Note

Rapid determination of vitamin D in fortified skim milk*

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Most of the skim milk for retail sale in the United States is fortified with vitamins A and D. Because these vitamins are toxic when consumed in excess amounts, careful control of the fortification procedure must be assured. Thus, determination of vitamins A and D in milk products should be a part of a routine quality-control program.

It is clear that cow's milk is not a good source of vitamin D[1-4]. For this reason, fortification of milk is common. The antirachitic compounds present in unfortified milk include vitamin D, 25-OH-D, and 1,25-OH$_2$-D. The report of a water-soluble vitamin D sulfate in milk[5] has not been substantiated[1,6]. Fortified milk may contain, in addition, either vitamins D$_2$ or D$_3$ and perhaps their pre-vitamins, formed after pasteurization or other heating steps.

Estimates of adult human daily requirements of vitamin D range from 5 to 10 μg[7] and the RDA is 10 μg[8]. Vitamin D is toxic when as little as 50–120 μg is taken per day[9]. There are reports of commercial milks having vitamin D levels well above the label claims[10,11] and that both vitamins D$_2$ and D$_3$ have been added while only one was declared on the label[12].

Methods of vitamin D determination have been reviewed by Parrish[13]. Since that time, many reports have been published of vitamin D analysis in dairy products. Most recent reports of vitamin D determinations in milk products require extensive time-consuming cleanup procedures before high-performance liquid chromatography (HPLC) with UV absorbance detection. Cleanup procedures include enzyme hydrolysis[14], room-temperature[10,15-21] and hot alkali saponification[12,22-27]. Sample hydrolysis was not employed by some workers[11,28]. Other cleanup steps include digitonin precipitation of sterols[18,27], silica[3,16,28] or C$_{18}$[25] cartridges, alumina columns[18,21,27], and semi-preparative HPLC using silica[3,10,19,27], nitrile[22], amino[11], amino–cyano[26,28], gel filtration[11], or C$_{18}$ (ref. 4) columns. The choice of columns for the final determination depends upon the separation required. Reversed-phase columns are necessary to resolve vitamins D$_2$ and D$_3$. This resolution allows the use of vitamin D$_2$ as an internal standard for vitamin D$_3$ and *vice versa*. However, cholesterol should be removed before C$_{18}$ HPLC[17]. Normal-phase HPLC clearly separates pre-vitamin and *trans*-vitamin (which have 35 and 5% of the activity of the

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cis isomer, respectively) from the *cis*-vitamin[26,29]. Although the use of the various cleanup procedures assures a good separation of vitamin D from interfering compounds and may improve column life by eliminating materials that may be difficult to remove from the stationary phase, the amount of time needed to determine the vitamin D level is greatly increased. The time needed for analysis is a concern in dairies because pasteurized milk has a short shelf life, and the milk should be held until results are obtained. Time is less of a concern for infant formulas, ultra high temperature (UHT) sterilized milks or dried milk powders for which shelf life is not a problem.

We present a simple method, suitable for routine screening of vitamin D levels in fortified skim milk, that avoids saponification or other cleanup steps and relies on the separation power of HPLC.

EXPERIMENTAL

Milk samples

Commercial fortified milk samles were purchased from local stores. The milk was fortified with vitamin D_3 at a claimed level of 2.5 μg per cup (10 μg per quart).

Lipid extraction

Milk (50 ml) was measured, by using a volumetric flask, into a 500-ml separatory funnel, which contained 30 ml ethanol (95%) and 15 ml ammonium hydroxide (28–30% ammonia). The volumetric was washed with 30 ml water, and this water was added to the funnel. The funnel was shaken. Peroxide-free diethyl ether (50 ml) was added, the funnel shaken, and hexane (50 ml) was added with gentle swirling. The ether–hexane extraction was repeated four times. The combined organic phase was washed with water (50 ml), and dried by using anhydrous sodium sulfate. The solvent was removed by evaporation under vacuum with slight heating (40°C) just until dryness. The lipid was transferred to a test tube by using portions of diethyl ether. The ether was evaporated by using a slight stream of nitrogen, and the residue was transferred to a 2-ml volumetric tube with portions of mobile phase. The extract was then filtered through a 0.45-μm filter into an autosampler vial. This sample was injected (50 μl) onto the HPLC system.

High-performance liquid chromatography

A Model 110B pump, Model 163 variable-wavelength detector and Model 427 integrator were obtained from Beckman (Berkley, CA, U.S.A.). A Model SP8780XR autosampler (Spectra Physics, San Jose, CA, U.S.A.) was employed with a 50-μl loop. A Zorbax silica column (10 μm, 25 cm × 4.6 mm I.D.), obtained from DuPont, was preceded by a Brownlee silica Spheri-10 guard column (3 cm × 4.6 mm I.D.) (Rainin Scientific, Woburn, MA, U.S.A.). The mobile phase consisted of hexane–isopropanol (99:1, v/v) at a flow-rate of 1 ml/min. The detector was set to 265 nm and 0.01 a.u.f.s. The column was cleaned after every twenty samples by using the following procedure. Isopropanol–hexane (1:4, v/v; 50 ml) was followed by hexane (300 ml) and mobile phase (300 ml) at a flow-rate of 3 ml/min. Activation of the silica column was done by using the method of Bredeweg *et al.*[30].

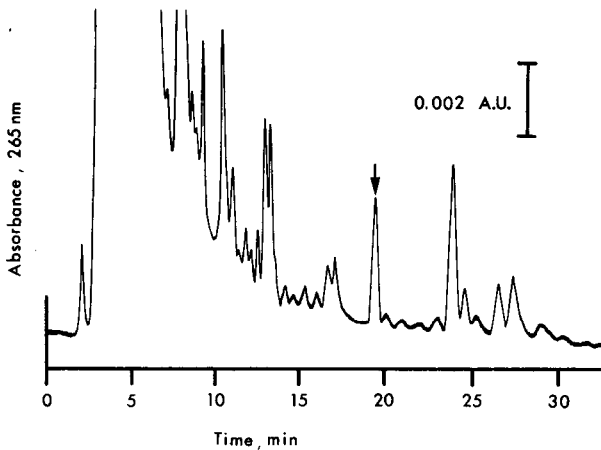Vitamin D_3 standard was purchased from Sigma (St. Louis, MO, U.S.A.). A

Fig. 1. Chromatogram of skim milk, fortified with 10 μg cholecalciferol (vitamin D_3) per quart, from a normal-phase silica column. Operating conditions: mobile phase, hexane–isopropanol (99:1); flow-rate, 1.0 ml/min. Vitamin D_3 peak is indicated by the arrow.

first stock solution (A) was prepared by dissolving 250 mg in 250 ml mobile phase. A second stock solution (B) was made by diluting 10 ml of stock A to 250 ml. The working solution ws prepared by bringing 5 ml of stock B to 500 ml. The working solution had 0.4 ng vitamin D_3 per μl mobile phase. A standard curve was prepared by using 4–20 ng vitamin D_3. Pre-vitamin D_3 was formed by heating a vitamin D_3 standard on a boiling water bath for 20 min.

RESULTS AND DISCUSSION

A typical chromatogram is illustrated in Fig. 1 The vitamin D_3 peak was well separated from other components and eluted at retention time 19.0 ± 0.6 min. Retinol palmitate eluted close to the solvent front and contributed to the massive peak at retention time 4–8 min. The purity of the vitamin D_3 peak was determined by collecting the D area from six 50-μl injections of a lipid extract from fortified milk, reducing the solvent with a stream of nitrogen and collecting a second time from normal phase to obtain the vitamin D peak. The ratio of UV absorbance at 254/280 nm was determined for both the collected vitamin D and an authentic standard. The values for the standard and collected peaks were 1.36–1.42 and 1.36–1.39, respectively. These compare well with the value of 1.35 ± 0.05 reported by Idyk and Woollard[17] but are slightly higher than the numbers reported by Landen[11], which were 1.24–1.31 for D peak and 1.26–1.30 for standard. The discrepancy may be due to differences in detector calibration, wavelength slit width, or solvents employed. The collected vitamin D peak was dried, dissolved in methanol, and chromatographed on a reversed-phase C_{18} column eluted with 100% methanol. The D peak co-eluted with an authentic standard on this column with only minor amounts (< 10% of total area) of other UV absorbing compounds. As further evidence, the collected vitamin D peak was heated in a boiling water bath for 15 min alongside an authentic standard of a similar concentration. The ratio of vitamin D to pre-vitamin (formed during heating) was determined. The D to pre-D ratio for the authentic

TABLE I

RECOVERY OF SPIKED VITAMIN D$_3$

Amount spiked (μg)	Amount found (μg)	Recovery (%)
0	0.750	—
1	1.702	95.3
1	1.723	97.3
2	2.714	98.2
2	3.070	106.4
3	3.554	93.5
3	3.679	98.0
Mean		98.1*

* Standard error of the mean: 1.81%.

standard was 6.4–6.6, compared to 6.3–6.9 for the collected peak. From these studies we are confident that significant interference does not take place under the conditions employed. Thompson et al.[20] were unable to separate vitamin D from interfering compounds in a saponified milk sample by using a 15 cm silica column [mobile phase: hexane–cyclohexane–isopropanol (50:50:.5), flow-rate 1 ml/min]. This is likely because they employed a shorter column (retention time under their conditions was 9.5–10 min whereas it was 18.4–19.6 for our work) and because we examined skimmed milk (the type of milk was not identified by Thompson et al.[20] so it is assumed that it was whole milk).

We have not seen evidence of column deterioration after analysis of almost 200 samples. This is likely because of the presence of a guard column and the cleaning procedure used after every twenty samples. The retention time of vitamin D was not altered appreciably by this cleaning step (for one typical case, before and after cleaning, retention times were 18.4 ± 0.06 and 18.7 ± 0.09 respectively). The low lipid content of skim milk makes the injection of large amounts of lipid unnecessary.

The recovery of vitamin D added to the milk was investigated, and the results are shown in Table I. As can be seen, the recovery was good over the range studied, with an average recovery of 98.1 (standard error of the mean 1.81). The coefficient of variation of four replications of one sample was 2.2% (data not shown).

Vitamins D$_2$ and D$_3$ co-elute on the silica column. However, vitamin D$_2$ was not observed in the reversed-phase chromatography of the collected vitamin D peak. Either vitamin D$_2$ or D$_3$ could be determined by using our method but a mixture of the two would lead to error because of the differences in the molar absorbtivities of the two vitamins. Pre-vitamin D$_3$ eluted around 12–13 min under the conditions described but was not be observed because of interfering compounds. Because of the speed of analysis, skim milk can be analyzed for vitamin D before and after fortification to ensure that significant interference does not take place on the silica column. Some vitamin D$_3$, however, is present in unfortified milk at levels around 0.28–0.334 μg per quart[2] and lower levels than these have been reported (ca. 0.05 μg per quart[3]).

The range of values that we have found in commercial vitamin fortified milks ranged from 14.16 μg per quart (42% overfortification) to one sample that did not seem to be fortified at all. It is unlikely that an overfortificationn of skim milk by ca.

40% would result in chronic toxicitty to the average consumer. However, the forti-fication must be done carefully, and scrupulous attention given to mixing to avoid "hot spots" with exccessive levels of vitamin.

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