Assay for Vitamin D2 and Vitamin D3 in Plasma of Dairy Cows: Changes after Massive Dosing of Vitamin D3

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ABSTRACT

A sensitive, precise assay for vitamin D in plasma is described. Three to five milliliters of plasma were extracted with methanol: methylene chloride (2:1). The lipid extract was chromatographed on Sephadex LH-20 and then on Lipidex-5000 columns. After high pressure liquid chromatography with a reverse phase chromatographic system, vitamin D2 and vitamin D3 were quantitated by ultraviolet absorbance.

We used this assay system for monitoring daily changes of vitamin D3 in plasma of two Jersey cows after four intramuscular doses (15 × 10^6 IU) of vitamin D3 administered at weekly intervals. Basal vitamin D in plasma was 3.2 ± .99 ng/ml with a range of 1.7 to 4.9 ng/ml. Vitamin D3 in plasma remained relatively low (10 to 45 ng/ml) the week after the first vitamin D3 injection. Vitamin D3 was high (130 to 234 ng/ml) after the second, third, and fourth injections. Vitamin D3 decreased steadily to 88 ng/ml by 38 days after the fourth vitamin D3 injection.

Phosphorus in plasma increased sharply to a plateau at 9.5 mg/100 ml during the week after the second vitamin D3 injection and returned to normal (4.5 mg/100 ml) at the end of the experiment. Calcium, however, gradually increased to 14.0 mg/100 ml 20 days after the fourth vitamin D3 injection. Both animals remained hypercalcemic (calcium 11.5 mg/100 ml) during the experiment.

INTRODUCTION

The prohormone, vitamin D3, is an important factor in calcium (Ca) and phosphorus (P) metabolism. Active transport of Ca and P in the intestinal mucosa and Ca and P resorption from bone both depend upon vitamin D3 (4).

Before vitamin D3 becomes active, it first must be converted in the liver to 25-hydroxyvitamin D3 (25-OHD3) which is transported to the kidney and converted to 1,25-dihydroxyvitamin D3 [1,25-(OH)2D3] (4). Although 1,25-(OH)2D3 is the physiologically active form of vitamin D3 and is normally at 25 to 50 pg/ml in the cow, 2 × 10^3 times greater concentrations of precursor 25-OHD3 and 2 × 10^4 times greater concentrations of vitamin D3 (in vitro) will simulate 1,25-(OH)2D3 in resorbing bone (13). Also, 25-OHD3 and vitamin D3 can mimic the binding of 1,25-(OH)2D3 to the intestinal receptor protein when at 10^3 and 10^6 times greater concentrations (5). Therefore, the possibility that vitamin D3 acts directly to stimulate absorption of Ca from intestine or Ca resorption from bone appears likely especially when massive doses of the vitamin are given to prevent milk fever in dairy cows (6, 12) or to treat vitamin D-resistant rickets in children (9).

Suitable physical chemical assays for the evaluation of the vitamin D status of animals have not been described. Our study describes a new assay for vitamin D2 in plasma and evaluates concentrations in plasma of vitamin D3 in two dairy cows given intramuscular injections of 15 × 10^6 IU of vitamin D3 weekly for 4 wk.
MATERIALS AND METHODS

Animals and Diets

Two nonpregnant and nonlactating Jersey cows (4 yr old) were fed a basal diet of alfalfa hay cubes plus 6 kg of grain daily. This diet supplied 50 to 60 g of Ca and 25 to 30 g of P daily.

Lab Plasma Internal Standard

One liter of blood was collected by venipuncture from a Holstein cow. The plasma was harvested and divided into 5-ml aliquots, frozen, and stored at -15 C.

Treatment and Blood Sampling

Each cow was treated with four weekly injections of 15 x 10^6 IU of vitamin D_3 (im in the gluteus medius) in 1.5 ml of ethanol. Blood samples were taken frequently before and after injections. Blood was collected in heparinized (5 to 10 U/ml of blood) syringes, immediately centrifuged at 4 C, and plasma was stored at -15 C.

Apparatus

The chromatograph was a Waters Model ALC/GPC 204 equipped with a Model 6000A solvent pumping system and a U6K injection valve. Optical density was monitored by a Model 440 ultraviolet (UV) fixed wavelength (254 nm) detector and recorded by a Houston Instruments Model B-5000 strip chart recorder. This apparatus was used for all the high pressure liquid chromatography (HPLC).

Quantitation of Vitamin D, Calcium, and Phosphorus

The eluant from the Lipidex 5000 column containing the vitamin D was dried under N_2 and was prepared for HPLC by addition of 50

Sterols

Crystalline vitamins D_2 and D_3 were from Sigma Chemical Company, St. Louis, MO. The [1α,2α-3H] vitamin D_3 (15 Ci/mM) was purchased from New England Nuclear, Boston, MA and was purified by HPLC before use.

Extraction of Plasma Lipids

The [1α,2α-3H] vitamin D_3 (1000 cpm) was added to 5 ml of plasma in a 25 x 125-mm glass culture tube. The lipids were extracted by adding 15 ml of cold (4 C) methanol:methylene chloride (2:1) and thoroughly mixed for 15 s with a vortex. After 60 min at 4 C, the phases separated after adding 5 ml of cold methylene chloride to each tube, vortexing for 15 s, and then centrifuging at 800 x g for 5 min. The methylene chloride layer was removed with a 50-ml glass syringe equipped with an 8-cm stainless-steel needle, and the rest of the aqueous layer was reextracted with 5 ml of cold methylene chloride. The two methylene chloride layers that contained the plasma lipids were combined and dried at 40 C under N_2. This lipid residue was solubilized in .5 ml of hexane:chloroform:methanol (9:1:1).

Preparative Chromatography of Lipid Residue

The lipid residue was chromatographed on a Sephadex LH-20 column (.6 x 15.5 cm) in a solvent system of hexane:chloroform:methanol (9:1:1) (Figure 1). The lipid residue was applied to the column in .5 ml of the column solvent and rinsed with an additional .5 ml. After the sample was applied, 3.0 ml of solvent were added to the column, and the resulting column eluant was collected. The 3.0-ml fraction was dried under N_2 and chromatographed on a Lipidex 5000 (Packard) column (.6 x 14.5 cm) in hexane:chloroform (95:5) (Figure 2). The sample was applied in .5 ml with a .5-ml wash. After the sample was applied and washed onto the column, the first 6.0 ml of column eluant were discarded. The next 4.0 ml of column eluant contained the vitamin D.

Figure 1. Elution of vitamin D_2 and vitamin D_3 and other vitamin D metabolites from a .6 x 15.5-cm Sephadex LH-20 column developed in a solvent system of hexane:chloroform:methanol (9:1:1).
Figure 2. Elution of vitamin D₃ and vitamin D₃ from a 0.6 × 14.5-cm Lipidex 5000 column developed in a solvent system of hexane: chloroform (95:5).

μl of methanol:water (96:4). Vitamin D₂ and vitamin D₃ of plasma were quantitated finally and resolved by reverse-phase HPLC on a Zorbax-ODS column (Figure 3) developed in a solvent system of methanol:water (96:4) with a flow of 2.0 ml/min. Vitamin D₃ standards were prepared from a solution containing a known amount of vitamin D₃, usually in 100% ethanol, and then by adding 50 cpm of purified [1α,2α-³H] vitamin D₃ per ng. This solution then was dried under N₂ and redissolved in 500 μl of methanol:water (96:4). Varying amounts of vitamin D (10, 20, 30, 40, and 50 ng) were subjected to HPLC, and the region corresponding to the elution of vitamin D₃ was collected in scintillation vials, the eluant evaporated, scintillation cocktail added, and the vials counted in a liquid scintillation counter to determine the recovery of the [1α,2α-³H] vitamin D₃. The resulting peak heights (in absorbance units) of the standards and unknowns were corrected for radioactive recovery before they were used in the final calculations. The concentration (ng/ml) of vitamin D₃ in plasma samples was calculated from the equation:

\[
\text{ng/ml} = \sum_{i=1}^{N} A \times \frac{R_s \times 1}{C_s} \times \frac{C_u}{R_u} \times \frac{1}{D}
\]

where

- \(N\) = the number of standards,
- \(A\) = ng of standard injected,
- \(C_s\) = peak height of standard in absorbance units,
- \(R_s\) = fraction of standard recovered after HPLC,
- \(C_u\) = peak height of unknown in absorbance units,
- \(R_u\) = fraction of unknown recovered after HPLC,
- \(D\) = milliliters of plasma extracted.

Correcting standards and unknown peak height for recovery after HPLC avoids the assumption that the standard and sample preparations are recovered with the same efficiency and also allows for continuous monitoring of the purity of the [1α,2α-³H] vitamin D₃.

Calcium was measured by atomic absorption spectrophotometry (10) and total inorganic phosphorus by the method of Fiske and Subbarow modified for an AutoAnalyzer (1).

RESULTS

Extraction and Recovery

The use of methanol:methylene chloride (2:1) offered the advantage of extracting less lipid than methanol-chloroform (2:1) extracted (2). The recovery of [1α,2α-³H] vitamin D₃ after extraction, purification, and analysis on Zorbax-ODS was 85 ± 10% (± SD) (n = 60).

Purification of Vitamin D for HPLC Analysis

In efforts to remove adequately the interfering UV peaks, the regions containing vitamin D collected from the Sephadex LH-20 column...
was chromatographed on a Lipidex 5000 column. The typical elution profile of vitamin D$_2$ and vitamin D$_3$ on the Lipidex 5000 column is in Figure 2. Vitamin D$_2$ and vitamin D$_3$ were collected in a single fraction from this column because these two forms of vitamin D did not separate.

The vitamin D was quantitated by HPLC with a Zorbax-ODS column developed in a solvent system of MeOH:H$_2$O (96:4). This system resolves vitamin D$_2$ from vitamin D$_3$. When the same amount of the two vitamins was applied to the Zorbax-ODS column, the resulting UV peak heights of the vitamin D$_3$ standards were 87% of the vitamin D$_2$ standards (Figure 4).

After the initial purification steps, the purified lipid extracts of plasma from cows in our dairy herd on a low vitamin D intake displayed no UV absorbing peaks coeluting in the vitamin D$_2$ and vitamin D$_3$ regions (Figure 5a). However, purified lipid extracts of plasma from cows exposed to sunlight showed a major peak of absorbance comigrating with vitamin D$_3$ and a minor vitamin D$_2$ peak (Figure 5b).

**Variability and Sensitivity of Assay**

When the lab plasma internal standard was analyzed five times over 4 mo, the interassay coefficient of variability was 5.71%. The minimum sensitivity for the vitamin D assay was 5 ng. Therefore, the least detectable plasma concentration for 85% recovery and assaying 5 ml of plasma was 1.15 ng/ml.

![Figure 4](image)

**Figure 4.** Standard curve of vitamin D$_2$ and vitamin D$_3$ expressed as nanograms of vitamin against peak height in absorbance units. Vitamin D$_3$ standard UV peak heights were 87% of the vitamin D$_2$ standards.

![Figure 5](image)

**Figure 5.** High pressure liquid chromatogram of purified lipid extracts from plasma of a) cows on low vitamin D intake (top) and b) cows exposed to sunlight while grazing (bottom). The ultraviolet (UV) plasma profiles of cows on a low vitamin D intake had no UV absorbing peaks in the region of vitamin D$_3$ and vitamin D$_2$. Animals receiving daily exposure to sunlight had a large vitamin D$_3$ peak and a minor vitamin D$_2$ peak.
Plasma Ca, Inorganic Phosphorus (Pi), and Vitamin D in Treated Cows

During the first wk after the initial vitamin D3 injection, Ca and Pi of plasma tended to be normal (Figure 6). However, after the second injection of vitamin D3, plasma Pi increased from 4.5 to 9.5 mg/100 ml and Ca increased from 9.0 to 11.5 mg/100 ml. Plasma Pi remained at 9.5 mg/100 ml; and 38 days after the last vitamin D3 injection, Pi of plasma fell to near normal 4.5 mg/100 ml. Plasma Ca peaked at 14 mg/100 ml 20 days after the fourth injection and remained elevated (∼11.5 mg/100 ml) to the end of the experiment.

After the first injection, vitamin D3 of plasma increased from a basal concentration of 3.2 ng/ml to 45 ng/ml of vitamin D3 (Figure 7). One day after the second injection, vitamin D3 of plasma increased to 160 ng/ml. Vitamin D3 remained below 250 ng/ml following the third and fourth injections and gradually declined to 80 ng/ml by the end of the experiment.

Vitamin D2 was not detected in plasma of these animals throughout the experiment.

DISCUSSION

Assay

This assay offers a fast, accurate, reproducible method for assaying vitamin D2 and vitamin D3 in plasma. However, circulating vitamin D was little, and approximately 50% of the plasma we analyzed from "normal" cows from other experiments had undetectable amounts. The lack of detectable vitamin D in the plasma did not indicate D deficiency, however, because further analysis of the "normal" plasma revealed concentrations of 25-OHD between 30 and 60 ng/ml, which is normal for other species. A more sensitive vitamin D assay and probably a procedure for more extensive lipid extract purification will be needed to study vitamin D of plasma in the "normal" or deficient state in the cow.

Plasma Ca, Pi, and Vitamin D

During the first wk after the first vitamin D3 injection, Ca and Pi in plasma remained normal or below normal. This was comparable with results in a similar experiment (7). In contrast, however, Seekles and Hendricks (11) reported elevations in Ca to 11.5 to 12.0 mg/100 ml and Pi to 7 to 8 mg/100 ml from basal 8.5 and 6.0 mg/100 ml within a week after injection of 10 × 10⁶ IU of vitamin D3. Dietary Ca and P consumption in the Seekles and Hendricks experiment appeared to be similar to that in our experiment. The different response of Ca and Pi in plasma is probably due to a difference in vitamin D3 preparation or breed of dairy cows, or to both.

Vitamin D3 of plasma increased from 3.2 ng/ml to 45.0 ng/ml 7 days after the first treatment with vitamin D3 and never exceeded 230 ng/ml. Even the maximum vitamin D was about 1/75 of that postulated by Hollis et al. (7), who estimated 15,000 ng/ml during the
week after a vitamin D₃ injection of 15 x 10⁶ IU. The relatively small amount of vitamin D₃ in the plasma of cows receiving a total of 60 x 10⁶ IU (1.5 g) of crystalline vitamin D₃ was unexpected. Apparently, the cow has an effective system for excretion or storage, or both, of huge amounts of vitamin D.

There was a marked difference in the pattern of increase in vitamin D₃ after repeated injections of vitamin D₃ (Figure 7). The reason for this difference is unknown. In contrast to the slow moderate increase of vitamin D₃ in plasma after the first injection, increase in vitamin D₃ was sharp in response to the second injection, which suggests that the tissue storage and plasma binding capacity of vitamin D became progressively saturated after the first and second vitamin D₃ injections. At this point, excretion or metabolism processes, or both, prevented further elevation of vitamin D₃ in plasma from subsequent massive injections of the vitamin.

Intestinal receptor binding and resorption of Ca from bone have been observed with 1,25-(OH)₂D₃ in physiological concentrations (40 pg/ml) (8) in vitro experiments (5, 13). To evoke the same responses with vitamin D₃, however, required 40,000 ng/ml and 1,000 ng/ml. Therefore, we probably can assume safely that similar concentrations are needed in vivo. If this assumption is true, then the vitamin D₃ of plasma in our experiment (<235 ng/ml) were not enough to simulate 1,25-(OH)₂D activity at the bone or intestine and, therefore, are probably not responsible for the observed hypercalcemia and hyperphosphatemia.

REFERENCES
1 AutoAnalyzer, Technicon Instruments Corporation.