Some Problems Associated with Assay of 25-Hydroxycholecalciferol in Human Serum

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Methods available for the assay of 25-hydroxycholecalciferol in human serum are evaluated and compared to one another. Ethanol was chosen for use in the initial extraction procedure and rat-kidney cytosol as the binding protein, although good alternative methods are also available. We used silicic acid for chromatography and found this an essential step. Reproducibility was increased when, after "bound" and "free" material were separated, an aliquot of the supernate was pipetted into the counting vial instead of the entire supernatant fluid being decanted. Beta-lipoprotein added to the assay system was of no advantage; added bovine serum albumin interfered with the assay by giving rise to high blank values. With ethanol extraction, silicic acid chromatography, rat kidney cytosol and separation on dextran-coated charcoal, sera from normal subjects showed a mean 25-hydroxycholecalciferol concentration of 28.5 μg/liter (range, 13.1 to 43.9) during the fall season. Coefficients of variation for a control serum were 4.9% (intra-assay) and 10.9% (interassay).

Additional Keyphrases: vitamin D₃ • Intermethod comparison • variation, source of

Since 1971, when Haddad and Chyu (1) and Belsey et al. (2) described competitive protein-binding assays for 25-hydroxycholecalciferol (25-hydroxy vitamin D₃, 25-OH-D₃), the major circulating metabolite of vitamin D, several modifications of this assay have been published (3–12). Here, we describe attempts to evaluate the various methods that are available for routine clinical use.

Materials

25-Hydroxycholecalciferol (25-OH-D₃), donated by Upjohn Pty. Ltd. and Roussel UCLA, was diluted with absolute ethanol (spectroscopic grade) to a concentration of 1 mg/liter and stored in a light-proof bottle under nitrogen at −20 °C. Serial dilutions of standard were made for each assay.

[26,27-³H]-25-Hydroxycholecalciferol (Radiochemical Centre, Amersham; spec. acty., 12.2 kCi/mol) was stored at 4 °C in benzene/ethanol (9/1 by vol), both of spectroscopic grade, in a light-proof bottle, under nitrogen. Its radiochemical purity was checked every three months by chromatography on a Sephadex LH20 (Pharmacia, Sweden) column, 0.5 × 48 cm, with chloroform/hexane (equal volumes) as the eluent.

Vitamin D₃ (crystalline) was purchased from Sigma Chemical Co., St. Louis, Mo. 63178 and stored at 4 °C.

Solvents (analytical grade unless otherwise stated) were used as purchased, except for chloroform, which was passed through a column containing alumina just before use, and ether, which was regularly checked for peroxides.

Buffers. Phosphate buffer (50 mmol/liter, pH 7.5) was used for assays with cytosol binding protein. Diethyl barbiturate/acetate buffer (2), pH 8.6, was used in assays involving serum binding proteins.

Silicic acid (Mallinckrodt) was extracted with ethanol and activated before use by heating overnight in an oven at 180 °C.

Beta-lipoprotein was prepared according to the method of Belsey et al. (2) and diluted 400-fold in phosphate or barbital/acetate buffer (5) (as appropriate for the relevant binding protein).

Bovine serum albumin (Cohn Fraction V; Commonwealth Serum Laboratories, Melbourne) was used as a 10 g/liter solution in barbital/acetate buffer.

Assay tubes were of Pyrex, 13 X 75 mm unless otherwise stated.

Weanling or adult rats were kept in the dark and allowed free access to water and food devoid of Vitamin D (13). After four weeks they were anesthetized with diethyl ether, killed by exsanguination, and kidney cytosol fraction was prepared according to the method of Haddad and Chyu (1).

Dextran-coated charcoal. Dextran T20 or T70 (Pharmacia, Sweden) was mixed with 10-fold its weight of charcoal (Norit A; Calatonic, Los Angeles, Calif.) in the appropriate buffer for 45 min. Fines were removed by centrifugation and the precipitate was resuspended in buffer to give concentrations ranging from 8 to 30 g/liter. The charcoal suspension was stirred continuously at 4 °C while 200-μl aliquots were dispensed into tubes containing the incubation mixture.
Radioactivity was measured in a Packard Tri-Carb Model 3320 liquid-scintillation counter with 10 ml of "Instagel" (Packard Instrument Co.) in each vial. Quench corrections, where necessary, were made according to an external standard.

Methods

Extraction

One milliliter of serum, 1 ml of distilled water, and 6 ml of diethyl ether were mixed on a rotary mixer at room temperature for 45 min. The ether phase was pipetted into a round-bottomed flask and the aqueous phase was extracted twice more, each time with 6 ml of ether. The combined ether extracts were evaporated under a stream of nitrogen at 45 °C. Extractions were also performed with two volumes of ether instead of three, or at 4 °C, or without added water.

In an alternative procedure, 0.3 ml of serum and 1.5 ml of ethanol were agitated on a vortex-type mixer for 30 s. After standing at 4 °C for 30 min, the tubes were centrifuged at 3000 rpm for 30 min. The ethanolic extract was pipetted into a 15-mm diameter test tube and dried under nitrogen in a water bath at 45 °C. In other experiments, 0.5 ml of serum was extracted with 1 ml of ethanol. The temperature of the water bath was varied from 45 to 55 °C.

We also tried a third type of extraction, with a dichloromethane/methanol mixture according to the method of Bouillon et al. (5), modified by our use of a rotary mixer for 30 min.

Extraction efficiency was monitored as follows: Tritiated 25-OH-D₃ (1500 dpm) in 10 μl of ethanol was added to the serum sample and the mixture allowed to stand for 10 min before extraction. After extraction, silicic acid chromatography (when used), and reconstitution, the radioactivity of a 0.2 aliquot of each sample was measured; the remainder was used for incubation with the binding protein.

Chromatography

Activated silicic acid was slurried in diethyl ether/hexane (1/4 by vol) and poured into 5-mm i.d. columns to a height of 3 cm. The extracted evaporated sample was dissolved in 1.5 ml of the ether/hexane and applied to the column, followed by a further two 1.5-ml washes. The ether/hexane eluate was discarded. The 25-OH-D₃ was eluted with 5.5 ml or 3 ml of ether. The recovery of 25-OH-D₃ from columns was estimated by adding a known quantity of tritiated 25-OH-D₃ in ether/hexane to the columns, then collecting the ether eluate directly into scintillation vials and evaporating these before adding "Instagel."

After chromatography, the ether eluate was dried under nitrogen and redissolved in 0.4–2 ml of ethanol, the volume depending on the expected 25-OH-D₃ content. When no chromatography was used, the initial extract was dissolved in ethanol immediately after evaporation. It was shown in a separate series of experiments (Mason and Lawrence, unpublished) that use of different volumes of ethanol at this stage did not affect assay values.

Incubation

The incubation volume was 2.46 ml, consisting of 2.3 ml of phosphate buffer, 3500 dpm of tritiated 25-OH-D₃ in 50 μl of ethanol, 100 μl of ethanol containing either standard (from 1.56 to 25 μg/liter) or reconstituted serum extract, and 10 μl of rat cytosol preparation or 10 μl of phosphate buffer without added protein.

When serum was used as binding protein, the incubation volume was 1.0 ml, consisting of 0.7 ml of barbital/acetate buffer, 50 μl of ethanol containing 3500 dpm of tritiated 25-OH-D₃, 50 μl of ethanol containing either standard (from 3.12–50 μg/liter) or reconstituted extract, and 0.2 ml of 2000-fold diluted rachitic rat serum or 1000-fold diluted human serum in barbital/acetate buffer or 0.2 ml of barbital/acetate buffer without added protein.

All samples and standards were assayed in triplicate with binding protein and in duplicate in "blank" tubes (containing no binding protein). All tubes were agitated on a vortex-type mixer for 10 s after the addition of the reagents and incubated for 2 h at 4 °C.

In some assays, standards were evaporated, reconstituted with rachitic rat serum, and extracted with ethanol, so that their treatment resembled that of sera.

In other modifications of the incubation procedure beta-lipoprotein or bovine serum albumin (10 g/liter) was included in the assay buffer.

The apparent adsorption of tritiated 25-OH-D₃ to the walls of the incubation tubes was assessed for containers made of glass, plastic, or siliconized glass. The "cytosol mixture" (2.3 ml of phosphate buffer, with and without beta-lipoprotein or bovine serum albumin, 3500 dpm of tracer 25-OH-D₃ in 50 μl of ethanol, 100 μl of ethanol, and 10 μl of cytosol protein solution) was incubated for 2 h at 4 °C and decanted into a scintillation vial without further treatment. The counts were compared with those of the same mixture directly pipetted into "Instagel."

Separation

After incubation, 200-μl aliquots of dextran-coated charcoal suspension, containing from 1.6–6 mg of charcoal (constant within each assay), were added to each tube. The mixture was agitated for 5 s, kept at 4 °C for a further 15 min, then centrifuged at 3000 rpm for 30 min. In one experiment, the time interval between addition of charcoal and centrifugation was varied from 1 to 30 min. Aliquots of 0.8 or 1.6 ml of the supernatant fluid were counted. In some assays, we tried the effect of decanting the entire supernate into the scintillation vial. A flow chart of the selected procedure is shown in Table 1.

Calculation of Results

The percentage of tritiated material bound to protein was calculated according to the formula \((x - b)/(y - b)\).
Table 1. Flow Chart for the Assay

Serum sample (usually 0.3 ml)
Add tritiated 25-OH D₃, stand for 10 min.
Add ethanol, mix, incubate at 4 °C for 30 min, centr. 3000 rpm 30 min.
Remove ethanol layer, evaporate under N₂ at 45 °C.
Redissolve in ether/hexane (1/4), apply to silicic acid column.
Remove 1/5 to monitor extraction efficiency.
Add dextran-charcoal, wait 15 min, centr.
Redissolve in ethanol (volume depends on expected assay value).
Elute with 5.5 ml ether, evaporate.
Pipet aliquot of supernate into counting vial containing 10 ml “Instagel.”
Add to incubation mixture 4 °C, 2 h.
Evaporation: Bath for evaporating ethanolic extracts was 55 °C, the assay value for a test serum was 15.5 ± 0.3 µg/liter; at 50 °C the value was 17.4 ± 1.3 and at 45 °C or below the value was 19.8 ± 1.2.

Chromatography: Tracer recovery through the silicic acid columns depended on the volume of ether used for elution. It was 97 ± 3% with 5.5 ml of ether, less if smaller volumes were used. The mean analytical recovery of 25-OH-D₃ added to serum samples was 95.3 ± 15% with chromatography, but 110 ± 41% without it. The mean blank value (i.e., “bound counts” in the absence of binding protein) was 3.5 ± 1.3% if chromatography was used and 18.6 ± 18.8% if this step was omitted. The mean blank value for standards was 3.2 ± 2.6%. There was no significant interference by cholecalciferol added to serum to give a final concentration of 300 µg/liter, regardless of whether or not chromatography was performed.

When a single test serum was assayed with a range of procedures a mean value of 36 ± 7 µg/liter was obtained with prior chromatography compared with a mean value of 156 ± 136 µg/liter when this step was omitted. The ratio of assay values obtained for individual sera without and with chromatography ranged from 0.7 to 4.3 for sera from normal subjects and from 0.8 to 2.7 for sera with high 25-OH-D₃ content.

Binding proteins: Standard curves were obtained after use of adult-human serum, weanling-rat-kidney cytosol, adult-rat-kidney cytosol, or adult-rat serum as binding proteins (Figure 1). Under appropriate conditions, each of these bound about 50% of tracer counts and all displaced in the range 0.156–5 ng/tube. Results obtained with test sera and with four of these binding proteins are shown in Table 2.

Buffer additives: Addition of beta-lipoprotein to the buffer containing rat-serum binding protein increased mean zero binding from 34 to 54% of total counts; the blanks remained at 7%. However, when the points on standard curves were expressed as percentages of the zero binding, the curves were not significantly different.

Results

Analytical Variables

Extraction: When ethanol was used under optimal conditions the mean (n = 60) extraction efficiency for the initial extraction step was 83% with a within-assay CV of 6% and a between-assay CV of 7%. When ether was used under optimal conditions, the mean (n = 60) extraction efficiency was 84%, with an intra-assay CV of 11% and an interassay CV of 12%. The mean extraction efficiency with dichloromethane/methanol was 63%, but we did only six extractions with this solvent. The extraction procedure required about 3 h when ether and dichloromethane/methanol were used, 90 min when ethanol was used.

In a separate series of experiments it was shown that the extraction efficiency of ether could be improved 44% by adding 1 ml of water to the serum sample,¹ 26% by extracting at room temperature rather than at 4 °C, and 10% by repeating the extraction procedure three times. The efficiency of ethanol extraction was improved from a mean of 65.7% to a mean of 79.3% when ethanol serum ratios of 5/1 rather than 2/1 were used.

Evaporation: When the temperature of the water

¹ Kent, G. N., Retallack, R. (personal communication).
from those obtained in experiments not involving the use of beta-lipoprotein. Values obtained with a test serum had a mean CV of 12.6% with beta-lipoprotein present, as compared with 7.8% when it was omitted.

Increasing concentrations of bovine serum albumin caused a significant increase in “zero binding” values, whether or not a specific binding protein was present in the assay system. However these “bound counts” were unaffected by the presence of unlabeled 25-OH-D₃.

Extracting standards from osteomalacic serum: When standards were extracted from osteomalacic rat serum before assay, the displacement curves obtained were identical with those obtained with standards made up in ethanol. Assay values for test serum, as expected, were identical with both curves.

Test tubes used for assay: When 12 mm × 75 mm glass test tubes were used, 26 ± 4% of the tracer counts were lost, presumably owing to adsorption of tritiated material to the walls of the tubes. This loss was 25% if beta-lipoprotein was in the buffer, but only 4% if bovine serum albumin (10 g/liter) was in the buffer. Larger glass tubes (15 × 100 mm) adsorbed 47% of counts; smaller ones (10 mm × 65 mm) adsorbed 23%. Siliconizing the 12 mm × 75 mm glass tubes increased the loss to 45%. Polyethylene tubes, (12 × 75 mm), adsorbed 48%; polypropylene tubes (12 × 80 mm) adsorbed 18%.

Phase separation: Similar displacement curves and values for control sera were obtained with charcoal coated with either Dextran 20 or Dextran 70. If the amount of charcoal per tube was kept constant within an assay, similar curves were obtained with quantities ranging from 1.6–6.0 mg/tube.

A zero binding value of 74.6 ± 5.2% was obtained when tubes were centrifuged for 1 min after adding the charcoal. As the time interval between addition of charcoal and centrifugation was increased to 5, 10, 15, or 30 min, the binding value declined to 66.3 ± 2.4%, 62.2 ± 3%, 63.5 ± 3.1%, and 60.2 ± 5%, respectively.

Normal Values

Using ethanol extraction, silicic acid chromatography, rat-kidney cytosol binding protein, and dextran-coated charcoal T₂₀ separation (3 mg/tube), we obtained a mean of 28.5 ± 7.7 µg/liter of serum for normal subjects. The specimens were taken during the Southern Hemisphere fall season. This value is almost identical with values obtained by Haddad and Chyu (1) and Belsey et al. (3). A control serum with a mean value of 19.3 µg/liter showed an intra-assay CV of 4.9% (n = 7) and an inter-assay CV of 10.9% (n = 7).

Discussion

Before assay, 25-OH-D₃ has to be extracted from serum or plasma into some organic solvent such as ether (1), chloroform/methanol (2), ethanol (3), or dichloromethane/methanol (4, 5). We found extraction into ethanol to be the simplest and most convenient method; extraction efficiencies compared favourably with those of other solvents, especially in terms of reproducibility.
The ethanol extract should not be evaporated at temperatures above 45 °C, otherwise results are lower. This effect is attributed to denaturation of the 25-OH-D₃ molecule so that it does not displace tritiated 25-OH-D₃ from binding sites.

In earlier assays silicic acid (1, 2), thin-layer chromatography (4) or chromatography on Sephadex LH 20 (6) was used to purify the extracts containing 25-OH-D₃. Some recent publications (3, 7–9, 12) advise omitting this step. In our experience it has been possible, occasionally, to achieve similar values with and without chromatography, but in general, the omission of chromatography resulted in large interassay variations in blank values, in irregular analytical recoveries of added 25-OH-D₃, and in widely fluctuating assay values. We therefore believe that some form of chromatography is necessary for the assay of 25-OH-D₃ in human sera. The need for such a procedure appears to have nothing to do with the presence of cholecalciferol, which does not significantly displace tritiated 25-OH-D₃, even when concentrations are 100-fold those of 25-OH-D₃. Rather it would seem that some factor, possibly a serum protein, is incompletely separated during the initial extraction procedure and that unless this is removed during a subsequent purification step it binds 25-OH-D₃ in a capricious manner.

Binding proteins used for 25-OH-D₃ assay include kidney cytosol obtained from rachitic weanling rats (1, 7, 11), serum from rachitic rats (2, 3, 5, 8, 10, 11), normal rats (5), osteomalacic patients (4, 9), or normal humans,² and partially purified rat-serum protein binding rat (6). We have used kidney cytosol from rachitic adult and weanling rats, rat serum, and normal human serum and have been able to obtain similar binding, displacement curves, and assay values.

The low solubility of 25-OH-D₃ in aqueous buffer is overcome by using ethanol (70–100 ml/liter) in the assay system. The inclusion of beta-lipoprotein (2, 3, 5) was advocated so as further to enhance the solubility of the 25-OH-D₃ molecule. We found that while “zero binding” with rat serum certainly increased with the addition of beta-lipoprotein, displacement values expressed as percentages of the zero value were similar to those obtained without beta-lipoprotein. In addition, we confirmed the finding of Garcia-Pascual et al. (8) that use of beta-lipoprotein is associated with greater variabilities in assay results.

Preece et al. (10) suggested that bovine serum albumin be included in the assay buffer to prevent the loss of 25-OH-D₃ from the solution. Although this does decrease tracer adsorption to the test tube wall, at a concentration of 10 g/liter it significantly interferes with reversible binding by the assay protein.

Offermann and Dittmar (7) compared standards made up in ethanol with standards extracted from osteomalacic serum and obtained nonparallel standard curves. We obtained identical displacement curves with both types of standards and we consider extraction of standards from osteomalacic serum unnecessary. We also believe that the need for chromatography is not obviated by the use of standards extracted from osteomalacic sera.

Phase separation has been achieved with use of Florisil (4), and with dextran-coated charcoal T₂₀ (1, 10, 11), T₇₀ (3), or T₇₀ (5–9, 12) in amounts ranging from 1 mg (3) to 6 mg (9) per assay tube. We were unable to demonstrate any advantage of one type of dextran over another and found that the amount of charcoal (constant within an assay) within the range 1.6 to 6 mg/tube was not critical. However it seems necessary to wait for 10–30 min between the addition of charcoal and centrifugation.

Erratic blank values were our major problem when the supernate was decanted into scintillation vials. We believe this is due to non-sedimenting charcoal and found that the problem disappeared when aliquots were pipetted into the vials.

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References


² Larkins, R. G. (personal communication).