A method for the determination of the circulating concentration of vitamin D

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Overview

The vitamin D assay has been of value in determining intestinal malabsorption of vitamin D and has proven useful in the study of the photobiology of vitamin D.[1-3] The strategy for determining the circulating concentrations of vitamin D (vitamin D_2 and/or D_3) is a lipid extraction of serum or plasma followed by the separation of vitamins D_2 and D_3 from lipid contaminants and vitamin D metabolites by a rapid reverse-phase cartridge chromatography. The fraction that contains both vitamins D_2 and D_3 is then chromatographed by normal-phase high-performance liquid chromatography (HPLC) which separates UV-absorbing lipid contaminants from the UV-absorbing vitamins D_2 and D_3, which is then quantitatively determined by integration of its UV absorbance. In this paper, we provide the experimental protocol used in our laboratory for the determination of circulating concentrations of vitamin D. In subsequent papers in this journal, we describe the assays for 25-hydroxyvitamin D[4] and 1,25-dihydroxyvitamin D.[5]

Reagents

[1,2(n)-^{3}H] Vitamin D_3, 10 to 20 Ci/mmol, Amersham TRK.346 or New England Nuclear NET-180, stored at −20°C.
Vitamin D_3, (Duphar) HPLC purified.
16 × 125 mm Borosilicate glass tubes with teflon-lined screw caps.
20 × 125 mm Borosilicate glass tubes with teflon-lined screw caps.
13 × 100 mm Borosilicate glass tubes.
20-ml Glass scintillation vials.
Glass Pasteur pipets.
Pipetman (P-1000, P-200, and P-20; Gilson).
Disposable pipet tips.
Sep-pak rack, Waters Associates.
Bond-Elut C-18 cartridges, Analytichem International.
Absolute ethanol.
HPLC grade solvents: acetonitrile, ethyl acetate, hexane, methanol, and isopropanol.

Supported in part by grants M01-RR00533, R37-AG06079, and R01-AR36963 from the National Institutes of Health.
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Scintillation cocktail.
Vortex mixer.
Dubnoff shaker or equivalent.
RT-6000C Sorvall centrifuge or equivalent.
Nitrogen gas.
N-evap evaporator, Organomation Associates, Northborough, MA, USA.
Scintillation counter.
HPLC system, with Zorbax 5-μm silica column, 4.6 × 250 mm, or equivalent.

Procedure

Extraction

1. Pipet 1.0 ml of control, patient serum, or plasma into 16 × 125 mm glass tubes with screw cap.
2. Add 100 μl (approximately 5,000 cpm) of ^{3}H-vitamin D_3 to all tubes. With the same pipet, add 100 μl of the tracer to scintillation vials in duplicate. Add 10 ml of scintillation cocktail to each vial and set aside (this represents the total counts for recovery calculations).
3. Add 100 μl (25 ng) of vitamin D_3 standard to one of the control tubes.
4. Add 100 μl of ethanol to all of the remaining tubes.
5. Vortex-mix all of the tubes, and allow to equilibrate at room temperature for 30 minutes.
6. Add 6 ml of ethyl acetate to each tube.
7. Vortex-mix each tube for 15 seconds.
8. Place tubes at a 45-degree angle on a shaker and shake at medium speed for 30 minutes at room temperature.
9. Centrifuge at 3,000 rpm for 15 minutes at 4°C.
10. Transfer the supernatant to a clean 20 × 125 mm glass tube with screw cap. Be sure not to aspirate the emulsion that forms between the aqueous and organic phases.
11. Add 6 ml of ethyl acetate to each tube containing the aqueous phase (original tubes).
12. Vortex-mix each tube for 15 seconds.
13. Place at a 45-degree angle on a horizontal shaker and shake at medium speed for 30 minutes.
14. Centrifuge at 3,000 rpm for 15 minutes at 4°C.
15. Combine the organic phase of the second extract with that of the first extract.
16. Dry under a stream of nitrogen at 37 to 45°C.

C-18 Solid phase chromatography

1. When samples are dry, add 1 ml of methanol to each tube, vortex-mix, and allow to stand for 30 minutes.
2. Set up the C-18 cartridges on the Sep-pak rack.
3. Condition the cartridges by running the following solvents through in order:
   a. 5 ml hexane,
   b. 5 ml methanol, and
   c. 5 ml distilled, deionized water (ddH_2O).
4. Add 1 ml of ddH_2O to each sample, vortex-mix, and transfer to the appropriate cartridge.
5. Elute to waste.
6. Add 5 ml of ddH_2O to each cartridge and elute to waste.
7. Add 10 ml of 70% methanol in ddH_2O to each cartridge and elute to waste.
8. Add 5ml of acetonitrile to each cartridge and elute to waste.

9. Add 5 ml of methanol to each cartridge and collect eluant in appropriately labeled 13 × 100 mm glass tubes.
10. Dry eluant under a stream of nitrogen and store overnight in 1 ml of isopropanol at 4°C.
11. The cartridges can be regenerated for repeated usages by eluting the following solvents through the cartridges in order:
    a. 5 ml methanol,
    b. 5 ml hexane,
    c. 5 ml methanol, and
    d. 5 ml ddH_2O.
12. Go to step 4.

High-performance liquid chromatography

1. Prepare HPLC solvent: hexane:isopropanol (99:1). Make approximately 1 liter for each set of eight samples to be analyzed.
2. Prepare standards. Dissolve approximately 0.1 mg of crystalline vitamin D_3 in 5 ml of absolute ethanol and determine the concentration according to the following formula:

$$Concentration\ (\mu g/ml) = \frac{OD_{265nm} \times 384(\mu g \cdot \mu mol^{-1})}{18.2(ml \cdot \mu mol^{-1} \cdot cm^{-1}) \times 1\ cm\ light\ path}$$

An aliquot is taken and diluted with absolute ethanol such that a concentration of 250 ng/ml is obtained. This solution is the working standard.

Pipet 10, 20, 40, 80, and 160 µl of working standard (equivalent to 2.5, 5, 10, 20, and 40 ng of vitamin D_3) into five separate, labeled 12 × 75 mm glass tubes and dry under N_2 gas.

Both the stock and working standard solutions should be stored under N_2 gas, in the dark, and at − 20°C (or lower).

3. Add 100 µl of HPLC solvent to each standard tube, vortex-mix, and cap. Keep refrigerated in the dark if not used within 2 hours.
4. When the column has been equilibrated with solvent, run the standard curve (*Figure 1*) by injecting each standard at 2-minute intervals.
5. Dry each unknown sample under N_2 during the run just prior to its injection. Add 100 µl of HPLC solvent, vortex-mix, and cap.
6. Collect the vitamin D peak in a 13 × 100 mm glass tube.
7. Dry the collected fraction under N_2, redissolve in 1.0 ml of ethanol, vortex-mix, and allow to stand for at least 30 minutes.

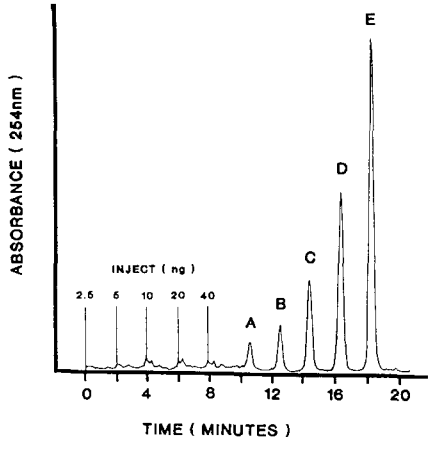

Figure 1 Chromatography of increasing concentrations of standard vitamin D_3 by HPLC on a Zorbax-silica column. Points A, B, C, D, and E represent absorbance peaks after the injection of 2.5, 5, 10, 20, and 40 ng of standard vitamin D_3.

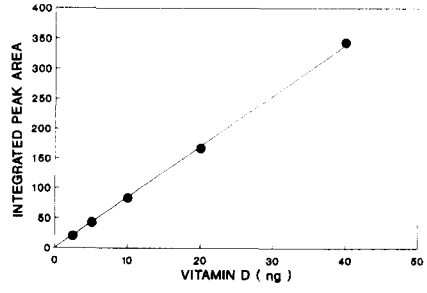

Figure 2 Calibration curve for the quantitation of vitamin D_3 obtained by HPLC of known amounts of each standard. The peak areas were integrated and are plotted against the amount of standard injected.

8. Transfer 100 μl of the reconstituted fraction to a scintillation vial containing 10 ml of scintillation cocktail. Count each vial, along with the recovery total count vials, for at least 10 minutes.

$$\%\ recovery = \frac{cpm\ in\ 100\ \mu l \times 10}{total\ counts\ for\ recovery} \times 100$$

9. Draw the standard curve obtained from running standards (*Figure 2*) and calculate the concentration of vitamin D in the unknowns as follows:

$$concentration\ (ng/ml) = \frac{ng\ (from\ standard\ curve)}{\%\ recovery \times serum\ volume\ (ml)}$$

10. To convert to mmol/l, multiply ng/ml by 2.6.

Discussion

It is well recognized that vitamin D is a hormone, not a vitamin.[6] There are two major forms of vitamin D in nature: vitamin D_2 in fungi and plants and vitamin D_3 in animals.[7] In animals, 7-dehydrocholesterol (provitamin D_3) is transformed to previtamin D_3 on the exposure of skin to sunlight. Previtamin D_3, a thermally unstable molecule, then undergoes a temperature-dependent internal isomerization to form vitamin D_3. Similarly, vitamin D_2 can be synthesized from ergosterol (provitamin D_2) by ultraviolet irradiation followed by thermal isomerization. The only structural difference between vitamins D_2 and D_3 is in their respective side-chains; the side-chain in vitamin D_2 contains a double bond between C_{22} and C_{23} and a methyl group at C_{24}.[6] Usually, casual exposure to sunlight is adequate to provide the body with its vitamin D requirement. However, when the skin is for any reason unable to provide sufficient quantities of this prohormone, dietary supplementation with either vitamin D_2 or vitamin D_3 is required.[6] Vitamin D (D_2 or D_3) is biologically inactive and must undergo two successive hydroxylation steps before its full biologic effects can be demonstrated. First, vitamin D is hydroxylated in the liver to produce 25-hydroxyvitamin D (25-OH-D), which is subsequently metabolized in the kidney to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the active form of vitamin D. Although vitamins D_2 and D_3 show comparable biologic activity in rats and humans, it is well recognized that vitamin D_2 is discriminated against in chickens and New World monkeys.[7]

The procedure described here offers a reasonably fast and reproducible method for the quantitation of vitamin D in serum or plasma. The intra- and inter-assay coefficients of variation are approximately 5% to 10% and 10% to 15%, respectively. Prepurification of the lipid extract using a Bond-Elut C-18 cartridge is an important step in removing contaminants prior to vitamin D analysis by normal-phase HPLC. A second HPLC system using reverse-phase chromatography on Zorbax-ODS can be used to separate and quantitatively determine the circulating concentrations of vitamins D_2 and D_3.[8] The analysis of vitamin D by HPLC with UV quantitation eliminates the inherent variability due to interfering compounds associated with competitive binding assays.[9]

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