High-Performance Liquid Chromatographic Analysis of Vitamins I: Quantitation of Cholecalciferol or Ergocalciferol in Presence of Photochemical Isomers of the Provitamin and Application to Cholecalciferol Resins

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Abstract □ A high-performance liquid chromatographic (HPLC) procedure was developed for the quantitative determination of cholecalciferol or ergocalciferol in the presence of the photochemical isomers of the provitamin. Separation is also achieved from various common reaction products encountered in the vitamin synthesis as well as other fat-soluble vitamins. The method was applied to the routine analysis of cholecalciferol resins, and experimental data are set forth. A comparison of the HPLC method to the AOAC biological and chemical procedures shows that the HPLC method most closely approximates the antirachitic activity of a cholecalciferol sample. The specificity, sensitivity, and reproducibility of the method make it applicable to various vitamin samples containing cholecalciferol or ergocalciferol.

Keyphrases □ Cholecalciferol—high-performance liquid chromatographic analysis and separation from photochemical isomers and reaction products of provitamin □ Ergocalciferol—high-performance liquid chromatographic analysis and separation from photochemical isomers and reaction products of provitamin □ Vitamins—ergocalciferol and cholecalciferol, high-performance liquid chromatographic analysis and separation from photochemical isomers and reaction products of provitamins □ High-performance liquid chromatography—analysis and separation, ergocalciferol or cholecalciferol

A method utilizing high-performance liquid chromatography (HPLC) was developed which quantifies ergocalciferol (vitamin D_2) or cholecalciferol (vitamin D_3) after separation from the previtamin and the photochemical isomers of the provitamin.

In the preparation of synthetic ergocalciferol or cholecalciferol, the specific provitamin, ergosterol or 7-dehydrocholesterol, respectively, is irradiated at low temperatures, and the resultant previtamin (precholecalciferol or preergocalciferol) is isolated and thermally converted to vitamin D[1] (1, 2). The irradiation sequence of the provitamins, Scheme I (3–5), was thoroughly investigated and results in a mixture of the previtamin, lumisterol, and tachysterol (6–17).

Any method for vitamin D analysis must be capable, therefore, of distinguishing the vitamin from these isomers as well as the provitamin. The HPLC method described in this paper accomplishes these goals. Vitamin D is also separated from the *trans*-isomers, isotachysterol, and dihydrotachysterol. However, this method does not separate ergocalciferol from cholecalciferol.

The biological activity of three of the photochemical isomers of the provitamin was established previously. Lumisterol has no antirachitic activity, tachysterol exhibits slight antirachitic activity, and the activity of precholecalciferol is 35% of that of cholecalciferol (18–21). Precholecalciferol in itself is inactive, and any biological activity exhibited by this isomer can be attributed to its *in vivo* conversion to cholecalciferol (18).

Since previtamin D and vitamin D are well separated in this system, the "actual" vitamin D content as well as the "potential" vitamin D content can be quantified. The actual vitamin D content of the sample is determined by measuring the area of the vitamin D peak; the potential vitamin D content is determined through measurement of both the previtamin D and the vitamin D peak areas. A correction factor is necessary for the calculation of previtamin D since its absorptivity at 254 nm is 40% of that of vitamin D (22). A further correction is needed to convert the amount of previtamin D present to potential vitamin D since, as already stated, it possesses only 35% of the activity of vitamin D.

In addition, the HPLC technique is carried out at room temperature, thereby reducing the possibility of thermal breakdown or of shifting of the vitamin D ⇄ previtamin D equilibrium.

EXPERIMENTAL

Equipment—A liquid chromatograph[2], operated at ambient temperature and equipped with a UV detector for monitoring the column effluent at 254 nm, was used. The column was a 30-cm × 4-mm (i.d.) commercially available microparticle silica column[3]. The liquid chromatographic detector was connected to a programmed digital computer-based system[4] for obtaining the areas of the chromatogram peaks.

Reagents—USP cholecalciferol reference standard and *p*-dimethylaminobenzaldehyde[5], 99+% pure, were the reference and internal standards, respectively. Spectroquality grade chloroform[6] and *n*-hexane[6], as well as analytical reagent grade tetrahydrofuran[7], were used for the preparation of the mobile phase. A GLC analysis of the chloroform was performed to assure that it was essentially free of ethanol and water.

Mobile Phase—A mixture of chloroform–*n*-hexane–tetrahydrofuran (70:30:1 v/v) was used.

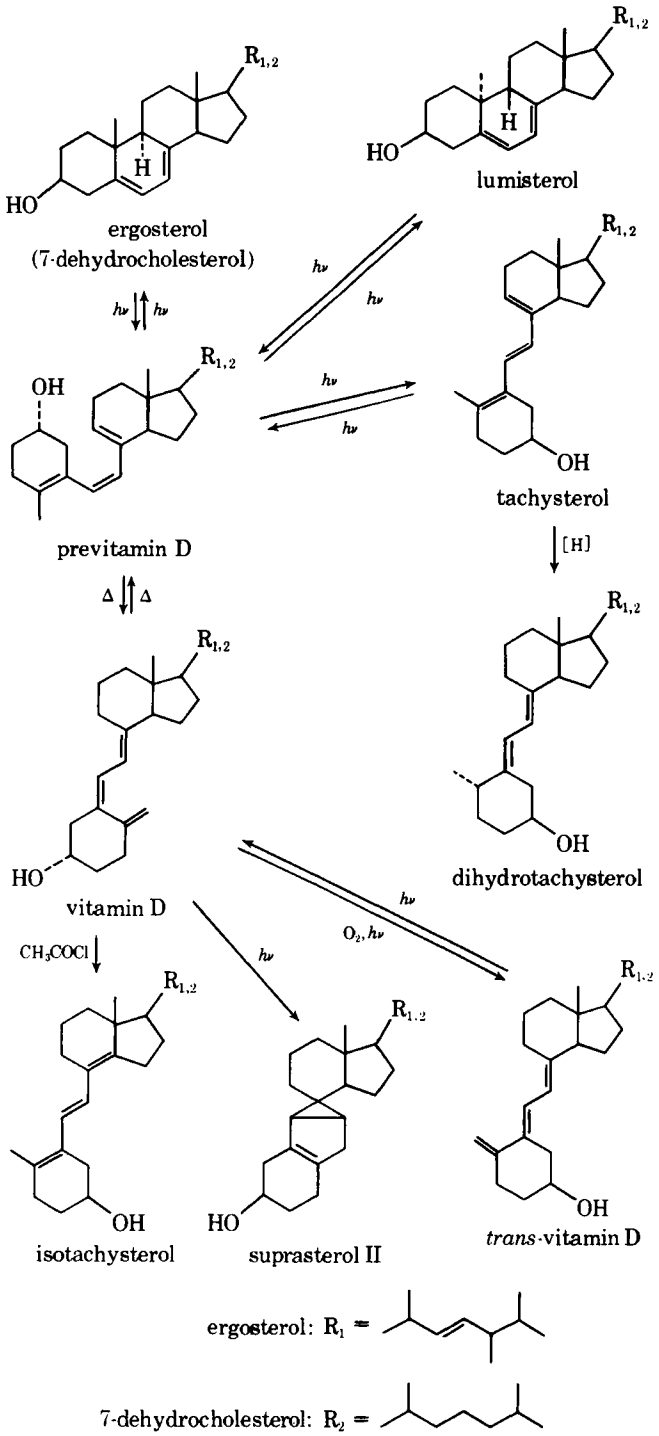
Preparation of Internal Standard Solution—Accurately weigh approximately 30 mg of *p*-dimethylaminobenzaldehyde into a 100-ml volumetric flask and dissolve in and dilute to volume with the mobile phase.

Preparation of Standard Solution—Accurately weigh approximately 12 mg of cholecalciferol standard into a 50-ml amber volumetric flask and dissolve in and dilute to volume with the mobile phase. Prepare a mixture (1:1) of the cholecalciferol standard and the *p*-dimethylaminobenzaldehyde solution in a suitable amber vessel and mix well.

Preparation of Sample Solution—Accurately weigh a quantity

[1] The term vitamin D will be used throughout to mean either ergocalciferol or cholecalciferol.

[2] Dupont 830.
[3] μPorasil, Water's Associates.
[4] PACE, Electronic Associates.
[5] Aldrich Chemical Co.
[6] Matheson, Coleman and Bell.
[7] Fisher.



Scheme I—Effect of UV radiation and heat on the synthesis and isomerization of vitamin D. (The $R_{1,2}$ group signifies that the chain can be either R_1 or R_2. When the group is R_1, the chain for ergosterol is specified and the reaction scheme is for the synthesis of ergocalciferol. Similarly, when the group is R_2, the chain for 7-dehydrocholesterol is specified and the reaction scheme is for the synthesis of cholecalciferol.)

of the resin sample, equivalent to 30 mg of cholecalciferol, into a 25-ml amber volumetric flask and dissolve in and dilute to volume with mobile phase. Pipet 5 ml of the resultant solution into a 25-ml amber volumetric flask and dilute to volume with the mobile phase. Prepare a 1:1 mixture of the final sample solution and the *p*-dimethylaminobenzaldehyde solution in a suitable amber vessel and mix well.

Chromatography—Condition the column with the mobile phase at a flow rate of approximately 1.0 ml/min. Inject 5 µl of the standard solution and adjust the flow rate to give retention times of 13–15 and

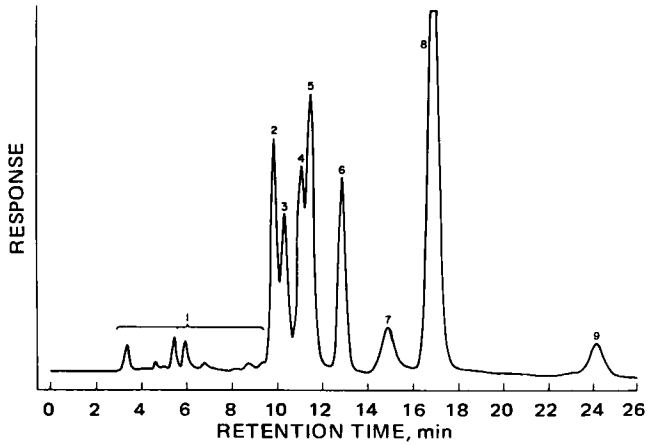


Figure 1—*Chromatogram of a synthetic mixture of photochemical isomers and reaction products of vitamin D_3. Key: 1, unknowns; 2, trans-vitamin D_3; 3, previtamin D_3; 4, lumisterol$_3$; 5, isotachysterol$_3$; 6, p-dimethylaminobenzaldehyde (internal standard); 7, tachysterol$_3$; 8, vitamin D_3; and 9, 7-dehydrocholesterol.*

16–18 min for *p*-dimethylaminobenzaldehyde and cholecalciferol, respectively. Determine the response ratio by replicate injections of the standard solution. When the deviation of the response ratio is less than 2%, make duplicate injections of the sample solution. Use the peak areas obtained to calculate the cholecalciferol content of the resin.

Calculations—Determine the areas of the peaks and calculate the ratio of the area of the cholecalciferol peak to the area of the *p*-dimethylaminobenzaldehyde peak for each chromatogram. Use the average ratio obtained for the standard replicates and the ratio for each sample duplicate for the calculation of cholecalciferol in the samples as shown in the following equations:

$$\text{response ratio (R.R.)} = \frac{A_D\ (\text{standard}) \times C_{IS}\ (\text{standard})}{A_{IS}\ (\text{standard}) \times C_D\ (\text{standard})} \quad \text{(Eq. 1)}$$

$$\text{IU of cholecalciferol/g} = \frac{A_D\ (\text{sample}) \times C_{IS}\ (\text{sample}) \times 250 \times 40{,}000}{A_{IS}\ (\text{sample}) \times \text{R.R.} \times \text{sample weight, g}} \quad \text{(Eq. 2)}$$

where:

A_D = area of cholecalciferol peak
A_{IS} = area of internal standard peak
C_D = concentration of cholecalciferol standard in milligrams per milliliter
C_{IS} = concentration of internal standard in milligrams per milliliter
250 = dilution factor
40,000 = number of International Units of cholecalciferol per milligram of USP reference standard

RESULTS AND DISCUSSION

Typical cholecalciferol resin samples contain various minor impurities. Although there is quantitative variation from different manufacturers, most impurities are photochemical isomers of 7-dehydrocholesterol and various reaction products such as *trans*-cholecalciferol and isotachysterol$_3$. The chromatographic system employed for the analysis of cholecalciferol resins must separate these impurities from cholecalciferol. A synthetic mixture of several interfering compounds plus cholecalciferol was prepared and well resolved by the HPLC procedure (Fig. 1). In addition, this system also separates dihydrotachysterol$_3$ from cholecalciferol. The retention time of this impurity, not shown in Fig. 1, was 10.5 min.

Figure 2 shows a chromatogram of a typical cholecalciferol resin. Again, separation of the resin impurities from cholecalciferol is well evidenced. There is also baseline separation of the internal standard, cholecalciferol, and tachysterol$_3$, one of the most difficult isomers to separate from cholecalciferol.

To evaluate the precision of this method, six replicate sample

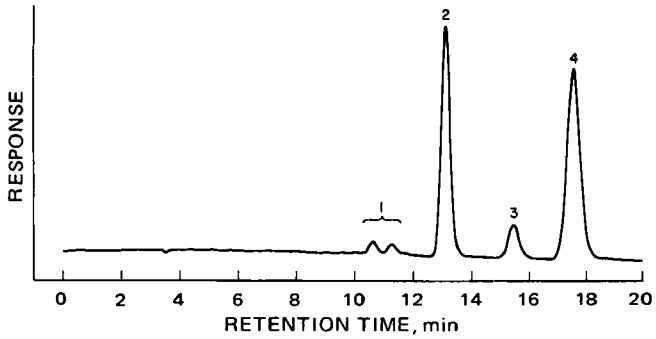


Figure 2—*Chromatogram of a typical vitamin D_3 resin. Key: 1, resin impurities; 2, p-dimethylaminobenzaldehyde (internal standard); 3, tachysterol$_3$; and 4, vitamin D_3.*

preparations of a cholecalciferol resin and six replicate injections of one sample solution were analyzed (Table I). The relative standard deviations for the replicate sample preparations and the replicate injections were 1.37 and 0.36%, respectively. These data indicate the low variability between test results.

In addition, the method was evaluated for linearity of the cholecalciferol response at concentrations ranging from 3 to 580 μg/ml. A plot of concentration *versus* absorbance was found to obey Beer's law. The sensitivity limit of this method was 3 μg/ml.

In an effort to establish the correlation between the HPLC method and biological activity, 10 samples of cholecalciferol resin were sent to two independent laboratories, A and B, for bioassay by the Association of Official Analytical Chemists (AOAC) three-level chick method (23). The data summarized in Table II show the large deviations in the bioassay results. Interlaboratory assays on the same sample differed by 1–41%, with most results differing by greater than 20%. This wide range in results attests to the lack of precision of the bioassay method.

The same samples were also assayed by the AOAC chemical (24) and HPLC methods. The results obtained by these two methods were compared to the average of the bioassay obtained by Laboratories A and B. As seen in Table III, both methods correlated well with the bioassay. However, the AOAC chemical method generally overestimated the biological activity and was found to be 114.3% of the mean of the bioassay. On the other hand, the HPLC assay closely approximated the biological activity and was 99.2% of the mean of the bioassay. The relative standard deviations of the AOAC and the HPLC methods, as compared to the bioassay, were essentially equal, 12.7 and 12.8%, respectively.

The current USP chemical method (25) for the analysis of cholecalciferol or ergocalciferol results in an overestimation of the amount of vitamin D present in a sample since it is not specific enough to distinguish between vitamin D and interfering sterols (24, 26, 27). With the USP method, the sample is pretreated and then reacted with Nield's reagent; vitamin D-type compounds produce a pink color measured at 500 nm. The amount of vitamin D is determined by comparison to a standard.

It has already been shown that vitamin D is not the only compound that produces color with Nield's reagent (24, 26, 27). Several photochemical isomers that may be present in a vitamin D sample, which have little or no antirachitic activity, are quantified as vitamin D. The AOAC chemical method of vitamin D analysis was subsequently developed to eliminate the interference of one of these isomers, tachysterol. With the AOAC chemical method, the sample is treated with maleic anhydride and a Diels–Alder adduct is formed with the tachysterol, thereby making it inactive toward Nield's reagent. Thus, the AOAC chemical method is more specific than the current USP method but still overestimates the amount of antirachitic activity present in a resin sample.

This result suggests that there may yet be another interfering substance present, such as isotachysterol, that is reacting with Nield's reagent to give measurable color at 500 nm. The HPLC method eliminates this problem since the method is specific for vitamin D and there is no interference from the various impurities. Because of the separation capability, only the desired compounds are measured and quantified. From the results, it was concluded that the HPLC method most closely relates the cholecalciferol content of a resin to its antirachitic activity.

Cholecalciferol and ergocalciferol are used in many products and are often found in combination with other fat-soluble vitamins. Therefore, mixtures of cholecalciferol and other fat-soluble vitamins were investigated (Fig. 3). The high efficiency of this HPLC system allows for the separation of cholecalciferol from vitamin A acetate, vitamin A palmitate, alpha tocopherol, and alpha tocopheryl acetate. Vitamin A alcohol, phytonadione, and antioxidants are also separated from cholecalciferol by this system (not shown in Fig. 3).

Table I—Precision Study of Cholecalciferol Analysis on Cholecalciferol Resin

Run Number	Cholecalciferol × 10^{-6}, IU/g: Replicate Sample Preparations	Cholecalciferol × 10^{-6}, IU/g: Replicate Injections of One Preparation
1	20.32	20.38
2	20.34	20.36
3	20.97	20.34
4	20.69	20.25
5	20.57	20.38
6	20.22	20.21
Mean	20.52	20.32
SD	±0.28	±0.07
RSD, %	±1.37	±0.36

Table II—Results of AOAC Bioassay[a] from Two Independent Laboratories for the Analysis of Cholecalciferol in Resin Samples

Sample	Cholecalciferol × 10^{-6}, ICU[b]/g: Laboratory A	Laboratory B	Average
1	21.8	21.6	21.7
2	23.5	19.9	21.7
3	20.3	12.0	16.2
4	18.8	24.1	21.5
5	26.6	16.5	21.6
6	22.8	17.6	20.2
7	29.4	20.6	25.0
8	22.2	18.3	20.3
9	19.0	25.9	22.5
10	22.6	24.3	23.5

[a] Three-level chick method. [b] International Chick Units.

Table III—Comparison of HPLC, AOAC Chemical, and AOAC Bioassay Results for the Determination of Cholecalciferol in Resins

Sample	Cholecalciferol × 10^{-6}, IU/g: HPLC[a] Method	AOAC[a] Chemical Method	HPLC as Percent of Mean of Bioassay[b]	AOAC as Percent of Mean of Bioassay[c]
1	21.0	22.6	96.8	104.1
2	20.6	22.0	94.9	101.4
3	20.9	23.7	129.0	146.3
4	19.9	22.9	92.6	106.5
5	21.3	23.1	98.6	106.9
6	22.2	25.7	109.9	127.2
7	21.8	26.0	87.2	104.0
8	21.3	25.8	104.9	127.1
9	19.6	24.4	87.1	108.4
10	21.4	26.1	91.1	111.1
Mean			99.2	114.3
SD			±12.7	±14.5
RSD, %			±12.8	±12.7

[a] Average of two sample weights. [b] The percentage was obtained by comparing the HPLC method to the average of the AOAC bioassay (Table II). [c] The percentage was obtained by comparing the AOAC chemical method to the average of the AOAC bioassay (Table II).

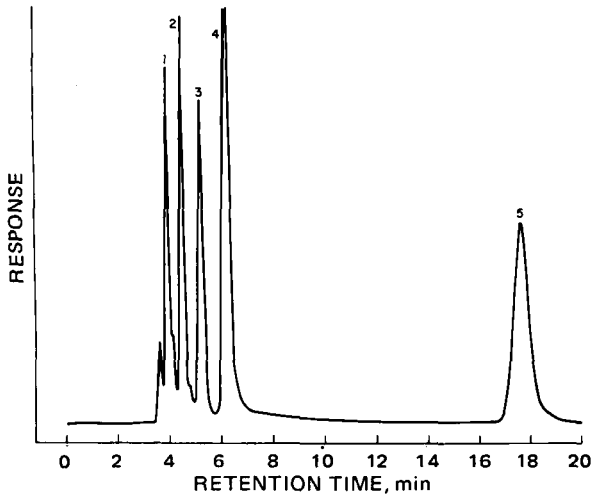


Figure 3—*Chromatogram of a synthetic mixture of fat-soluble vitamins. Key: 1, vitamin A palmitate; 2, vitamin E acetate; 3, vitamin A acetate; 4, vitamin E alcohol; and 5, vitamin D_3.*

Methodology also has been developed for the determination of ergocalciferol in vitamin A and D_2 gelatin-protected beadlets, cholecalciferol premix formulations, and commercial multivitamin formulations and will be published later.

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