# A method for the determination of the circulating concentration of 1,25-dihydroxyvitamin D

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## Overview

Measurement of 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] in serum or plasma is of particular value in the evaluation of disease in patients with either acquired or inherited disorders of 25-hydroxyvitamin D (25-OH-D) metabolism, including (1) renal osteodystrophy, (2) for the differential diagnosis of vitamin D dependent rickets, type I and type II, (3) for the evaluation of metabolic bone disease, and (4) for hypercalcemia associated with chronic granulomatous disorders.[1] The assay can be broken down into three principal parts: (1) the solvent extraction of 1,25$(OH)_2$D [1,25$(OH)_2D_2$ and/or 1,25$(OH)_2D_3$] from serum or plasma; (2) separation from other vitamin D metabolites utilizing some form of column chromatography; and (3) quantitation of 1,25$(OH)_2$D by a radio-receptor assay, (RRA) using either chicken intestinal receptor,[2] or calf thymus receptor,[3] or by means of a radio-immunoassay (RIA).[4] The method discussed in this paper is used in our laboratory for the determination of 1,25$(OH)_2$D in serum and plasma.[5] The method for vitamin D determination was described previously in this journal.[6] The method for measurement of 25-hydroxyvitamin D was reported in the preceding paper.[7]

## Reagents

*$^3$H-1,25$(OH)_2D_3$ [1,25-dihydroxy(26,27-methyl-$^3$H)cholecalciferol],* Amersham, Arlington Heights, IL, USA (TRK 656), or New England Nuclear, Boston, MA, USA (NET 626). Specific activity 160–180 Ci/mmol.

*HPLC grade hexane, isopropanol, acetonitrile, methanol, and dichloromethane,* Fisher Scientific, Medford, MA, USA.

*C-18-OH solid-phase extraction columns,* Analytichem Inc., Harbor City, CA, USA.

*Sep-Pak cartridge rack system,* Waters Associates, Milford, MA, USA.

*N-Evap nitrogen evaporation system,* Organomation Associates, Northborough, MA, USA.

*Scintillation counter.*

*Sorvall RT-6000C centrifuge* (or equivalent).

*Micropipetors with tips.*

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*Vortex-mixer.*
*12 × 75 mm borosilicate glass tubes.*
*13 × 100 mm borosilicate glass tubes.*
*Pasteur pipets.*
*Nitrogen gas.*
*Absolute ethanol.*
*Scintillation cocktail,* Liquiscint, National Diagnostics, Manville, NJ, USA, or equivalent.
*Temperature-controlled water bath.*
*1,25(OH)$_2$D calf thymus receptor,* Nichols Institute Inc., Cat. No. 300600, San Juan, Capistrano, CA, USA, or IncStar Inc., Cat. No. 20307, Stillwater, MN, USA.

## Chemicals, reagent grade

*$K_2HPO_4$.*
*KCl.*
*Dithiothreitol* (store dessicated in freezer).
*Tris-HCl, Ultra-pure.*
*EDTA.*
*Boric acid.*
*Sodium molybdate.*
*Sodium azide.*
*Bovine Serum Albumin, Fraction V (BSA)* (store in dessicator at 4°C), Sigma, St. Louis, MO, USA.
*Norit A charcoal,* Fisher, Medford, MA, USA.
*Dextran T-70,* Pharmacia, Piscataway, NJ, USA.
*Gelatin,* Sigma, St. Louis, MO, USA.
*KOH.*

## Preparation of reagents

1. Assay Buffer

| | |
|---|---|
| 450 mM KCl | 33.56 g |
| 1.5 mM EDTA | 0.56 g |
| 50 mM Tris-HCl | 7.88 g |
| 10 mM $Na_2MoO_4$ | 2.42 g |
| 0.1% $NaN_3$ | 1.0 g |
| 0.1% Gelatin | 1.0 g |

Dissolve in 900 ml ice-cold distilled deionized water (dd $H_2O$) and adjust pH to 7.5 at 4°C. Bring final volume to 1 liter and store in the refrigerator at 4°C. Shelf-life is 2 to 3 months. Immediately prior to running the assay, add 0.077 g/100ml of dithiothreitol to the buffer.

2. Calf Thymus Receptor

Calf thymus receptor is prepared according to the procedure described by Reinhardt et al.[3] or can be obtained commercially. The receptor preparation is reconstituted according to the manufacturer's directions, or in the case of an in-house preparation, as follows.

Resuspend the receptor pellet in an appropriate amount of buffer. Gently vortex-mix, until the pellet is fully suspended in the buffer. Dilute further with buffer to a concentration of receptor which will bind 25 to 35% of added tritiated 1,25(OH)$_2$D$_3$. This dilution factor should be predetermined by a titer test. The procedure for a titer test is similar to the 1,25(OH)$_2$D radioreceptor assay described later in this manuscript, except that serial dilutions of receptor solution are used to determine binding in both non-specific binding (NSB) and total binding ($B_0$) tubes. It is mandatory to keep the receptor solution on ice **at all times.**

3. Assay Tracer

Dry 20 to 25 μl of stock $^3$H-1,25(OH)$_2$D$_3$ under $N_2$ and reconstitute with a volume of ethanol which will yield 2000 to 2200 cpm/10 μl of solution. Store the stock tracer under nitrogen at or below −15°C.

4. Recovery Tracer

Follow steps outlined in (3), but make a solution containing 700 to 800 cpm/10 μl.

5. Dextran-Coated Charcoal Suspension (DCC)

a. Prepare 1 liter of 0.1 M boric acid buffer

| | |
|---|---|
| Boric acid | 6.18 g |
| BSA | 0.50 g |
| $NaN_3$ | 0.20 g |
| dd$H_2O$ | 0.90 L |

Adjust pH to 8.6, make up volume to 1 liter, and store at 4°C for not more than 2 months.

b.

| | |
|---|---|
| Norit A charcoal | 6.0 g |
| Dextran T-70 | 0.6 g |

Suspend the charcoal and dextran in 500 ml of 0.1M boric acid buffer and stir overnight at 4°C. Centrifuge the suspension at 2500 rpm for 20 minutes. Discard the supernatant and resuspend the pellet in 500 ml of 0.1M boric acid buffer. Stir the suspension at 4°C for 15 minutes prior to use in the assay. Make fresh every 6 to 8 weeks.

## Preparation of standards

Crystalline 1,25(OH)$_2$D$_3$ is stored at −20°C. A stock concentration is prepared as follows: Dissolve about 100 μg of crystalline 1,25(OH)$_2$D$_3$ in 5 ml of absolute ethanol. Its concentration is determined according to the following formula:

$$\text{Conc. } (\mu g/ml) = \frac{OD_{265nm} \times 416}{18.2}$$

An aliquot is then taken and diluted with absolute ethanol until a concentration of 1.0 μg/ml is obtained (stock standard).

Non-Specific Binding (NSB) Standard: 6.4 ng/40 μl. Take 200 μl of stock standard (1 μg/ml) and add 1025 μl of absolute ethanol. The concentrated stock and NSB standard solutions should be stored under $N_2$ gas, in the dark, and at or below −70°C.

6. Working Standards

Take 10 μl of NSB standard and dilute to 3.2 ml with absolute ethanol. This will yield a solution with a concentration of 20 pg/40 μl. Make serial dilutions by taking 200 μl of standard and diluting with 200 μl of absolute ethanol. The standards thus obtained will contain the following concentrations: 20, 10, 5, 2.5, and 1.25 pg/40 μl. Prepare fresh working standards for each assay.

7. Solvent Systems

Make 1 liter of each of the following:

a. 70% methanol in dd$H_2O$
b. 10% methylene chloride in hexane
c. 1% isopropanol in hexane
d. 6% isopropanol in hexane

8. 0.4M $K_2HPO_4$ pH 10.5

   To prepare 500 ml:

   Weigh out 34.84g $K_2HPO_4$ (anhydrous) and dissolve in 400 ml of distilled deionized water. Adjust to pH 10.5 with 1M KOH and bring the volume up to 500 ml. Store the solution at 4°C for no longer than 8 weeks.

## Procedures

### *Extraction*

1. Allow all samples, including quality control (QC) samples which have known concentrations of $1,25(OH)_2D$, to thaw completely; vortex-mix each sample.
2. Pipet 1.0 ml of unknown or QC samples into labeled 13 × 100 mm borosilicate glass tubes.
3. Add 10 µl of recovery tracer to each tube. Using the same pipet, add 10 µl of recovery tracer to two scintillation vials containing 10 ml of scintillation cocktail.
4. Add 1.0 ml of acetonitrile to each tube and vortex-mix intermittently for 10 minutes.
5. Centrifuge for 15 minutes at 3000 rpm at 4°C.
6. While the samples are being centrifuged, prepare the C-18-OH columns for sample application as follows:

   Assemble the Sep-Pak rack. Before proceeding, ensure that the C-18-OH columns are in position A, and that labeled 13 × 100 mm borosilicate glass tubes are in position B. The Sep-Pak rack is now ready for use.

   a. Add 5 ml hexane to each column.
   b. Turn on the vacuum by using the vacuum control handle at the side of the apparatus. Allow the solvent to pass completely through the column. Turn the vacuum off.
   c. Add 5 ml isopropanol to each column.
   d. Repeat step b.
   e. Add 5 ml methanol to each column.
   f. Repeat step b.
   g. Add 5 ml $ddH_2O$ to each column.
   h. Repeat step b.

The columns are now ready for sample application.

### *Sample application*

7. After centrifugation, pour off the supernatant into labeled 12 × 75 mm borosilicate glass tubes containing 1.0 ml 0.4M $K_2HPO_4$, pH 10.5, and mix. Discard the pellet.
8. With the columns in position A, apply the sample to the prepared column reservoir.
9. Turn on the vacuum, and allow the liquid to pass completely through the column. Turn the vacuum off.
10. Add 5 ml of $ddH_2O$ to each column.
11. Repeat step 9.
12. Add 5 ml of 70% methanol in $ddH_2O$ to each column.
13. Repeat step 9.
14. Add 5 ml of 10% methylene chloride in hexane to each column.
15. Repeat step 9.
16. Add 5 ml of 1% isopropanol to each column.
17. Repeat step 9.
18. Rotate C-18-OH columns to position B for eluate collection, according to directions provided by the manufacturer.

19. Add 5 ml of 6% isopropanol to each column.
20. Turn on the vacuum and collect the eluate in 13 × 100 mm borosilicate glass tubes. Make sure that all of the eluate has passed out of the column. Turn off the vacuum.
21. Remove the top plate assembly, and remove the sample tubes containing the eluate. Dry the eluate with a gentle stream of nitrogen in a water bath set at 37 to 45°C (in a fume hood).

    Remove the tubes as soon as they are dry, and reconstitute them with 200 µl of ice-cold absolute ethanol. Vortex-mix gently, cap, and keep on ice until assayed. Allow all samples to sit on ice for at least 30 minutes, but for no longer than one hour.

### Column regeneration

It is possible to reuse the C-18-OH columns after a washing and regeneration step has been performed. It is possible to use each column a total of five times.

The regeneration procedure is as follows:

1. Add 5 ml of isopropanol to each column reservoir.
2. Turn on the vacuum and allow the solvent to pass completely through the column.
3. Add 5 ml of hexane to each column reservoir.
4. Repeat step 2.
5. Add 5 ml of isopropanol to each column reservoir.
6. Repeat step 2.
7. Add 5 ml methanol to each column reservoir.
8. Repeat step 2.
9. Add 5 ml of distilled deionized water to each column reservoir.
10. Repeat step 2.

The columns are now ready to be re-used.

### 1,25-Dihydroxyvitamin D radioreceptor assay

1. Set up labeled 12 × 75 mm borosilicate glass tubes, in duplicate, for the standard curve, consisting of NSB, $B_0$, and 7 concentrations of standard, unknowns, and controls.
2. Keep all reagents on crushed ice and set up the assay in an ice/water bath.
3. Add 40 µl of ethanol to $B_0$ tubes.
4. Add 40 µl of NSB standard to NSB tubes.
5. Add 40 µl of appropriate standard to standard tubes.
6. Add 40 µl of extracted sample or control to appropriate tube.
7. Add 100 µl of extracted sample or control to scintillation vials containing 10 ml of scintillation cocktail for recovery purposes. This represents one-half (100 µl/200 µl) of the reconstituted extract.
8. Add 500 µl of receptor solution to all tubes.
9. Mix well and incubate in a water bath at 25°C for 1 hour.
10. Place assay tubes in an ice-water bath for 5 minutes.
11. Add 10 µl of assay tracer solution to all tubes, as well as to 2 scintillation vials containing 10 ml of scintillation cocktail (total count tubes).
12. Mix well and incubate in a 25°C water bath for 1 hour.
13. Place assay tubes in an ice-water bath for 5 minutes.
14. Add 200 µl of cold DCC suspension to each tube. (DCC should be mixed thoroughly prior to and during addition.)
15. Mix well and incubate on ice for 30 minutes.
16. Centrifuge at 3000 rpm for 15 minutes at 4°C.
17. Carefully pour the supernatant into scintillation vials containing 10 ml of scintillation cocktail. Touch the rim of the tube against the surface of the cocktail to ensure transfer of all of the supernatant. Be careful to

avoid the accidental transfer of any charcoal particles into the scintillation vial.
18. Count each vial for at least 10 minutes in a scintillation counter.

## Calculations

### A. *Calculating % $B/B_0$*

1. Calculate the average CPM for each standard, control, and unknown.
2. Subtract the average CPM of the NSB tubes from all of the counts.
3. Divide the corrected CPM of each standard, control, or unknown by the corrected CPM of the $B_0$ tubes and multiply by 100.

$$\% \, B/B_0 = \frac{(\text{CPM of standard or unknown} - \text{CPM of NSB}) \times 100}{\text{CPM of } B_0 \text{ tube} - \text{CPM of NSB}}$$

### B. *Standard curve plot and unknown calculation*

1. Using 3-cycle semi-log paper, plot percent $B/B_0$ for the standards (vertical axis) against the standard concentration (horizontal axis).
2. Draw the best fit sigmoidal line through the points (Figure 1).
3. Read the unknown concentration from the graph.
4. Divide the concentration from (3) by the recovery (see below).
5. Multiply the result from (4) by five (5). This multiplication is necessary because one-fifth of the sample extract (40/200 μl) was taken for assay.
6. The final result is expressed in pg/ml. To convert to pmol/l, multiply pg/ml by 2.4.

### *Sample calculation*

A sample has 5.4 pg in the 40 μl aliquot assayed (from the standard curve). The recovery is 71%. What is the final concentration?

$$\frac{5.4 \times 5}{0.71} = 38 \text{ pg/ml}$$

To calculate recovery:
Count the recovery total count vials and the sample recovery vials for 10 minutes.

$$\text{Recovery} = \frac{\text{Sample recovery CPM} \times 2}{\text{Total count recovery CPM}}$$

Example:
Total count recovery = 800 CPM
Sample recovery = 284 CPM

$$\text{Recovery} = \frac{284 \times 2}{800} = 0.71$$

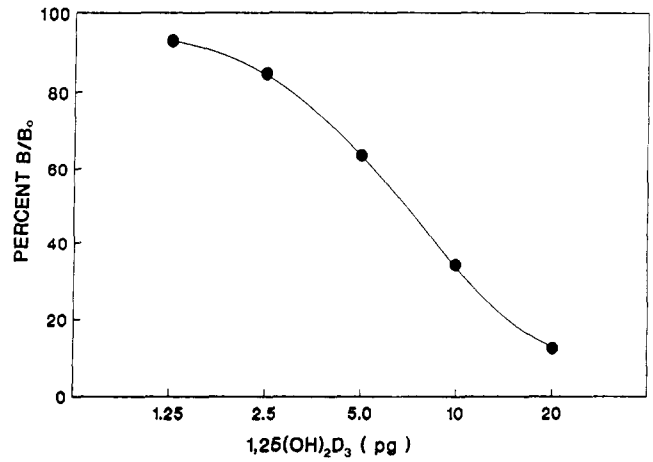


**Figure 1** Standard curve for the $1,25(OH)_2D$ assay. Each point represents the mean of two determinations.

## Discussion

The recognition that vitamin D requires activation by two successive hydroxylation steps to form $1,25(OH)_2D$ before its biologic effects can be realized prompted many laboratories to develop specific assay methods for $1,25(OH)_2D$.[8] These methods include bioassay,[9] isotope dilution-mass fragmentography technique,[10] radioimmunoassay,[4,11,12] and radioreceptor assay.[2,3,5,13-15]

To date, only one bioassay technique to measure $1,25(OH)_2D$ has been reported.[9] This assay is based on the ability of $1,25(OH)_2D$ to cause the release of $^{45}$calcium from fetal rat bone to the culture media. Disadvantages of this method include the need for tissue culture facilities, advanced tissue culture techniques, variability of results due to intrinsic biological variation between animals, and the fact that this technique is very time-consuming.

Isotope dilution-mass fragmentography[10] has received little attention because of its complexity and the necessity of expensive equipment. In addition, this technique is specific for $1,25(OH)_2D_3$ and would therefore underestimate the total circulating concentration of $1,25(OH)_2D$ which includes $1,25(OH)_2D_2$ derived from dietary sources.

Both radioimmunoassay[4,11,12] and radioreceptor assay[2,3,5,13-15] utilize the same principle; two isotopic forms of the same ligand, one radioactive and the other non-radioactive, compete for binding sites on either antibodies or receptor protein. Interest in the development of radioimmunoassays stems from the desire to eliminate cumbersome procedures such as column chromatography, by generating antibodies which would recognize both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ with equal affinity and specificity. Unfortunately, none of the antibody preparations available to date are able to accomplish this goal. For this reason, radioimmunoassay is not used widely at the present time. However, it remains a potentially useful method for $1,25(OH)_2D$ measurement.

Since the first successful demonstration of a radioreceptor assay for $1,25(OH)_2D$ in 1974,[13] most efforts have been directed toward improving assay sensitivity and simplification of the procedures involved.[8] The utilization of thymus receptor represents one of the most significant areas of progress. In contrast to chick intestinal receptor, the thymus receptor has very little affinity for other dihydroxyvitamin D metabolites and is relatively insensitive to other lipid interference.[3,5] These characteristics are primarily responsible for the elimination of the HPLC purification step prior to the binding assay. In addition, thymus receptor recognizes $1,25(OH)_2D_2$ as well as $1,25(OH)_2D_3$, unlike the chicken intestinal receptor which recognizes $1,25(OH)_2D_3$ much better than $1,25(OH)_2D_2$. Further improvement in assay sensitivity is accomplished by using a non-equilibrium incubation technique[16] and tritiated $1,25(OH)_2D_3$ of higher specific activity.[17] The intra- and interassay coefficients of variation for this assay are approximately 5 to 10% and 10 to 15%, respectively. Although the ammonium sulfate precipitated thymus receptor is stable for up to 1 year when stored at $-70°C$ to $-140°C$, the addition of dithiothreitol to the assay buffer greatly enhances the maximum binding of the ligand to the receptor, thereby enhancing the sensitivity and accuracy of the assay. For reasons of cost containment, we suggest that the C-18-OH columns can be regenerated and reused repeatedly. Care must be taken to assure complete washing of the columns to avoid any cross-contamination of samples, especially important when picogram levels of $1,25(OH)_2D$ are being measured.

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