Improved Method for Quantifying Vitamin D in Proprietary Infants' Formulas and in Breast Milk

L. Ballester, E. Cortes, M. Moys, and M. J. Campello

In this new method for quantifying vitamin D in infants' formulas and breast milk, after repeated lipid extraction, samples are further purified by passage through Sep-Pak cartridges, followed by liquid chromatography, then quantified by competitive protein-binding assay. Analytical recovery is estimated by use of added $^{3}$H-labeled vitamin D. For repeated assays of a reconstituted proprietary powdered milk formula in two runs, the intra-assay vitamin D values were 9.0 (SD 1.4) (n = 6) and 8.9 (SD 1.6) μg/L (n = 7) (t = 0.07). Assay of a proprietary liquid formula yielded values of 14.9 (SD 0.9) μg/L (n = 6). For each, the results agreed with the vitamin D content shown on the label. Vitamin D concentrations in breast milk from two groups of lactating mothers of different social class and nutritional status were 2.3 (SD 1.4) (n = 7) and 2.0 (SD 1.7) μg/L (n = 7). Overall recoveries ranged from 65 to 75%. Only 2 mL of milk is required, which facilitates sample collection, and the assay is less time-consuming than other current methods.

Additional Keyphrases: chromatography, reversed-phase competitive protein-binding assay, nutrition, newborns, osteoporosis

Many newborns, especially premature infants, are at risk to develop rickets because of too low a vitamin D intake (1). At the other end of the age spectrum, vitamin D deficiency may contribute to involutional osteoporosis (2). Thus, estimation of vitamin D and its polar metabolites is important.

Measurements of polar metabolites of vitamin D in plasma are based on competitive protein-binding assay (CPBA) or radioimmunoassay, both of which are highly sensitive and reliable (3, 4). However, the techniques for quantifying vitamin D itself in human breast milk or infants' formulas have several drawbacks and involve tedious procedures (5-7). Nonetheless, estimation of vitamin D is often required. Because the vitamin D content of cows' milk is clearly insufficient to meet an infant's requirements of 400 international units or 10 μg per day (8), infants' formulas have been variously fortified with vitamin D. Moreover, vitamin D in breast milk can be scanty and varies with the mother's nutritional status and her exposure to sunlight (9).

Here we describe a fast, more convenient method to measure vitamin D in breast milk, raw cows' milk, and infants' formulas, to determine whether they can supply recommended intakes, especially during the first postnatal months.

Materials and Methods

Materials

Vitamin D metabolites: We obtained 25-hydroxy-[26,27-methyl-$^{3}$H]cholecalciferol (specific activity 20.6 kCi/mol) and $^{3}$H-labeled vitamin D$_{3}$ (specific activity 18.3 kCi/mol) from Amersham International, Amersham, Bucks, U.K. Liquid scintillation cocktail ("Ready-Solv EP") was from Beckman, Brea, CA. Unlabeled 25-hydroxy-cholecalciferol was kindly supplied by the Upjohn Co., Kalamazoo, MI, and by Hoffmann-La Roche, Basel, Switzerland. Cholecalciferol (vitamin D$_{3}$) was from E. Merck, Darmstadt, F.R.G.

Solvents: All solvents were "Merck analytical" or "HPLC" grade. They were filtered through a FH 0.5-μm (pore size) filter supplied by Millipore Corp., Bedford, MA, and were sonicated before use.

Apparatus: For chromatography we used a system from Waters Associates, Milford, MA, consisting of a Model 590 pump, a UK injector, a 30 cm × 3.9 mm reversed-phase μ-Bondapak C18 column, a Lambda-max SE 120 ultraviolet detector (254 nm), and a data module. We measured radioactivity with a beta counter (Model LS 2800, Beckman).

Samples: We assayed two marketed brands of formula that are fortified with vitamin D according to the recommendations of the European Society for Pediatric Gastroenterology and Nutrition (10). One (formula A) is supplied in powdered form. Formula B is liquid and bottled for direct use. Breast milk was sampled as described by Picciano (11) from mothers who had delivered term babies (Perinatal Unit, Hospital Provincial of Alicante). These women differed in social class, nutritional status, nutritional supplements during pregnancy, and probably in their exposure to sunlight.

To compare the described method, in five instances we also assayed the same breast milk, obtained during late winter and pooled from different mothers, by the Hollis method (12).

Methods

To quantify vitamin D in any liquid (formula or milk), we followed four steps:

(a) Extraction, according to the method of Nabholz and Herforth (13), slightly modified by adding 4000 dpm (50 μL) of $^{3}$H-labeled vitamin D$_{3}$ to 2 mL of milk, to assess analytical recovery for the whole procedure. We deproteinized the 2-mL samples with 0.5 mL of concentrated NH$_{4}$OH and 2.5 mL of absolute ethanol.

To extract all lipids from the samples, we washed the deproteinized samples four times, alternately with 5 mL of diethyl ether and 5 mL of petroleum ether (bp 40–60 °C), then dried them in a rotary evaporator system. Next, the fat was saponified by treatment with 0.5 mL solution of 0.1% KOH and 1 mL of absolute ethanol at 50 °C for 15 min. To avoid oxidation, we included resorcinol and maintained a continuous flow of nitrogen. The unsaponified fraction was extracted five times with 5-mL portions of petroleum ether, then washed several times with distilled water to eliminate soap and residual KOH.

(b) Purification on Sep-Pak silica cartridges (Waters Associates) was performed according to Adams et al. (14), with some modifications. The mobile phase was ethyl acetate/n-hexane (7/93 by vol). After conditioning the cartridge with 4 mL of the mobile phase, we applied samples consisting of the
extracted fraction, redissolved in 200 μL of the same phase, followed by 400 μL used to rinse the tube that had contained the extract. We then applied 18 mL of the mobile phase to the cartridge. The first 6 mL of effluent was discarded. The next 12 mL, which contained the vitamin D, was collected and dried under a stream of nitrogen.

(c) The previously dried sample, redissolved in 200 μL of water, was purified by means of a μ-Bondapak column, a mobile phase of methanol/water (90:10 by vol), and a flow rate of 1.3 mL/min. Absorbance was monitored at 254 nm (sensitivity 0.005 A full-scale). The retention time for vitamin D, both labeled and unlabeled, was 21 min under these conditions. The fraction containing vitamin D (that emerging from the column between 19.5 and 22.5 min) was collected and dried under a stream of nitrogen.

(d) For the CPBA we redissolved the dried chromatographic fraction in 500 μL of methanol and assayed two 100-μL aliquots and two 50-μL aliquots by CPBA. The remaining 200 μL was used in calculating the analytical recovery of vitamin D. The CPBA we used is based on a constant time reaction not reaching equilibrium (15). We placed 100 μL of standard or sample in each test tube, except those for total counts, nonspecific binding, and Bn, to which we added 100 μL of methanol each. We then added 500 μL of a binding-protein solution: serum from osteomalacic humans diluted 10 000-fold in sodium barbital buffer (50 mmol/L, pH 8.4).

All samples were stirred, then incubated at 4 °C for 48 h, after which we added 400 μL of 3H-labeled 25-OHD3 (7 × 10−8 Ci/L). After stirring, we incubated the samples plus label at 4 °C for 2.5 h, then added 200 μL of a mixture of activated charcoal and dextran T70 (2 g and 0.2 g, respectively, in 100 mL of the sodium barbital buffer), stirred briskly, and re-incubated the mixture at 4 °C for 15 min. Finally, after we centrifuged at 1300 × g, we placed 900 μL of each supernate in vials containing 5 mL of scintillation liquid, and counted their radioactivity. Concentrations used for standard curve ranged from 0.005 to 7.4 μg/L.

Results

Assay Evaluation

Extraction. The extraction method, used with six samples, yielded a vitamin D recovery of 96% (SD 2%). The tritiated vitamin D3, added before extraction, was used as internal marker throughout the whole procedure.

Elution by silica cartridges. The elution profiles obtained by use of the internal marker were in all instances similar (Figure 1). In this figure it is evident that the first 6 mL of eluate can be discarded, and the following 12 mL should be collected; this was similar for all six. About 90% of the vitamin D was present in this 12-mL fraction (Figure 1).

Chromatographic purification. We tested different proportions of methanol and water in the mobile phase, ranging from 85/15 to 95/5 by vol. Elution flow rates were varied from 0.8 to 1.5 mL/min. The 90/10 ratio of methanol/water and the 1.3 mL/min flow rate were finally selected. In chromatograms for milk formulas the vitamin D peak was not resolved, whereas in some samples of breast milk the peak appeared more clearly. Still, liquid chromatography alone would not suffice to quantify vitamin D in milk. Figure 2 shows typical chromatograms of a formula (part A) and of a pure standard of vitamin D (part B). Clearly, the retention time of 3H-labeled vitamin D is identical regardless of whether it has been added to a formula submitted to the extracting procedure or to a standard that is assayed directly. When the absorbance at 254 nm is used to monitor the effluent, the labeled and unlabeled vitamin D of the standard are seen to have the same retention time, but this is not the case for the formulas, in which the peak of beta-radiation corresponds to no particular chromatographic peak.

CPBA. We tried different simultaneous incubation times (1.5–3 h) for 3H-labeled vitamin D, samples, and binding protein. The longer the incubation time, the better was the binding to protein. Nevertheless, we discarded this type of incubation, because the variation in the binding values, even between the extreme points of the curve, was negligible. Therefore, we tested a non-simultaneous binding. The union between 25-OHD (from samples or standards) and protein was complete by 48 h. After that, we added 3H-labeled vitamin D and incubated for 2.5 h. Under these conditions, when the bound protein was titrated, we obtained for a 10 000-fold dilution a 50% binding. On the other
radiation received by the mother (9, 12); and third, resolution of these two compounds would require another chromatographic run, prolonging the procedure (5, 6, 12).

In our procedure, these fatty acids are precipitated as soaps during aqueous extraction.

Use of liquid chromatography alone to quantify vitamin D is not advisable, for the following reasons: proprietary formulas show well-defined peaks, with retention times similar to those of the pure standards (Figure 2) but the concentrations measured were 10-fold those by cPBA (and those printed on the label). These peaks thus include other substances co-migrating with vitamin D. In chromatograms of breast milk, concentrations measured from the peak area were more in accord with those expected. Nevertheless, in many instances (samples from nursing mothers in winter and springtime) vitamin D concentrations approach the sensitivity of the method and thus, for the same reasons that applied to proprietary formulas, chromatography as a single method was never used.

The vitamin D concentrations we found in breast milk agree with results from some others (19) but are higher than those reported by Hollis (12) and Reeve et al. (16). However, our value coincides well with that of Hollis' subgroup after receiving ultraviolet radiation (12). The relatively high exposure to ultraviolet radiation from sunshine on the Mediterranean coast could be the reason for the slightly higher values we found in this group of nursing mothers, particularly if we take into account that the breast milk was collected in late autumn. Some of the mothers in our group show very high amounts of vitamin D in breast milk, usually those in a higher social class, who take close care during pregnancy. The intake of 400 int. units daily, as has been pointed out (20), plus the sunshine could be responsible for such high amounts of vitamin D in breast milk (9). When we assayed a pooled specimen of breast milk collected during early spring, to compare this method with others, we found very similar vitamin D concentrations by both assays, but the values were significantly lower than those obtained for mothers during summer or autumn. Thus one could speculate that, counting only on ultraviolet radiation, vitamin D repletion in this population could be very irregular.

The reliability of the method was assessed by the intra-assay measurements. The percentage of error ranged between 6 and 16% for a level of 95%. The inter-assay variation led to similar means, with no significant differences.

References


