Determination of Potential Content of Vitamin D_2 (Ergocalciferol) and Vitamin D_3 (Cholecalciferol) in Gelatin-Coated Beadlets, Oil-Based Mixtures, and Multivitamin Tablet and Capsule Formulations

GUSTAV A. WALKER ˣ, BRUCE E. CARPENTER, and DAVID L. TUESCHER

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Abstract □ A high-performance liquid chromatographic procedure was used to determine routinely the potential vitamin D content in raw materials and multivitamin formulations. The method employs a microparticulate silica column to separate vitamin D from its degradation products as well as other fat-soluble vitamins. Sample preparation is simple, and the chromatographic time is <20 min when progesterone is added to the injection mixture as an internal standard. Replicate analyses of complex multivitamin formulations demonstrate precision with a relative standard deviation of <4%. Spiked placebos typically show 98–100% recovery and a linear chromatographic response. The use of bulk drug as a working reference standard is recommended for the determination of the potential vitamin D concentration in pharmaceutical multivitamin preparations.

Keyphrases □ Vitamin D—high-performance liquid chromatographic determination of potential content of vitamins D_2 and D_3 in gelatin-coated beadlets, oil-based mixtures, and multivitamin tablet and capsule formulations □ Ergocalciferol (vitamin D_2)—high-performance liquid chromatographic determination of potential content in gelatin-coated beadlets, oil-based mixtures, and multivitamin tablet and capsule formulations □ Cholecalciferol (vitamin D_3)—high-performance liquid chromatographic determination of potential content in gelatin-coated beadlets, oil-based mixtures, and multivitamin tablet and capsule formulations □ High-performance liquid chromatography—analysis, potential content of vitamins D_2 and D_3 in gelatin-coated beadlets, oil-based mixtures, and multivitamin tablet and capsule formulations

High-performance liquid chromatography (HPLC) has been established as a rapid, reproducible, and selective method for the separation and analysis of vitamin D in pharmaceutical raw materials. In contrast, current compendial procedures (1) are imprecise and dependent on extensive, time-consuming sample preparation to confer tolerable specificity. For example, the compendial procedure is not suitable for formulations containing vitamin E without the removal of vitamin E from the sample preparation by TLC.

The HPLC methodology allows the separation of the D vitamins from the provitamins, previtamins, and photochemical isomers such as lumisterol, tachysterol, and *trans*-vitamin D. Isocratic separations with adsorption or reversed-phase chromatography have been reported as have gradient elution methods for the determination of vitamin D only or simultaneously with vitamins A and E (2–7).

BACKGROUND

The analysis of vitamin D in bulk drugs as in gelatin-coated beadlets or in oil by HPLC usually is easy and straightforward with minimum sample preparation. However, quantitation of vitamin D in multivitamin products with frequently complex matrixes is difficult without optimum chromatography. The variation in chromatographic cleanliness of excipient components from different suppliers constitutes a special challenge to HPLC methodology. Therefore, few reported HPLC procedures have been equally amenable to all vitamin formulations.

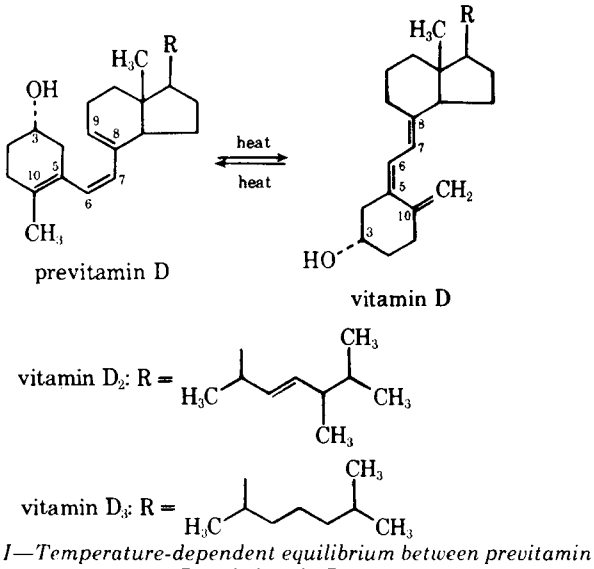
The analysis of vitamin D in pharmaceutical preparations is complicated further by whether this determination should account for the previtamin D content, which is in equilibrium with vitamin D (Scheme I). To allow for conversion of the previtamin into vitamin D, it is necessary to estimate the potential vitamin D content that can be determined through measurement of both the previtamin D (with the appropriate absorptivity correction) and the vitamin D areas. Unfortunately, previtamin D is not always adequately resolved from excipient peaks in the chromatography of products with complex matrixes. Under such chromatographic conditions, it is not quantitated easily.

To account for the previtamin D–vitamin D equilibrium in various products without measuring the previtamin directly, some pharmaceutical manufacturers have recommended a heat equilibration step wherein standards and samples are treated for the same time and at the same temperature (8). Only the vitamin D would have to be measured since the previtamin D should be the same in both the standard and the sample. However, when applied to some multivitamin preparations, this technique is imprecise, and its use frequently results in erroneous estimations of vitamin D content.

The applicability of HPLC methodology is demonstrated in this paper in the determination of vitamin D in bulk drug and in various multivitamin preparations. Sample preparation and chromatographic conditions, modified from previously reported procedures (3, 5, 8), are simple and require minimal changes to accommodate samples with different matrixes. In addition, a practical and reliable approach is recommended for the determination of potential vitamin D content by the use of a bulk drug working standard for samples treated similarly.

EXPERIMENTAL

Reagents and Solvents—Stabilizer-free chloroform[1], *n*-hexane[1], tetrahydrofuran[1], acetic acid[2], and diatomaceous earth[3] were obtained



Scheme I—Temperature-dependent equilibrium between previtamin D and vitamin D

[1] Spectroquality grade, Burdick & Jackson Laboratories, Muskegon, Mich.
[2] Analytical grade, Mallinckrodt, St. Louis, Mo.
[3] Florex (Fuller's earth), Floridin Co., Quincy, Fla.

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commercially. Water was deionized. Ergocalciferol USP (vitamin D_2) and cholecalciferol USP (vitamin D_3) were used as reference standards. Progesterone USP served as the internal standard.

Apparatus and Column—The liquid chromatograph[4] was equipped with a fixed-wavelength UV detector[5] (254 nm). A microparticulate silica column[6] (250 × 4.6 mm i.d.) was used. The mobile phase was pumped at a flow rate of 1.5 ml/min and 1200 psi. The column was maintained at room temperature. Chromatograms were traced on a strip-chart recorder[7].

Mobile Phase—A mixture of chloroform–water-saturated *n*-hexane–dry *n*-hexane–tetrahydrofuran–acetic acid (60:15:25:1.5:0.4 v/v) was used. This ratio may be adjusted to achieve satisfactory system suitability.

Internal Standard Solution—The internal standard was prepared by adding ~6 mg of progesterone to 200 ml of mobile phase.

Reference Standard Solution—Approximately 18 mg of vitamin D USP reference standard was weighed accurately, transferred to a 100-ml low actinic volumetric flask, diluted to volume with isooctane, and mixed well. This was reference standard Solution A and could be kept for 1 week if maintained at 0° or below. Exactly 5.0 ml of Solution A was transferred into a 100-ml low actinic volumetric flask and diluted to volume with isooctane. This was reference standard Solution B and was prepared daily.

Prior to chromatography, a 1:1 mixture of Solution B and the mobile phase or the internal standard solution was prepared in a low actinic container and mixed well.

Reference standard Solution B was used to characterize the working standard.

Working Standards—The working standards were vitamin D beadlets USP or cholecalciferol USP in oil.

Approximately 8.0 mg of vitamin D gelatin beadlets was weighed accurately and transferred into a 250-ml centrifuge tube fitted with a 24/40 ground-glass joint. Duplicate weighings of a characterized lot were used. The analysis was continued as described under *Procedure A*.

With a syringe, cholecalciferol in oil equivalent to ~2.6 mg of vitamin D_3 was weighed accurately into a 250-ml low actinic volumetric flask. The flask was filled to volume with hexane, and the contents were mixed well. Duplicate weighings of cholecalciferol were used. The analysis was continued as described under *Procedure B*.

Sample Preparation—*Coated Compressed Tablets*—The weight of 25 tablets was measured accurately, and an average tablet weight was determined. Approximately 12 tablets at a time were ground to a fine powder with a mill[8] and were mixed. A sample equivalent to 20 tablets was weighed accurately and transferred into a 250-ml centrifuge tube fitted with a 24/40 ground-glass joint. The analysis was continued as described under *Procedure A*.

Soft Elastic Capsules—Eighteen capsules were transferred into a suitable container such as a 250-ml centrifuge cup fitted with a 24/40 ground-glass joint. The analysis was continued as described under *Procedure B*.

Oil-Based Mixtures—With a syringe, an accurately weighed sample equivalent to ~260 μg of vitamin D_3 was placed in a 25-ml low actinic volumetric flask. The flask was filled to volume with hexane, and the contents were mixed well. The analysis was continued as described under *Procedure B*.

Procedure A—To each centrifuge cup was added 80 ml of a 3:1 solution of dimethyl sulfoxide and water, which had been cooled to room temperature. The headspace was flushed with nitrogen. The centrifuge cups were stoppered tightly, sonicated for 10 min, and shaken vigorously for 1 hr on a mechanical horizontal shaker. The samples must be protected from heat and prolonged exposure to UV irradiation; contact with oxygen should be minimized.

To each centrifuge cup was added an exact volume of hexane (*e.g.*, 25 ml), and the cups were shaken for 45 min as described previously. The samples were centrifuged at ~1500 rpm for 5 min. For the final preparation of tablet formulations or gelatin-coated beadlets, an aliquot of the hexane layer was transferred into a suitable low actinic container and diluted 1:1 with the internal standard solution. The containers were capped tightly, and the contents were mixed well.

Samples with soft elastic capsules were analyzed for vitamin D as described under *Procedure B*.

Procedure B—Without exposing the samples to UV irradiation, an exact volume of the sample (hexane solution) was pipetted onto the column of diatomaceous earth. Then the column was washed with 100 ml of hexane, and the effluent was discarded. Vitamin D was eluted with the addition of 150 ml of toluene and collected in a suitable low actinic boiling flask containing 3.0 ml of the internal standard solution. The toluene was evaporated using a suitable evaporator and a water bath at 37° under vacuum. The residue was dissolved in 6.0 ml of the mobile phase.

Chromatography—The HPLC column was conditioned with the mobile phase until a relatively stable baseline was obtained. A vitamin D beadlet reference standard and not more than four sample preparations were injected alternately.

A chromatographic run should include at least two beadlet reference standard preparations injected in duplicate. The injection volume usually was 40–50 μl for both reference and sample analysis. Retention times of 8–9 and 14–16 min should be obtained for vitamin D and progesterone, respectively.

Calculations—The amounts of potential vitamin D_2 in micrograms per tablet (or capsule) and vitamin D_3 in micrograms per gram were determined from:

$$\text{amount} = \frac{PR_s}{PR_{std}} \frac{C_{std}}{WT_s} SV \times TW_t \times P_{std} \qquad \text{(Eq. 1}a\text{)}$$

or:

$$\text{amount} = \frac{PR_s}{PR_{std}} \frac{C_{std}}{SN} SV \times P_{std} \qquad \text{(Eq. 1}b\text{)}$$

where PR_s is the peak response ratio of the vitamin D peak to the internal standard peak in the sample preparation, PR_{std} is the peak response ratio of the vitamin D peak to the internal standard peak in the standard preparation, C_{std} is the concentration of the vitamin D reference standard [vitamin D_2 in gelatin beadlets (milligrams of beadlets per milliliter) or cholecalciferol USP in oil solution (milligrams per milliliter)], WT_s is the weight of the sample (grams), SN is the sample number (number of capsules, if applicable), SV is the sample dilution volume [equal to the volume of added hexane in the sample preparation (*e.g.*, 25 ml)], TW_t is the weight of one tablet in grams (factor not applicable when sample number is used), and P_{std} is the assigned potency of the vitamin D beadlet reference standard or cholecalciferol USP in oil in micrograms per milligram.

Calculation of Previtamin D—Previous studies showed that previtamin D and vitamin D form an equilibrium dependent on temperature (9). A solution of reference standard vitamin D_3 dissolved in isooctane and contained under nitrogen in a low actinic flask was heated overnight at 80°. Upon cooling, the solution was injected into the HPLC system, and the amounts of previtamin and vitamin D were determined by peak area. Comparisons were made between the vitamin D concentrations in the freshly prepared solution, which had no detectable traces of previtamin, and the vitamin D concentrations in the heat-treated solution. Since no other degradation products were observed, the amounts of previtamin D formed could be calculated from the decrease in the vitamin D peak. The expected areas of the previtamin (assuming an absorptivity identical to vitamin D) were compared with the areas observed, and the differences were equated with the differences in the respective absorptivities of previtamin D and vitamin D. Results from samples treated as described produced an average value for the absorptivity (254 nm) of the previtamin of 40% of that of vitamin D. This value agrees with the value found by Havingo *et al.* (10).

The concentration of previtamin D_2 was calculated from:

$$\frac{\text{area of previtamin } D_2}{\text{area of vitamin } D_2} \times \frac{\text{vitamin } D_2 \text{ (micrograms per tablet)}}{CF} = \text{micrograms of previtamin } D_2 \text{ per tablet} \qquad \text{(Eq. 2)}$$

and:

$$\frac{\text{previtamin } D_2 \text{ (micrograms per tablet)}}{\text{previtamin } D_2 \text{ (micrograms per tablet)} + \text{vitamin } D_2 \text{ (micrograms per tablet)}} \times 100 = \text{percent of previtamin } D_2 \qquad \text{(Eq. 3)}$$

where CF is the correction factor (0.4).

RESULTS AND DISCUSSION

The chromatographic system used separates vitamin D from its major degradation products. Figure 1 shows the resolved elution profile of the products of partially degraded vitamin D_2. The vitamin D_2 solution was

[4] Model ALC/GPC 244 equipped with a U6K injector, Waters Associates, Milford, Mass.
[5] Model 440, Waters Associates, Milford, Mass.
[6] Si-5A, Brownlee Laboratories, Santa Clara, Calif.
[7] Model B5000 Omniscribe, Houston Instruments, Austin, Tex.
[8] Spex mixer/mill, Metuchen, N.J.

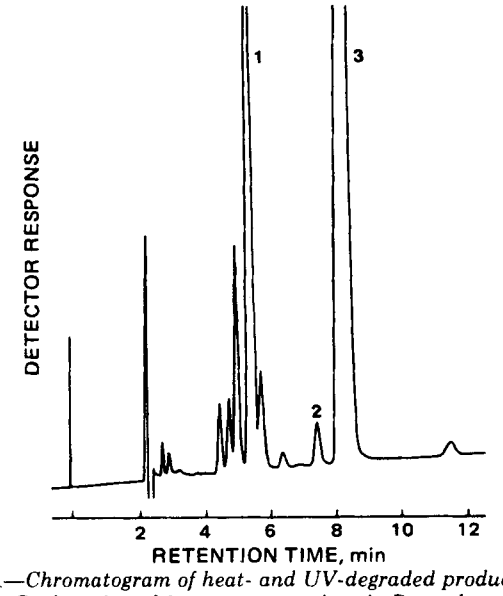

Figure 1—*Chromatogram of heat- and UV-degraded products of vitamin D_2. Peaks 1, 2, and 3 represent previtamin D_2, tachysterol, and vitamin D_2, respectively. The identity of the other peaks was not determined.*

degraded by 2 hr of heat treatment at 90° followed by irradiation for 2 hr at both long and short UV wavelengths. Approximately 6.2 μg of degraded vitamin D_2 was injected. The peaks of interest were associated with previtamin D_2, tachysterol, and vitamin D_2; the respective retention times were 5.4, 7.5, and 8.2 min. Degradation products of vitamin D_3 (not shown) showed a similar elution profile.

Figures 2 and 3 show representative chromatograms of multivitamin preparations. Included are chromatograms of vitamin D_2 in a multivitamin–mineral tablet formulation (Fig. 2) and in a multivitamin capsule formulation (Fig. 3). Chromatograms of placebos showed no interference with the vitamin D peak. Separation of vitamin D from interfering excipient peaks (due largely to degradation of vitamins A and E) and the previtamin D isomer was achieved in each example. Tachysterol was not detected in any preparation, although it was resolved from the vitamin D peak when the degraded D mixture was injected to determine system suitability. Progesterone, which served as an internal standard, was separated completely from vitamin D, its degradation products, and any excipient peaks.

Table I shows an example of a spike recovery study of vitamin D_2 from a multivitamin–mineral placebo formulation. The placebo was spiked with gelatin beadlets from 60 to 120% of theory and carried through the assay. The mean recovery for 10 samples was 99.4% with a relative standard deviation (*RSD*) of 2.2%.

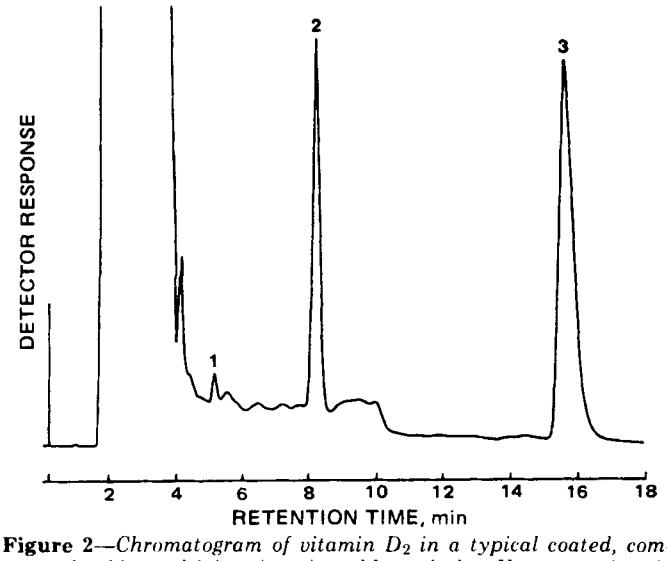

Figure 2—*Chromatogram of vitamin D_2 in a typical coated, compressed, tablet multivitamin–mineral formulation. Key: 1, previtamin D_2; 2, vitamin D_2; and 3, progesterone (internal standard).*

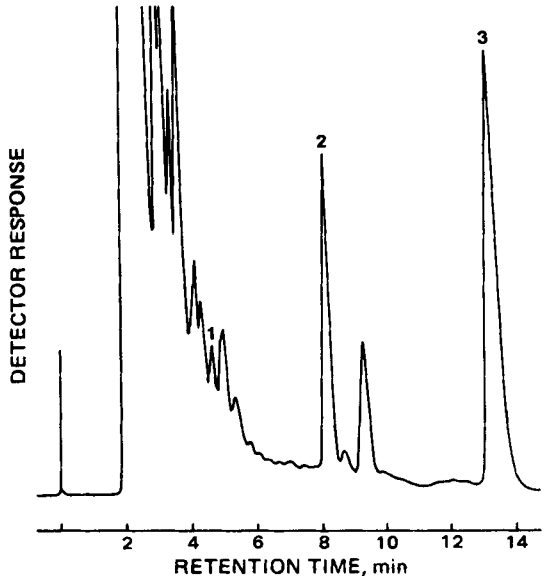

Figure 3—*Chromatogram of vitamin D_2 in a typical capsule multivitamin formulation. Key: 1, previtamin D_2; 2, vitamin D_2; and 3, progesterone (internal standard).*

The data from the recovery studies also provided a comparison of the previtamin D_2 concentration in the bulk drug and in the product formulation. A sample chromatogram (Fig. 2) shows that previtamin D_2 clearly was resolved from excipient peaks. The previtamin D_2 concentration in gelatin beadlets was constant. The mean value for the percentage of previtamin D_2 in seven lots of gelatin beadlets was 11.1% with a relative standard deviation of 3.2%. In comparison, the mean percentage of previtamin D_2 in 14 lots of product formulation was calculated to be 11.0% with a relative standard deviation of 4.2%. Although all product lots were assayed within the expiration date, the age of the samples varied across a range to the limit of expiration.

An alternative to the direct measurement of the previtamin is the use of a well-characterized lot of vitamin D in bulk drug (*i.e.*, gelatin beadlets) as the reference standard. As long as the bulk drug has been maintained at the same temperature as the product formulations and has not been exposed to prolonged UV irradiation, the percentage of previtamin D in the bulk drug is equal to that in the product formulation in accordance with the temperature-dependent equilibrium between these isomers. As indicated previously, the amount of previtamin D_2 in gelatin beadlets and in the formulations in which they had been added was found to be ~11% of the potential vitamin D content when both were stored at room temperature (25°). However, to maintain control of the assay, the potency of the bulk drug was determined periodically *versus* a ergocalciferol USP reference standard.

The use of a well-characterized lot of gelatin beadlets containing vitamin D_2 as a reference standard permits reproducible determinations of the potential vitamin D content in multivitamin formulations. For example, when 12 replicate samples of a multivitamin tablet formulation were assayed over 3 days, the relative standard deviation was 3.5%.

A comparison of assay results obtained by HPLC and the current compendial chemical procedure for the determination of vitamin D_2 in

Table I—Recovery of Vitamin D_2 in Gelatin Beadlets from a Multivitamin–Mineral Placebo Formulation

Day	Percent of Theory	Vitamin D_2 Added, μg	Vitamin D_2 Found, μg	Recovery, %
1	60	140.6	141.3	100.5
1	70	164.2	158.4	96.5
1	80	187.8	185.4	98.7
1	90	211.1	205.2	97.2
1	100	234.8	239.4	102.0
1	120	281.3	273.6	97.3
2	60	140.9	138.7	98.4
2	70	164.8	167.8	101.8
2	90	211.1	216.0	102.3
2	100	234.8	232.4	99.0
Mean				99.4
RSD				2.2

Table II—Potential Vitamin D Content for Several Lots of a Multivitamin–Mineral Formulation [a]

Lot	HPLC Assay Result, μg/tablet	Chemical Assay Result, μg/tablet
A	11.3	11.6
B	11.8	12.5
C	11.1	13.0
D	11.1	11.4
E	12.7	12.8
F	11.4	12.6
G	11.2	11.7
H	11.2	12.4
I	11.2	12.1
J	11.1	11.4
K	11.2	12.1
L	12.0	11.7
M	11.7	12.9
N	12.2	12.2
O	11.6	11.5
Mean	11.5	12.1

[a] Theoretical content was 11.7 μg/tablet.

15 lots of a multivitamin–mineral tablet formulation is shown in Table II. Vitamin D_2 in gelatin-coated beadlets was used as a reference standard for the HPLC analysis. Each assay result was the average of at least two determinations. The relative standard deviations associated with the HPLC and chemical assays were ~4 and 8%, respectively. For the 15 lots of multivitamin formulation, the mean value for the vitamin D content by the HPLC assay was 11.5 μg/tablet (98% of theory); by the chemical assay, it was 12.1 μg/tablet (103% of theory). The chemical assay results were statistically different at the 95% level of confidence from the results obtained by the HPLC assay.

If the photochemical isomers of vitamin D are held to a minimum, the assay values by the chemical procedure should be approximately equal to the sum of the values for previtamin D_2 and vitamin D_2 as determined by HPLC. However, if such isomers are present at a significant concentration, the results of the chemical assay should be significantly higher than those obtained by the HPLC assay because the chemical method is a colorimetric assay that does not distinguish between previtamin D_2, vitamin D_2, or any of the vitamin D_2 photochemical isomers, which, although separated by HPLC, would positively bias vitamin D_2 chemical analysis.

The use of bulk drug as a working reference standard for the determination of the vitamin D content in commercial multivitamin preparations is recommended when it is known that the bulk drug and product formulation have been handled and stored under similar conditions. These conditions preferably are met by storage and sample preparation at room temperature in the absence of UV irradiation. This approach in the quantitation of the potential vitamin D content in product formulations by HPLC is practical and reliable and does not require direct measurement of the previtamin concentration or time-consuming, frequently imprecise, heat equilibration experiments. Sample preparation is quick, with a single extraction step for most formulations. Assay time is <20 min/sample, and the assay can be automated easily. Chromatography is specific and selective. The assay results are reproducible and within 4% of manufacturing theory for the potential vitamin D content.

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