Methods for the determination of the circulating concentration of 25-hydroxyvitamin D

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Overview

The circulating concentration of the combination of 25-hydroxyvitamin D_2 (25-OH-D_2) and 25-hydroxyvitamin D_3 (25-OH-D_3) is a summation of the vitamin D that comes from sunlight and the diet.[1] This measurement is considered to be the best indicator of vitamin D status.[2] To determine its concentration, 25-OH-D (25-OH-D_2 and/or 25-OH-D_3) is first extracted from serum or plasma with absolute ethanol, followed by a protein-binding assay using the serum vitamin D-binding protein which has high affinity for 25-OH-D.[3] To accurately determine circulating concentrations of 25-OH-D without other vitamin D metabolites, a silica Sep-Pak cartridge chromatography can be performed.[4] In this paper, we describe the method used in our laboratory for the assay of 25-OH-D in plasma and serum. The method for vitamin D determination was reported previously in this journal.[5] The assay for 1,25-dihydroxyvitamin D is described in the following paper.

Reagents

25-hydroxy(26,27-methyl-^{3}H)cholecalciferol (^{3}H-25-OH-D_3), approximately 5 to 15 Ci/mmol, obtained from Amersham (TRK 396) or New England Nuclear (NET-349). Store at −20°C. To make tracer solution, pipet an aliquot of stock tracer into a glass vial. Dry under N_2 gas and reconstitute with sufficient absolute ethanol to make a solution containing about 2,000 cpm in 10 μl.

Crystalline standard 25-OH-D_3. Crystals are stored at −20°C. A stock concentration of 10 μg/ml is prepared as follows: Dissolve about 0.1 mg of crystalline 25-OH-D_3 in 5 ml of absolute ethanol. Its concentration is determined according to the following formula:

$$\text{Concentration } (\mu g/ml) = \frac{OD_{265\ nm} \times 400\ (\mu g \cdot \mu mol^{-1})}{18.2\ (ml \cdot \mu mol^{-1} \cdot cm^{-1}) \times 1\ cm\ light\ path}$$

An aliquot is then taken and diluted with absolute ethanol until a concentration of 10 μg/ml is obtained. This solution is then further diluted to yield a working standard with a concentration of 64 ng/ml. Both the concentrated and the working standard solutions should be stored under N_2 gas, in the dark, at or below −20°C.

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Buffer. Barbital-acetate stock solution is prepared by dissolving 9.71 g sodium acetate and 15.74 g sodium barbital in 1 liter of distilled deionized water. Assay buffer is prepared by adding 400 mg bovine serum albumin (BSA, affinity purified, Pierce catalog no. 90004) to 20 ml stock buffer and bringing the final volume to 400 ml with distilled deionized water. Sodium azide (0.1 g) is added and the pH is adjusted to 8.6. Shelf life is 4 to 5 weeks.

Serum vitamin D-binding protein. Serum obtained from normal or vitamin D-deficient rats is diluted 100-fold with assay buffer *without* BSA. Aliquots (0.5 ml) are stored at or below −70°C and are stable for 2 to 3 years. The solution is further diluted for use in the assay to a concentration which will yield 30% to 40% specific binding of ^{3}H-25-OH-D_3 in the absence of added cold 25-OH-D_3 (see titer determination for binding assay).

Dextran-coated charcoal. A total of 0.3 g Dextran T-70 (Pharmacia) and 3.0 g "Norit GSX" charcoal (Hopkin and Williams) are mixed with 100 ml of assay buffer *without* BSA and gently stirred for 1 hour. The pH should be 8.6 and the suspension should be stored at 4°C. The suspension is stirred gently for 15 minutes prior to use in the assay.

12 × 75 mm Borosilicate glass tubes.

20-ml Glass scintillation vials.

Pipetman (P-1000, P-200, and P-20; Gilson) and disposable pipet tips.

Absolute ethanol.

Scintillation cocktail. Liquiscint or equivalent (National Diagnostics).

Vortex mixer.

Sorvall RT-6000C centrifuge or equivalent.

Nitrogen gas.

Scintillation counter.

Silica Sep-Pak cartridges (optional), Waters Associates.

Procedure: day 1

Extraction (done on ice)

1. Aliquot 100 μl of each serum or plasma sample into appropriately labeled 12 × 75 mm test tubes in duplicate. If vitamin D deficiency is suspected, add 200 μl of serum or plasma. If vitamin D intoxication is suspected, use 50 μl of serum or plasma.
2. Bring the total volume in the tubes to 1.0 ml with 100% ethanol.
3. Vortex-mix and let stand on ice for 20 minutes.
4. Centrifuge at 3,000 rpm for 20 minutes.
5. While extraction is proceeding, label 12 × 75 mm tubes in duplicate for each unknown serum sample. Label, in triplicate, nonspecific binding (NSB) tubes, B_o tubes (zero concentration of 25-OH-D_3), and tubes for standards 2a through 2g.

Silica Sep-Pak cartridge chromatography (optional)

1. Follow steps 1 through 4 of the extraction procedure, but add 10 μl (approximately 2,000 cpm) of ^{3}H-25-OH-D_3 to serum or plasma samples before ethanol extraction (for recovery purposes). With the same pipet, add 10 μl of the tracer to the scintillation vials in duplicate. Add 10 ml of the scintillation cocktail to each vial and set aside (these represent the total counts for recovery purposes).
2. Add 0.3 ml of 7% ethyl acetate in n-hexane to each tube, vortex-mix, and allow to stand for 30 minutes.
3. Set up the Sep-Pak cartridges on the Sep-Pak rack.
4. Condition the cartridges by washing with 4 ml of 7% ethyl acetate in n-hexane.
5. Apply samples to the appropriate cartridges. Rinse the tubes with an additional 0.3 ml of the same solvent and apply to the cartridges. Elute to waste.

6. Add 30 ml of 7% ethyl acetate in n-hexane to each cartridge and elute to waste.
7. Add 5 ml of 25% ethyl acetate in n-hexane to each cartridge and collect eluant in appropriately labeled tubes.
8. Repeat step 7 three times. A total of 20 ml of 25% ethyl acetate in n-hexane is required to completely elute 25-OH-D from the cartridge.
9. Combine the eluates and dry under a stream of nitrogen at 37 to 45°C.
10. When the samples are dry, add 1 ml of ethanol to each tube, vortex-mix, and allow to stand for 30 minutes.

Standard preparation

1. Label seven 12 × 75 mm tubes and add 400 µl of absolute ethanol to each tube.
2. Add 400 µl of working standard 25-OH-D_3 (64 ng/ml) to the standard a tube. Mix, then take 400 µl from the standard 1 tube and add to the standard b tube. Continue serial dilutions in this fashion through standard tube g. The concentrations in the respective standard tubes are as follows:
   a. 32 ng/ml,
   b. 16 ng/ml,
   c. 8 ng/ml,
   d. 4 ng/ml,
   e. 2 ng/ml,
   f. 1 ng/ml, and
   g. 0.5 ng/ml.

Tracer addition

1. Pipet 10 µl of ^{3}H-25-OH-D_3 in ethanol containing approximately 2,000 cpm into all tubes, as well as into three vials containing 10 ml of scintillation cocktail to determine the radioactivity added to each tube.

Sample addition (done at room temperature)

1. Pipet 50 µl of standards 1 through 7 into appropriately labeled tubes in triplicate.
2. Aliquot 50 µl of absoute ethanol into the "B_o" tubes.
3. Add 50 µl of concentrated (10 µg/ml) 25-OH-D_3 into the NSB tubes.
4. Transfer 50 µl of serum sample extracts into appropriately labeled duplicate tubes. At the same time, pipet 500 µl of the extract into counting vials containing 10 ml of scintillation cocktail and set aside if Sep-Pak cartridge chromatography is performed (for recovery calculations).
5. Add 500 µl of binding protein solution into *all* of the tubes.
6. Vortex-mix and incubate overnight at 4°C.

Procedure: day 2

Charcoal addition

1. Add 100 µl of dextran-coated charcoal from a stirring suspension to all but the total count tubes.
2. Vortex-mix and let stand for 20 minutes at room temperature.
3. Centrifuge at 3,000 rpm for 15 minutes.
4. Carefully transfer 500 µl of supernatant from each tube into the scintillation vials and add 10 ml of scintillation cocktail to each vial.
5. Count each vial for at least 5 minutes in a scintillation counter.

Calculation

Calculation of %B/B_o

1. Obtain the average cpm for each standard, control, and unknown.
2. Subtract the average cpm of the NSB tubes from each tube count.

3. Divide the corrected cpm (cpm − NSB cpm) of each standard, control, or unknown by the corrected cpm of the total binding (B_o) tube and multiply by 100.

$$\%B/B_o = \frac{cpm\ of\ standard\ or\ unknown - cpm\ of\ NSB}{cpm\ of\ B_o\ tube - cpm\ of\ NSB} \times 100$$

Standard curve plot and unknown calculation

1. Plot $\%B/B_o$ for the standards (vertical axis) versus the standard concentration (horizontal axis) on three-cycle semi-log paper (*Figure 1*).
2. Draw the best-fit sigmoidal curve through the points.
3. Read the unknown concentrations from the graph.
4. Multiply the result from step 3 by 20.
5. Divide the result from step 4 by the starting volume of serum or plasma in milliliters and by the % recovery if the optional chromatographic step is performed.

$$\%\ recovery = \frac{cpm\ in\ 500\ \mu l \times 2}{total\ counts\ for\ recovery} \times 100$$

6. The final result is expressed in ng/ml. To convert to nmol/l, multiply ng/ml by 2.5.

Titer determination for binding protein

1. Dilute stock serum binding protein solution an additional 100-, 200-, and 300-fold (the final dilution of the original serum is 1:10,000, 1:20,000, and 1:30,000).
2. Label 12 × 75 mm glass tubes B_01, B_02, B_03, NSB_1, NSB_2, and NSB_3, in duplicate, for the three different concentrations of binding protein.
3. Add 50 µl of absolute ethanol to all B_0 tubes.
4. Add 50 µl of 10 µg/ml 25-OH-D_3 solution to all NSB tubes.
5. Pipet 10 µl of ^{3}H-25-OH-D (approximately 2,000 cpm) in ethanol into all tubes, as well as into two scintillation vials containing 10 ml of scintillation cocktail (total count vials).
6. Pipet 500 µl of serially diluted binding protein solutions into their respective tubes; e.g., add 1:10,000 dilution to tubes NSB_1 and B_01, 1:20,000 dilution to tubes NSB_2 and B_02, etc.
7. Vortex-mix and incubate overnight at 4°C.
8. Follow steps 1 through 5 as described for the charcoal addition procedure.
9. Obtain the average cpm for each group, B_0 and NSB.
10. Subtract the average cpm of the NSB tubes from that of their respective B_0 tubes.

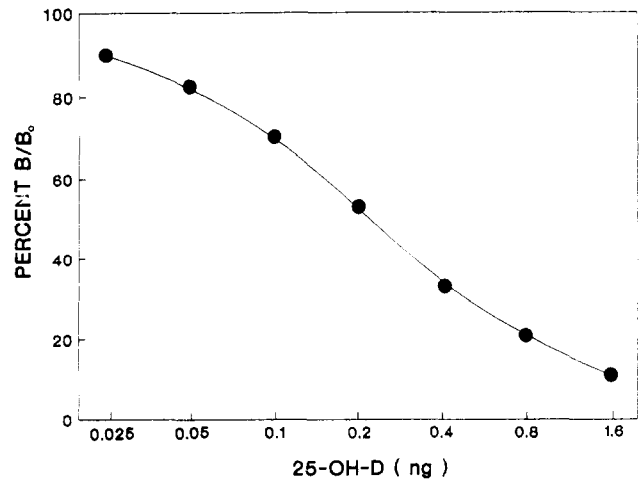

Figure 1 Standard curve for the 25-OH-D assay. Each point represents the mean of three determinations.

11. Divide the corrected cpm (B_0 cpm − NSB cpm) of each dilution by the background corrected total counts (total count cpm − background cpm) and multiply by 100. For example,

$$\%\ binding\ (1:10{,}000\ dilution) = \frac{B_0 l\ cpm - NSB_l\ cpm}{total\ count\ cpm - background\ cpm} \times 100$$

Discussion

For the analysis of 25-OH-D, we have found that direct determination with serum vitamin D-binding protein, without the addition of any chromatographic step, is adequate.[7] The reasons for this are that the vitamin D-binding protein has a much higher affinity for 25-OH-D than for vitamin D itself and that the serum contains much more 25-OH-D than vitamin D and all its other metabolites combined. For example, in normal subjects, the serum contains about 30 ng/ml of 25-OH-D, 3 ng/ml of vitamin D, less than 3 ng/ml of $24,25(OH)_2D$, and less than 0.1 ng/ml of $1,25(OH)_2D$.[8,9] If necessary, a separation of 25-OH-D from vitamin D and its hydroxylated metabolites by Sep-Pak cartridge[4] or Bond-Elut C_{18}-OH mini-column[10] may be incorporated prior to binding assay. The two-step HPLC purification described by Jones can also be applied in the case of separation of 25-OH-D_2 from 25-OH-D_3.[11] Once separation is achieved, the binding assay is conducted. Alternatively, HPLC UV detection can be applied to the direct quantitation of 25-OH-D_2 and 25-OH-D_3. This HPLC assay procedure is the most accurate method for the determination of 25-OH-D.[11] The protein-binding assay described here, however, offers the advantages of being able to process a large number of samples, as well as increased sensitivity. The intra- and inter-assay coefficients of variation are approximately 5% to 10% and 10% to 15%, respectively. To obtain optimal assay sensitivity and low nonspecific binding, the standard and radioactive 25-OH-D must be purified by HPLC prior to use.[11]

In addition to being an indicator of vitamin D status, serum 25-OH-D measurements have a variety of other clinical uses.[2]

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