24,25-Dihydroxyvitamin D₃ in Serum: Sample Purification with Sep-Pak C-18 Cartridges and Liquid Chromatography before Protein-Binding Assay

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We describe a precise, specific method for measuring 24,25-dihydroxyvitamin D₃ in human serum. A 2-mL serum sample is extracted with acetonitrile and passed through a Sep-Pak C-18 cartridge. The sample is further purified by "high-performance" liquid chromatography under isocratic conditions on a normal-phase column (Radial-Pak silica-gel cartridge), then subjected to a protein-binding assay. The mean concentration of 24,25-dihydroxyvitamin D₃ in serum from 22 normal adults (measured during the spring) was 2.9 μg/L (SD 1.9, range 6.3–0.42 μg/L). The intra-assay CV was 7.7%, the interassay CV 11.2%. Purification of the sample with Sep-Pak C-18 and liquid chromatography on normal plus reversed-phase columns leads to a mean value of 3.4 μg/L (SD 1.6 μg/L, n = 12), not significantly different from results with our method.

Additional Keyphrases: chromatography, reversed-phase • sample pretreatment • cholecalciferol • reference interval

Data on the concentration of 24,25-dihydroxy D₃ (DHD), a metabolite of vitamin D, in human serum are useful in several disorders—such as nephro lithiasis, chronic renal failure, and hyperparathyroidism (1, 2)—that are associated with mineral metabolism.

Several procedures described earlier for determination of DHD in serum are too laborious and time consuming. Here we report a relatively fast and reliable procedure for its determination. We measured the concentrations of DHD by a protein-binding assay after purifying the serum sample with Sep-Pak C-18 and "high-performance" liquid chromatography.

Materials and Methods

Apparatus. We used a chromatography system that included a Model 6000 A pump, U6K Injector, RCM-100 radial compression system, Model 440 ultraviolet fixed-wavelength (254 nm) detector, and M-730 data module, all from Waters Associates, Milford, MA 01757, as were the silica-gel (10-μm particle diameter) and C-18 Radial-Pak cartridges.

For scintillation counting we used a Model Mark III 6880 scintillation system (Tracor, Des Plaines, IL 60018).

A Unicam SP-1800 spectrophotometer (Pye-Unicam, Cambridge CI1 ZFX, U.K.) was used to measure concentrations of DHD in solution, which were calculated with use of the molar absorptivity value of 18 300 L·mol⁻¹·cm⁻¹ at 264 nm.

Solvents. All solvents were of analytical grade, as supplied by Merck, Darmstadt, F.R.G., and F.E.R.O.S.A., Barcelona, Spain. They were distilled or filtered through a Millipore filter, nominal pore size 0.5 μm (Millipore Corp., Bedford, MA 01730) before use. Vitamin D metabolites 25-hydroxy[28(27)-methyl-3H]cholecalciferol (3H-25OHD₃) and 24R, 25-dihydroxy[23,24(n)-3H] cholecalciferol [3H-24,25( OH)₂ D₃] were from Amersham International, Amersham, U.K. Unlabeled vitamin D metabolites were kindly supplied by The Upjohn Co., Kalamazoo, MI (25-(OH)D₃), and by Dr. W. Meier, Hoffmann-La Roche, Basel, Switzerland [25B,25(OH)₂ D₃ and 1a,25(OH)₂ D₃].

Cartridges of Sep-Pak C-18. (Waters Associates) were used to purify the samples before chromatography.

Serum samples. Serum samples were obtained from 22 apparently healthy adults during the spring and stored at −20 °C until assay (no longer than 15 days).

Extraction. Add about 2500 dpn (7·10⁻⁸ μg) of [3H]-DHD in 25 μL of methanol to 2 mL of human serum. Add 2 mL of acetonitrile, mix, and let stand for 30 min at 4 °C. Centrifuge at 1500 × g for 15 min at 4 °C. The supernate will contain the DHD and other vitamin D metabolites.

Pretreatment with Sep-Pak C-18: Inject the supernate from the acetonitrile extraction into a Sep-Pak C-18 cartridge. Discard the eluate. Wash the cartridge with 3 mL of methanol/water (50/50 by vol), and again discard the eluate, which contains lipids. Then elute the vitamin D metabolites with 3 mL of acetonitrile.

Chromatographic purification: For normal-phase liquid chromatography, evaporate, under nitrogen, the extracts eluted from Sep-Pak C-18, then dissolve each in 0.5 mL of hexane/ethanol (95/5 by vol). For further purification inject an aliquot of this solution into the chromatograph with a silica-gel cartridge. Elute the sample with hexane/ethanol (95/5 by vol) at a constant flow rate of 1.5 mL/min. Collect the DHD-containing fraction, as determined by retention times, and evaporate it under nitrogen.

For reversed-phase chromatography, reconstitute the dried extract from the normal-phase purification with 0.5 mL of methanol/water (85/15 by vol) and inject an aliquot of this solution into the chromatograph with a C-18 cartridge. Elute the DHD with methanol/water (85/15 by vol) at a constant flow rate of 1.5 mL/min. Collect the DHD-containing fraction and evaporate it under nitrogen. Before each chromatographic purification, check the retention time of DHD on the normal and reversed phases, to account for slight variations due to differences in solvent batch preparation and cartridges.

Quantification of DHD by protein-binding assay. In 500 μL of methanol reconstitute the dried extract from either the chromatographic normal phase or normal and reversed phase. Save a 200-μL aliquot to use in assessing the analytical recovery in a scintillation counter. After appropriately diluting the rest of the sample, quantify the vitamin metabolite by a competitive protein-binding assay. We used the method of Morris and Peacock (3), with slight modifications. Calculate the concentration of DHD in serum, taking in account the analytical recovery through the extraction and the purification procedure.

Results

Table 1 compares values for DHD in normal human serum obtained by the present method with values obtained.
through a dual liquid-chromatographic purification on normal and reversed-phase cartridges. There is no significant difference by Student's *t*-test between results by the two methods.

We evaluated the correlation between DHD values for normal human serum by protein-binding assay after one chromatographic purification (normal phase) and after dual purification (normal and reversed phase). Linear regression analysis gave the relationship $y = 1.076x - 0.2335$ ($r = 0.9206, p < 0.001, n = 14$).

The precision of our method, assessed by determining the intra-assay and interassay variation (CV) for six aliquots of the same serum pool from normal subjects, was 7.7% and 11.2%, respectively.

Analytical recovery of [$^3$H]DHD added to 2 mL of serum and taken through the extraction and one-step purification procedure was 45% (SD 4%, n = 22).

To assess overall analytical recovery, we added 7.5 μg of DHD per liter to five serum samples for which the DHD concentration was known: 4.2 ± 0.3 μg/L. We could account for 96.4% (SD 5.9%) of the added analyte.

**Discussion**

The values we found with this method agree with values reported previously (4–8). Moreover, the recoveries and the intra- and interassay variations are within the range found by other workers (7, 8).

Our sample extraction with acetonitrile and purification with Sep-Pak C-18 are based on previous work by our group (9) and a personal communication from S. Adam (Abstracts, XVI European Symposium on Calcified Tissues, Knokke, Belgium, 1981).

This approach is more economical, easier, and less time-consuming than the use of other extraction solvents and the widely used Sephadex LH20 (6–10). Twelve serum samples can be extracted with acetonitrile and purified through Sep-Pak C-18 cartridges in about 2 h by one person, and then are ready to be injected in the chromatograph.

A good separation of DHD from other vitamin D metabolites is critical, to avoid the cross reaction of these metabolites with the binding protein in the assay. Accuracy was not improved when we used a dual (normal plus reversed phase) chromatographic purification instead of only the normal phase. This finding, not in agreement with previous reports (11, 12), can be related to the before-chromatography purification with Sep-Pak C-18 that we used in our procedure.

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**References**