloride (10 mmol/L), KCl (0.3 mol/L), disodium ethylene-dinitrilotetraacetate (hydrate) (15 mmol/L), di-thiothreitol (0.5 mmol/L), pH 7.5; buffer D, tris(hydroxymethyl)aminomethane-HCl (10 mmol/L), KCl (0.1 mol/L), pH 7.5; buffer E, Triton X-100, 5 g/L in buffer D. Each day a buffer was used, its pH was checked and adjusted if necessary.

All solvents were “HPLC” or “spectroscopic” grade. Sephadex LH20 was obtained from Pharmacia, Uppsala, Sweden. Hydroxyapatite (DNA grade; Bio-Rad Labs., Richmond, CA 94804) was suspended in either buffer D (for 1,25diOHD assays) or buffer A (for 24,25diOHD assays). A slurry was made by adding one volume of the relevant buffer to one volume of the settled resin (8). The suspension was adjusted to a final pH of 7.2. Polyethylene glycol (M, 6000) (BDH, Poole, England) was prepared as a 400 g/L solution in buffer C.

Granular Celite columns ("Elutrol") were obtained from E. Merck, Darmstadt, F.R.G., and "Sep-pak" silica cartridges from Waters Associates, Milford, MA 01757. Stainless-steel "Swinnex" filter holders, 13 mm in diameter with 1-μm Teflon...
filters (Millipore Corp., Bedford, MA 01730), were used to filter samples. Conical glass tubes drawn out at the bottom were used to hold samples just before chromatography. All glassware was rinsed in methanol before use. We used 10 × 75 mm polypropylene tubes in the assays. An Altex (Altex Scientific Inc., Berkeley, CA 94710) liquid-chromatographic system was used, consisting of a Model 110 pump; a Model 905-42 loop injector with a 100-µL loop; a precolumn, 0.32 cm (i.d.) × 4 cm, filled with “Spherisorb” 20 µm (particle size), an analytical column 0.32 cm (i.d.) × 25 cm, filled with Spherisorb 5 µm; a Hitachi Model 100-10 spectrophotometer, wavelength, 265 nm; and a Cole-Parmer chart recorder. The precolumn was repacked after every 70 to 80 samples; the analytical column was repacked every three months (Edwards Instrument Co., Narellan, N.S.W., Australia). A separate column was used during purification of tracer material. An FC100 microfractionator (Gilsen Medical Electronics, Middleton, WI 53562) was used to collect sample fractions in conical glass tubes.

A Beckman L5-75 ultracentrifuge was used in preparing cytosol-binding protein, a Sorvall RC3 refrigerated centrifuge with an HLA rotor and an “Omni-carrier” system (capacity, 160 tubes) for all other centrifugations. Radioactivity was measured in a Tricarb Model 3320 liquid scintillation counter in glass vials containing “Instagel” scintillation fluid (Packard Instrument Co., Downers Grove, IL 60515). Quench corrections were made where necessary, according to an external standard.

Normal Subjects and Patients

Reference intervals were established by use of samples from symptomless laboratory workers, medical students, and blood donors 19 to 55 years old. Six patients with hypoparathyroidism (16, 17) who were receiving ergocalciferol were investigated on two occasions: once when they were normocalcemic, and again when they were hypercalcemic because of vitamin D intoxication (18).

We investigated 15 patients with normal renal function (serum creatinine <0.11 mmol/L) and steatorrhea (fetal fat >7% or >18 mmol/24 h) and (or) a history of previous gastric surgery. The patients had been admitted for iliac crest biopsy (19), and, for 12 of them, fetal fat estimation. Blood was taken for the measurement of vitamin D metabolites and other biochemical variables.

Methods

The assay essentially involves three steps (Table 1): (a) extraction of the relevant metabolites into appropriate solvents, (b) “high performance” liquid chromatography, and (c) competitive protein-binding assay.

Preparation of serum samples: A 2- to 5-mL aliquot of serum was incubated for 15 min at room temperature with radiolabeled 24,25diOHD3 and 24,25diOHD2 (about 1200 cpm of each). The sample was then diluted to 20 mL with distilled water and applied to an Extrelut column. After 15 min the column was washed with 50 mL of n-hexane, and the effluent discarded. The column was then washed with 50-mL of dichloromethane and the effluent was collected in a 25 × 150 mm glass tube and dried. This and all other evaporation steps were carried out under nitrogen and in a water bath at temperatures not exceeding 40 °C (20). The evaporated extract was then redissolved in 1 mL of isopropanol/n-hexane (8/92 by vol) and filtered through a Millipore filter, with two further 1-mL washes with this solvent mixture.

Liquid chromatography. After drying, the sample was redissolved in 40 µL of the above solvent mixture and aspirated into a Hamilton (Reno, NV 89510) 100-µL syringe. Two further washes with the same solvent brought the total volume to 90 µL.

The flow rate of the mobile phase (isopropanol/n-hexane) was 1.5 mL/min. Before each batch of samples, the system was calibrated with unlabeled 25-OHD3, unlabeled 24,25diOHD3 and labeled 1,25diOHD3. We collected eluates for 24,25diOHD and 1,25diOHD assays beginning 30–60 s before the times indicated by the relevant calibration markers. The collected fractions were partly dried and stored under nitrogen at −20 °C. After each batch of 12 samples the column was washed with at least 1 L of mobile phase.

Preparation of samples and standards for assay. The solvent in relevant sample fractions was completely evaporated on the day of assay and the residue redissolved in 200–400 µL of isopropanol/hexane. Aliquots of 80 µL were pipetted into assay tubes for 1,25diOHD measurements; 60-µL aliquots were used for 24,25diOHD measurements. The solutions were evaporated to dryness. Similar volumes were pipetted into glass vials containing 5 mL of Instagel, for determination of recovery rates.

A volume of isopropanol/n-hexane similar to that containing the 24,25diOHD or 1,25diOHD fractions (about 4.5 mL) was evaporated and reconstituted in 200–400 µL of the same solvent. Aliquots of 50 µL of this reconstituted solvent were pipetted into each of the assay tubes used for 1,25diOHD standards; 60-µL aliquots were pipetted into the assay tubes used for the 24,25diOHD standards. These solutions were evaporated to dryness.

Assay of 1,25diOHD. All samples were reconstituted in 10

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<td>Add 10 µL of ethanol, binding protein, and tracer; incubate at 4 °C, 2 h</td>
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<td>Count radioactivity</td>
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24,25diOHD

Redissolve in 400 µL of ethanol, pipette 60 µL to assay tube, dry
Remove aliquot to monitor extraction efficiency
Add 10 µL of ethanol, binding protein, and tracer; incubate at 4 °C, 2 h
Separation on hydroxyapatite, 2 washes of pellet
Add 1.5 mL of methanol, decant into counting vial containing 15 mL of “Instagel”

1,25diOHD

Redissolve in 270 µL of ethanol, pipette 80 µL to assay tube, dry
Add 10 µL of ethanol, binding protein, and tracer; incubate at 4 °C, 2 h
Separation on hydroxyapatite, 2 washes of pellet
Add 1.5 mL of methanol, decant into counting vial containing 15 mL of “Instagel”

Count radioactivity

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24,25diOHD

Redissolve in 400 µL of ethanol, pipette 60 µL to assay tube, dry
Remove aliquot to monitor extraction efficiency
Add 10 µL of ethanol, binding protein, and tracer; incubate at 4 °C, 2 h
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1,25diOHD

Redissolve in 270 µL of ethanol, pipette 80 µL to assay tube, dry
Add 10 µL of ethanol, binding protein, and tracer; incubate at 4 °C, 2 h
Separation on hydroxyapatite, 2 washes of pellet
Add 1.5 mL of methanol, decant into counting vial containing 15 mL of “Instagel”

Count radioactivity
μL of ethanol. Standards of 2.5 to 160 pg (6 to 384 fmol) per tube were added in 10 μL of ethanol. Labeled 1,25-dihydroxyvitamin D3 (3000 cpm) was added in 10 μL of ethanol. Binding protein was prepared in buffer C from the intestines of normal three-week-old chicks (21) according to a modification of the method of Eisman et al. (3, 4). The material was stored in 1-mL aliquots in liquid nitrogen at concentrations of 7 to 20 g of protein per liter (22), thawed immediately before use, and diluted eighthfold in buffer C. Of this diluted material, 200 μL was added to the assay tubes with a Hamilton repeating dispenser (0.2–0.5 mg of protein per tube). Nonspecific binding was determined in tubes containing either a 100-fold excess of unlabeled 1,25-dihydroxyvitamin D or 200 μL of buffer C without cytosol. The assay tubes were incubated at 23 °C for 50 min, then immersed in an ice bath. For phase separation with hydroxylapatite (9) we immediately added 400 μL of slurry, incubated for 15 min at 0 °C, agitating every 5 min, and centrifuged (3000 rpm, 4 °C, 5 min). The supernatant fluid was decanted. The hydroxylapatite pellet was washed twice with 1 mL of buffer E and extracted into 1 mL of methanol for 1 h. The pellet, the methanol, and a 0.5-mL methanol wash of the incubation tube were transferred to a scintillation vial containing 15 mL of Instagel.

Assay of 24,25-dihydroxyvitamin D. All dried eluates were dissolved in 10 μL of ethanol. Standards of 24,25-dihydroxyvitamin D3, 0.16 to 2.5 ng (0.38 to 6 pmol) per tube were added in 10 μL of ethanol. Radiolabeled 25-OHD3 (3000 cpm) was added in 10 μL of ethanol. Binding-protein solution consisted of 200 μL of normal human serum (20) diluted 2500-fold in buffer A. Nonspecific binding was estimated in tubes containing either a 700-fold excess of unlabeled 24,25-dihydroxyvitamin D2 or 200 μL of buffer A without serum. The mixture was incubated at 4 °C for 2 h. Separation was carried out in an ice bath with hydroxylapatite slurry added as described for 1,25-dihydroxyvitamin D, except that the hydroxylapatite was prepared in buffer A, and buffer B was used for washing. Pellet extraction was as for 1,25-dihydroxyvitamin D.

Calculation. We constructed standard displacement curves and derived values of samples in terms of percentage displacement after subtraction of counts due to nonspecific binding from standards and unknowns. After appropriate calculations to account for volume of serum extracted, aliquot size, and percentage recovery, results were expressed in nanomoles or picomoles per liter.

Alternative methods. In a separate series of experiments, samples were prepared by shaking the serum three times with dichloromethane (30, 15, and 15 mL, respectively). The bottom layers were pooled and evaporated. The residue was dissolved in 1 mL of diethyl ether/hexane (10/90 by vol) and placed on a Sep-pak silica cartridge, then washed twice more with 1-mL washes. The Sep-pak cartridge was fitted at the bottom with a Millipore filter. Vitamin D metabolites were eluted with 6 mL of isopropanol/dichloromethane (20/80 by volume). After evaporation of these samples, the residues were dissolved in isopropanol/hexane (8/92 by vol) and injected into the liquid chromatograph.

In another preparative method, the extraction on Extrelut columns was followed by chromatography on Sephadex LH20, according to the method of Eisman et al. (3, 4). The samples were then filtered, dried, redissolved in the isopropanol/hexane and injected into the liquid chromatograph.

A separation method involving polyethylene glycol (3, 4) was used in one set of experiments.

Standard biochemical and histological measurements. Serum creatinine, calcium, inorganic phosphate, and alkaline phosphatase were estimated as previously described (19). Three-day collections of feces were analyzed for fat by the method of Van de Kamer (23).

Decalcified and undecalcified sections of bone were prepared and the severity of osteomalacia and parathyroid osteopathy assessed as previously described (19). 25-Hydroxyvitamin D was assayed by the method of Mason and Posen (20), modified by the substitution of Sephadex LH20 for silicic acid chromatography.

Results
Choice of Preparative Method

When serum samples were extracted on Extrelut columns the mean extraction efficiency was 80% (SD 8%) for 1,25-dihydroxyvitamin D and 77% (SD 6%) for 24,25-dihydroxyvitamin D. Dichloromethane extraction recovered a mean of 90% (SD 2%) of labeled 1,25-dihydroxyvitamin D and 89% (SD 6%) of 24,25-dihydroxyvitamin D with no significant further loss during subsequent purification on Sep-pak silica columns. Extraction of 12 samples on Extrelut columns took 2 h, largely without operator involvement; conventional dichloromethane extraction and Sep-pak purification took 4.5 h and was labor-intensive. In addition, we occasionally encountered emulsification problems with dichloromethane. Thus we adopted Extrelut extraction as the method of choice.

As seen in Table 2, similar assay results were obtained with each extraction method. Filtration was necessary regardless of the extraction procedure; samples that were not filtered were difficult to aspirate into the Hamilton syringe before injection onto the chromatographic column.

Liquid Chromatography

Use of a 100-μL rather than a 50-μL sample loop reduced losses in the drawn-out conical glass tube from 7 to 3%, without significantly widening the calibration peaks, and permitted 5 μL of solvent and 5 μL of air to be drawn into the syringe before the sample. This procedure reduced losses...
during transfer into the chromatographic system from 10 to 3%. When injections were made into a port with a worn or insufficiently tightened needle seal, as much as 15% of sample was lost. This loss was reduced to 2% when port and needle seal were replaced. When serum was supplemented with 2.500 μmol of 25-OHD₃ per liter (1 mg/L), the assay values for 24,25-diOHD were unaffected if isopropanol/hexane (8/92 by vol) was used as the mobile phase. When isopropanol/hexane (10/90 by vol) was used, even 500 nmol of 25-OHD₃ per liter resulted in an increase in apparent 24,25-diOHD concentrations. The use of unlabeled 1,25-diOHD₃ in high concentrations (480 nmol/L) during calibration resulted in occasional contamination of the subsequent sample. Labeled 1,25-diOHD₃ (about 0.125 pmol/L) did not give rise to this problem.

When isopropanol/hexane (8/92 by vol) was used as the mobile phase, 25-OHD₃ eluted at 3 to 4 min, 24,25-diOHD₃ at 6 to 8 min, and 1,25-diOHD₃ at 17 to 21 min (Figure 1). When calibration markers were injected at the end of 12 samples, 24,25-diOHD₃ and 1,25-diOHD₃ eluted about 0.25 to 0.5 min earlier than at the beginning of the day. This effect disappeared after the columns were washed with a liter of solvent. Samples were stable for at least three days if kept in isopropanol/hexane (8/92 by vol) under nitrogen at −20 °C.

Assay of 1,25-diOHD. “Bound” 1,25-diOHD counts in assay tubes in which 1.5 mL of solvent had been evaporated were lower than in untreated tubes. The extent of the lowering (“solvent blank”) was inconsistent and was not related to whether binding protein was present, whether redistilled or spectroscopic-grade solvents were used, whether test tubes were glass or polypropylene, or whether hydroxylapatite or polyethylene glycol were used for phase separation. In solvent-treated tubes, samples and standards showed similar nonspecific binding.

Binding protein stored in liquid nitrogen was stable for at least seven months. When left at room temperature for 30 min and refrozen in liquid nitrogen, specific binding was decreased by 25%. Similar binding (approximately 45%) and similar displacement curves were obtained with rachitic and nonrachitic chick cytosol (Figure 2).

When hydroxylapatite and polyethylene glycol separations were compared, the specific binding values were 47% (SD 6%) and 45% (SD 6%), respectively. The nonspecific binding values were 5% (SD 0.3%) with hydroxylapatite and 18% (SD 3%) with polyethylene glycol. Similar nonspecific binding was observed regardless of whether the tubes contained excess unlabeled 1,25-diOHD₃ or no binding protein. In tubes containing binding protein and “zero” standards, the coefficient of variation for the separation step (n = 36) was 5.2% for hydroxylapatite and 7.7% for polyethylene glycol. Similar coefficients of variation were seen when nonspecific binding was measured. The limit of detection (that is, the least standard significantly different from zero) was 6 fmol (2.5 pg) per tube.

After Extrelut treatment and liquid chromatography the mean sample recovery (n = 56) was 70% (SD 10%). The intra-assay CV for a serum pool was 12% (n = 10), and the interassay CV was 22% (n = 13). Assay values for this pool were 72 (SD 13) pmol/L (30 ± 5 ng/L) in untreated samples and 192 (SD 12) pmol/L (80 ± 5 ng/L) when the pool was supplemented with 115 pmol (50 ng) of 1,25-diOHD₃ per liter. The observed range of 1,25-diOHD values in 47 apparently healthy individuals was 29–168 pmol/L (12–70 ng/L) with a mean of 86 pmol/L (36 ng/L) (Table 3). There were no age- or sex-related differences, and identical mean values were obtained in January and July.

Assay of 24,25-diOHD. There were no solvent effects on apparent binding during 24,25-diOHD assays. The limit of detection for this assay was 0.37 pmol (0.16 ng) per tube. The mean analytical recovery (n = 56) after all extraction and chromatographic procedures was 60% (SD 9%). The intra-assay CV for a serum pool was 11% (n = 10); the interassay CV was 22% (n = 12). Mean assay values for this pool were 8.0 (SD 0.9) nmol/L (3.3 ± 0.4 μg/L) in untreated samples and 14.6 (SD 0.5) nmol/L (6.1 ± 0.2 μg/L) when it was supplemented with 6 nmol (2.5 μg) of 24,25-diOHD₃ per liter. The observed range in 37 apparently healthy subjects was 2.9–16.1 nmol/L (1.2–6.7 μg/L) with a mean of 6.7 nmol/L (2.8 μg/L) (Table 3). No age- or sex-related differences were observed. The mean value in the middle of the southern-hemisphere summer was 29% higher than in mid-winter, but this seasonal difference could not be validated statistically.

Effects of Some Disorders

Renal failure. No detectable 1,25-diOHD was found in sera from two anephric sheep, but two control sheep had values of 86 and 96 pmol/L. In the sera of four patients with creatinine concentrations exceeding 1.1 nmol/L (120 mg/L), 1,25-diOHD was not detected. No 24,25-diOHD was detectable in three of these patients; 0.7 nmol/L (0.3 μg/L) was present in the fourth.

Hyperparathyroidism. The mean concentration of vitamin D metabolites in six hyperparathyroid patients is shown in Table 4. Hypercalcemia due to vitamin D intoxication in these patients was associated with a significantly increased mean serum 25-OHD concentration (to eight times the upper limit of normal), a significantly increased mean serum 24,25-diOHD

<table>
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<th>25-OHD</th>
<th>24,25-diOHD</th>
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<tr>
<td>Mean</td>
<td>73</td>
<td>6.7</td>
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<tr>
<td>Range</td>
<td>30–190</td>
<td>2.9–16</td>
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<td>n</td>
<td>73</td>
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Table 3. Vitamin D Metabolites in Serum of Normal Persons
concentration (to 1.5 times the upper limit of normal), and a significantly decreased mean serum 24,25diOHD concentration.

Malabsorption/postgastrectomy states. Mean concentrations of 25-OHD and 24,25diOHD in the sera of these patients were significantly subnormal (33 and 1.08 nmol/L, respectively) with a positive correlation between serum 25-OHD and serum 24,25diOHD concentrations (r = 0.65, p < 0.01). Mean serum 24,25diOHD concentrations (93 pmol/L) did not differ significantly from those of normal subjects. Only two patients had low serum 24,25diOHD concentrations and only one of these, whose serum 1,25diOHD was less than 12 pmol/L, was hypocalcemic (serum calcium 2.03 mmol/L or 81.2 mg/L). Inorganic phosphate concentrations in serum were in the normal range in all patients.

Concentrations of both 25-OHD and 24,25diOHD in serum were negatively correlated (r = 0.80, p < 0.001 and r = -0.64, p < 0.01, respectively) with the severity of osteomalacia. Serum 1,25diOHD values bore no relationship to the severity of osteomalacia, but were negatively correlated with the severity of parathyroid osteopathy (r = -0.64, p < 0.01).

Discussion

Most methods for estimating 1,25diOHD and 24,25diOHD in human serum require three preparatory steps: (a) extraction into an organic solvent; (b) removal of interfering materials, including lipids; and (c) some form of chromatography, usually “high-performance” liquid chromatography. The method described in this paper combines steps (a) and (b) in a liquid/liquid extraction on a commercially available granular Celite column (Extralut) without apparent loss of precision. The overall extraction efficiencies, the intra- and interassay CVs, and the range observed in healthy subjects agree with values obtained by other published methods (1–7, 9, 11).

It has been stated (1–4) that intestinal-cytosol binding protein for 1,25diOHD can be prepared only from vitamin D-deficient chicks. Figure 2 shows that vitamin D-replete chickens can be used equally well, thus putting the assay within the reach of most clinical chemistry laboratories. The volumes of sera required (2–5 mL) are acceptable.

The interpretation of results is complicated by the fact that vitamin D3 derivatives (25-OHD3, 24,25diOHD3 and 1,25diOHD3) are used as standards and tracers, whereas human serum contains both vitamin D2 and vitamin D3 derivatives, particularly in patients receiving pharmacological doses of vitamin D2 (2). However, in a chromatographic system (4) similar to that used in this study, 1,25diOHD2 and 1,25diOHD3 eluted close together. Furthermore, in a competitive protein-binding assay similar to ours, the binding affinities of 1,25diOHD2 and 1,25diOHD3 were almost identical (4, 13).

There is reason to believe that 24,25diOHD2 and 24,25diOHD3 also elute close together in this liquid-chromatographic system (24). Although there appear to be no experimental data concerning the relative affinities of 24,25diOHD2 and 24,25diOHD3 towards vitamin D-binding protein in human serum, it is established that the affinity of 24,25diOHD3 resembles that of 25-OHD3 (6), and the binding affinity of 25-OHD3 is similar to that of 25-OHD2 (25). It is therefore generally assumed (6, 13) that 24,25diOHD2 and 24,25diOHD3 have similar affinities for vitamin D-binding protein in serum.

With rare exceptions (26), compounds not containing the appropriate hydroxyl groups are unlikely to be measured as “1,25diOHD.” Specificity is provided by the chromatographic separation procedure (24) and by a binding protein with a much greater affinity for 1,25-dihydroxylated compounds than for other vitamin D metabolites (4, 8, 13).

The situation is less clear with the 24,25diOHD assay, which might, theoretically, measure at least two other metabolites. 25,26-Dihydroxyvitamin D2 (27) contaminates the 24,25diOHD fractions during Sephadex LH20 chromatography (27), but is almost certainly separated from these fractions in the type of liquid chromatography we used (24). A newly described vitamin D derivative, tentatively identified as 25-hydroxyvitamin D2 26,23-lactone (28), elutes near 24,25diOHD2 (29). This metabolite is not present in normal human serum (29), but its concentration increases markedly in sera of chicks fed large doses of vitamin D (28). Although the 25-hydroxyvitamin D2 26,23-lactone metabolite may compete for the binding protein used, it is separated from the 24,25diOHD fraction in this liquid-chromatographic system (30).

The hypercalcaemia of vitamin D excess appears to be a direct effect of 25-OHD. Increased absorption from the intestine and increased bone resorption can be demonstrated in vitro (14, 31, 32) at the concentrations of 25-OHD seen in these patients (Table 4). The inhibition of the 1-hydroxylase enzyme by hypercalcaemia (33) may be the cause of low 1,25diOHD concentrations in these patients (Table 4).

A positive correlation between serum concentrations of 25-OHD and 24,25diOHD has been previously described (6, 34; Mason et al., submitted for publication), and may be due to the induction of 24-hydroxylase by 25-OHD (35; MacIntyre and Spanos, personal communication) or to the availability of substrate during the 24-hydroxylase reaction.

We have found (Mason et al., submitted for publication) that the presence and severity of histological osteomalacia in uremic patients are related to the serum concentrations of 25-OHD and 24,25diOHD but not of 1,25diOHD. The demonstration of a similar relationship in patients with normal renal function (see Results) strongly suggests that whatever etiologic factors might be involved in the pathogenesis of osteomalacia, lack of 1,25diOHD is not one of them. This metabolite may be present in normal concentrations in patients with osteomalacia (36, 37) and at least in some systems (38, 39) it is incapable of healing established lesions.

This work provides further evidence for the current hypothesis that 1,25diOHD and 24,25diOHD have different physiological functions: 1,25diOHD with its powerful intestinal and skeletal effects prevents hypocalcemia, whereas 24,25diOHD (or some other vitamin D metabolite) constitutes the anti-rachitic factor. In cases of vitamin D deficiency, the available substrate (25-OHD) is preferentially hydroxylated to 1,25diOHD, so that normal serum calcium values are maintained despite low plasma concentrations of anti-rachitic factor(s). Only in states of gross or prolonged vitamin D deficiency does synthesis of 1,25diOHD become impaired, and only then do osteomalacic patients become hypocalcemic. On the other hand, a lack of 1,25diOHD (as in hypoparathyroidism), although associated with hypocalcemia, does not
lead to osteomalacia. The negative relationship between parathyroid osteopathy and serum 1,25(OH)2D concentrations, which is also seen in uremia (Mason et al., submitted for publication), is in keeping with the hypothesis of a negative feedback of 1,25(OH)2D on the parathyroids (40, 41).

It currently takes one technologist one week to process 24 serum samples (including quality-assurance samples) and to derive 48 assay results based on the following timetable:

Day 1—extract and filter; calculate and report previous assay (6 h). Days 2 and 3— chromatograph 12 samples each day (8 h). Day 4—assay 1,25(OH)2D (7 h). Day 5—assay 24,25OHD (8 h).

At a technologist's salary of $12,000 per annum, the approximate labor cost for one serum sample (two metabolites) is $13 (assuming four weeks annual leave and allowing five weeks per year for preparation of reagents, standards, binding protein, and tracer). When reagent costs ($7.50 a sample) and the cost of capital items and supervision are considered, the test is seen to be extraordinarily expensive.

We have previously demonstrated the clinical usefulness of 25-OHD assays (18, 19). In conditions that are liable to lead to vitamin D excess or deficiency, clinical decisions may be based on 25-OHD values, whereas measurements of serum dihydroxylated metabolites at present appear to add little additional clinical information. Although measurement of dihydroxylated vitamin D metabolites may be useful in monitoring patients receiving 1,25(OH)2D3 or 24,25(OH)2D3 therapy (42), we believe, in view of the expense of the tests, that their usefulness in diagnostic work requires further evaluation. Such studies are currently in progress.

This work was supported by grants from the Postgraduate Medical Foundation, the University of Sydney, the N.S.W. State Cancer Council and Roche Products Pty., Ltd., Australia. We gratefully acknowledge the technical and secretarial assistance of Margaret Wilkinson, Ann Potter, Deborah Reynolds, Karen Cranford, and Vicky Mullins. We thank Dr. E. Lumbers, University of New South Wales, and Dr. B. Morris, University of Sydney, for blood samples from healthy volunteers.

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