Microdetermination of Ergocalciferol (Vitamin D_2) in Pharmaceutical Preparations by Differential Spectroscopy Using Trifluoroacetic Acid in the Absence of Vitamin A

SAMIR A. GHARBO ˣ and LEO A. GOSSER

Abstract □ A differential spectroscopy method for the determination of microgram quantities of ergocalciferol, using trifluoroacetic acid and hydrogen peroxide, was applied to commercially available pharmaceutical preparations. Tablets were powdered and extracted with methanol after being made basic with diethylamine. The methanol was concentrated, chloroform was added, and the mixture was chromatographed on neutral alumina using chloroform as the eluent. Ergocalciferol was determined in an aliquot of the eluate by differential spectroscopy and was compared with a reference ergocalciferol standard treated similarly. The method applied to low potency formulations (2.5–6 μg/tablet) is simple and quantitative, needs less than 5 hr for completion, and has a precision of <±2%. A simple and accurate chromatographic method for the determination of ergocalciferol in stabilized powders and high potency tablets, which precludes the necessity of solvent–solvent extraction, was also developed. The results are comparable with those obtained by the rat bioassay and the USP XVIII chemical assay.

Keyphrases □ Ergocalciferol in pharmaceutical preparations—differential spectroscopic analysis using trifluoroacetic acid, absence of vitamin A □ Vitamin D_2 in pharmaceutical preparations—differential spectroscopic analysis using trifluoroacetic acid, absence of vitamin A □ Differential spectroscopy—analysis, ergocalciferol in pharmaceutical preparations not containing vitamin A

Ergocalciferol (vitamin D_2), one of the fat-soluble vitamins (1), is essential for the prevention of rickets. The determination of ergocalciferol in nutritional and pharmaceutical products and biological materials has been the subject of extensive research work. Studies on the metabolism of ergocalciferol, its toxicity when taken in excess (2), and its consideration as a drug if assimilated in quantities greater than 400 IU/day (40 IU = 1 μg) (3) have made its evaluation even more necessary.

The rat bioassay for ergocalciferol (4) is time consuming, expensive, and imprecise. Several chemical and physicochemical methods of determination are applicable to pure ergocalciferol (5), but a tedious cleanup is usually necessary before quantitation. Saponification in an alcoholic medium followed by partition with organic solvent (6) and column chromatography (7) is the usual route taken for the extraction and purification of ergocalciferol from the sample. The main difficulty in obtaining quantitative recoveries of the vitamin is its potential instability due to the effects of heat, oxygen, and water (8).

Kobayashi and Adachi (9) determined ergocalciferol and its isomers in mixtures by GLC after separation by TLC. Mulder *et al.* (10) evaluated the chemical analysis of ergocalciferol in concentrates (≥25,000 IU/g) by the most widely used antimony trichloride colorimetric method. Kneczke and Andersson (11) modified the USP XVIII chemical method, using hydroquinone as the antioxidant, and compared the results with and without saponification of different preparations containing ergocalciferol with a bioassay method.

"Reversed-phase partition" high-pressure liquid chromatography was used for the separation and quantitation of the fat-soluble vitamins and the D vitamins (12). The technique was applied to multivitamin capsules and tablets, but the study was for chromatographic reproducibility and not for the reproducibility of the extraction methods. Fat-soluble vitamins were separated by gel filtration, and vitamin D was determined in the presence of vitamin A and α-tocopherol (13).

Recently, a stable and sensitive differential spectroscopy method for the determination of ergocalciferol, using trifluoroacetic acid and hydrogen peroxide, was developed (14). This paper deals with the application of this procedure to the determination of ergocalciferol when present in microgram quantities in commercially available pharmaceutical preparations containing no vitamin A. A simple chromatographic procedure also was developed for preparations containing milligram quantities using the trifluoroacetic acid colorimetric method recommended by Clements *et al.* (15).

EXPERIMENTAL

Instrumentation and Apparatus—A spectrophotometer[1], a rotary evaporator, a stir-hot plate, an electric micromill, and a chromatographic glass column (250 × 25 mm i.d. with a 250-ml capacity reservoir) were used.

Reagents and Chemicals—The following were used: ethanol[2] (anhydrous), methanol[3] (anhydrous), chloroform[4], diethylamine[5], trifluoroacetic acid[6] (extremely corrosive acid), hydrogen peroxide (30%), aluminum oxide (neutral)[7], silicic acid[8], and ergocalciferol (USP reference standard). Hydroquinone[6] solution (0.1%) was prepared by dissolving hydroquinone in a minimal amount of ether and diluting to volume with chloroform.

Procedure A: For Low Potency Preparations—Carry out all operations in subdued artificial light.

Preparation of Alumina Column—Pour about 20 ml of methanol into the chromatographic column and lightly tamp a pledget of

[1] Coleman 124, Perkin-Elmer.
[2] U.S. Industrial Chemicals Co.
[3] Mallinckrodt, O.R.
[4] Mallinckrodt, A.R.
[5] Matheson Coleman & Bell, ACS.
[6] Matheson Coleman & Bell.
[7] Woelm.
[8] SilicAR CC7, Mallinckrodt.

cotton into the bottom, ridding the cotton of air bubbles. Weigh 15 g of neutral aluminum oxide and transfer to a small flask. Add about 20 ml of methanol, swirl to eliminate air bubbles, and transfer completely to the prepared column using 20–30 ml of methanol. Drain the methanol to about 0.5 cm above the alumina. Add 50 ml of chloroform, level the surface of the alumina by tapping the sides of the column, and drain the solvent to about 1 cm above the alumina. Wrap the stem of the column with aluminum foil and set aside until needed.

Preparation of Standard Solution—Accurately weigh about 20 mg of reference standard ergocalciferol from a freshly opened vial into a 100-ml volumetric flask. Dissolve in and dilute to volume with chloroform (Standard Solution A-I). Dilute 5.0 ml of this solution to 100 ml with chloroform (Standard Solution A-II). Prepare a third solution by diluting 5.0 ml of Standard Solution A-II to 100 ml with chloroform (Standard Solution A-III).

Determination of Ergocalciferol in Standard Solution—Accurately pipet 10.0 ml of reference standard ergocalciferol solution in chloroform (A-III, 0.5–0.7 μg/ml) in a small flask and add 1 drop of diethylamine. Evaporate the mixture completely on a rotary evaporator at 30–35°, add 5 ml of anhydrous ethanol, and evaporate again completely. Then add 5 ml of chloroform and evaporate completely.

Flush the flask for a few seconds with a stream of nitrogen to eliminate the last traces of chloroform. Pipet 1.0 ml of 0.1% hydroquinone solution to dissolve the residue. Using a fast delivery pipet, add exactly 1.0 ml of trifluoroacetic acid, swirl quickly two or three times, and transfer to a 1-cm cell within 30 sec (cover the cell).

Record the absorbance at 490 nm as a function of time, using a mixture of chloroform and trifluoroacetic acid (1:1) as the reference solution. Determine the highest absorbance value obtained within the first 3 min. Record the spectrum between 520 and 480 nm to ensure that the peak is at 490 ± 2 nm. Add 2 drops of hydrogen peroxide from a disposable pipet to the sample solution in the cell and shake once to mix. Record the absorbance value at 490 nm obtained 2 min ± 10 sec after the addition of the hydrogen peroxide. The difference between the two absorbance values (A_{std}) is proportional to the amount of ergocalciferol in the standard solution.

Preparation of Sample Solution and Determination of Ergocalciferol—Accurately weigh a number of tablets and determine the average tablet weight. Grind the tablets to a powder, using the electric micromill for 40–50 sec or a mortar and pestle. Accurately weigh sufficient powdered tablets to contain a total of about 25 μg of ergocalciferol and transfer to a 125–250-ml amber-colored erlenmeyer or extraction flask. Add 25 ml of methanol and 0.1 ml of diethylamine. Reflux the mixture in a water bath at 65–70° for 2 hr, using a magnetic stirrer for additional agitation. Remove the magnet and rinse it with chloroform by using a disposable pipet; add the rinse to the flask. Add 20 ml of chloroform and concentrate the mixture to about 10 ml with a rotary evaporator at 30–35°. Cool under the tap and add 30 ml of chloroform.

Transfer the mixture quantitatively through a wide mouth funnel to the prepared alumina column, rinsing the flask and funnel with about 10 ml of chloroform. Wash the sides of the column with about 5 ml of chloroform and then allow the mixture to stand for about 5 min to permit the powder to settle in the column. Allow the solvent to flow slowly (80–120 drops/min) into an amber-colored, 100-ml volumetric flask. Drain the column to the level of the powder and then carefully add 10-ml portions of chloroform, draining the column each time (use at least 30 ml of additional chloroform for elution). Collect exactly 100 ml of eluate. The colored zone, due to dyes in the tablets, if present should not have progressed more than halfway through the alumina.

Pipet 20.0 ml of the eluate into a 50-ml, amber-colored erlenmeyer flask and proceed as directed for Procedure A under *Determination of Ergocalciferol in Standard Solution*, starting with "Evaporate the mixture completely on a rotary evaporator at 30–35°, . . ." The difference between the two absorbance values (A_{spl}) is proportional to the amount of ergocalciferol present.

Calculations—The amount of ergocalciferol is calculated using:

$$\mu g \text{ ergocalciferol / tablet} = \frac{A_{spl}}{A_{std}} \times \text{weight of standard (mg)} \times \frac{\text{average tablet weight (g)}}{\text{sample weight (g)}} \times 1.25 \qquad \text{(Eq. 1)}$$

Procedure B: For High Potency Preparations—Carry out all operations in subdued artificial light.

Preparation of Silica Column—Pour about 40 ml of chloroform into the column and lightly tamp a small pledget of cotton into the bottom. Weigh 5 g of silicic acid[8] in a 50-ml erlenmeyer flask, add 15 ml of chloroform, mix well, and quickly pour through a funnel into the column. Wash the flask and the sides of the column with 2 × 10 ml of chloroform. Withdraw 40–45 ml of solvent, wrap the column with aluminum foil, and set aside until needed.

Preparation of Standard Solution—Accurately weigh about 35–40 mg of reference standard ergocalciferol from a freshly opened vial into a 100-ml volumetric flask. Dissolve in and dilute to volume with chloroform (Standard Solution B-I). Dilute 10 ml of Standard Solution B-I to exactly 100 ml with chloroform (Standard Solution B-II).

Determination of Ergocalciferol in Standard Solution—Accurately pipet 8.0 ml of reference standard ergocalciferol Solution B-II in a small flask and add 2.0 ml of trifluoroacetic acid. Swirl the mixture three or four times and transfer to a 1-cm cell. Record the absorbance at 403 nm as a function of time, using a mixture of chloroform and trifluoroacetic acid (4:1) as the reference solution. Determine the highest absorbance value (A_{std}) obtained within 10 min; the absorbance value will depend on the quality of the trifluoroacetic acid and may vary from day to day, which necessitates running a standard.

Determination of Ergocalciferol in Sample Solution—Weigh accurately an amount of sample (powder or powdered tablets) to contain 3.5–4.0 mg of ergocalciferol and transfer to a small flask. Add water (2.5 times the amount of sample) and mix well for 3–4 min to get a homogeneous slurry containing no lumps. Wash the sides of the flask with ethanol equal in volume to one-third the amount of water and mix gently for 2–3 min. Swirl the mixture, add silicic acid[8] in an amount equal to four times the volume of water, and mix quickly with a spatula to get a homogeneous mobile powder within 60 sec. Immediately add chloroform in an amount equal to four times the amount of the silica and mix well.

Transfer the slurry to the previously prepared silica column through a wide mouth funnel and wash the flask, funnel, and sides of the column with 2 × 10 ml of chloroform. Allow the solvent to drip fast into an amber-colored, 100-ml volumetric flask until it reaches the level of the adsorbent. Wash the flask and sides of the column with 10-ml portions of chloroform, draining each time. Collect exactly 100 ml of eluate. Pipet 8.0 ml of eluate in a small flask and continue as directed for Procedure B under the *Determination of Ergocalciferol in Standard Solution*, starting with "add 2.0 ml of trifluoroacetic acid."

Calculations—The amount of ergocalciferol is calculated as follows:

$$\text{mg ergocalciferol/g} = \frac{A_{spl}}{A_{std}} \times \frac{\text{standard weight (mg)}}{\text{sample weight (g)}} \times \frac{1}{10} \qquad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Due to the difference in the procedure for analysis, pharmaceutical preparations containing ergocalciferol can be divided into two categories: those containing vitamin A, which are numerous, and those devoid of it, which are few. The latter can be considered as high potency or low potency preparations according to the content of ergocalciferol and its administration per day relative to the daily requirement.

The problems of extraction of ergocalciferol in microgram quantities with minimal loss, especially from sugar-coated tablets (16), with subsequent purification and choice of a sensitive method of determination usually afford a challenge to the pharmaceutical analyst. In this laboratory the need arose for a simple quality control and stability assay for the determination of ergocalciferol in a vitamin–mineral supplement containing only 3.33–6 μg/sugar-coated tablet weighing 1.3–1.4 g (2.4–4.4 ppm).

Saponification with alcoholic potassium hydroxide or aqueous-alcoholic potassium hydroxide and extraction with ether or petro-

Table I—Ergocalciferol Content of Low Potency Preparations: Comparison of Results by Procedure A and the Biological Rat Assay

Product	Procedure A			Biological Assay[a]
	Number of Assays	Ergocalciferol, μg/Tablet	Average Deviation, %	Ergocalciferol, μg/Tablet
A[b]: Lot 1	4	5.32	0.45	5.63
2	4	4.28	1.29	5.06
3	2	4.46	0.00	4.26
4	2	4.31	0.39	5.06
5	2	4.53	1.47	6.09
6	2	4.68	1.07	—
B[c]	2	3.88	0.51	3.65
C[d]	3	3.58	1.92	3.15

[a] Assays carried out by private testing laboratories. [b] Theoretical amount 5 μg/tablet. [c] Label claim 3.12 μg/tablet. [d] Label claim 2.5 μg/tablet.

Table II—Ergocalciferol Content of High Potency Preparations: Comparison of Results by Procedure B, the Biological Rat Assay, and the USP Chemical Method

Product	Procedure B			USP Method[a]	
				Ergocalciferol, mg/g	
	Number of Assays	Ergocalciferol, mg/g	Average Deviation, %	Biological	Chemical
A[b]: Lot 1	6	2.98	1.23	3.22	2.75
2	2	2.91	0.86	3.05	2.92
3	2	3.17	1.89	2.68	3.02
B[c]	8	5.90	2.60	6.45	6.00

[a] Assays carried out by private testing laboratories. [b] Label claim >2.5 mg/g. [c] Label claim 6.25 mg/g.

leum ether, or a combination of both followed by subsequent purification by alumina and/or silica chromatography, proved to be tedious and gave erratic and nonquantitative results. The presence of water and the dye in the sugar coating of the tablets were the main sources of difficulties in obtaining an extract pure enough for spectral analysis. The application of Procedure B and many modifications of it proved unsuccessful for the determination, because substances in the eluate interfered by giving an absorbance near 403 nm. Moreover, Procedure B was inadequate for the analysis due to the small amount of the vitamin in comparison to the bulk of the material and thus the need for relatively large quantities of water and adsorbent. It was the development of the stable and sensitive differential spectroscopy method at 490 nm that paved the way for an adequate analysis.

Procedure A proved to be simple, quantitative, and adequate for quality control purposes, with a precision of <±2%. The application of the method for stability studies is currently under investigation. The small amount of diethylamine is added before extraction to ensure that the methanolic milieu is on the slightly basic side to avoid destruction of the vitamin. Concentration of the methanol before chromatography is recommended, because higher percentages of methanol in the solvent contributed to the increase of interfering substances in the eluate. Interferences were detected by the increase in the value of absorbance at 490 nm after the addition of hydrogen peroxide.

The preliminary cleaning of the alumina with methanol and then chloroform is necessary to remove substances that affect the colorimetry. The slow flow of the solvent through the alumina is essential in adsorbing the dyes used in the sugar coating of the tablets; these dyes appear as a colored band in the upper part of the column. Anhydrous ethanol is added to the residue of the aliquot from the eluate and evaporated to remove mainly all traces of diethylamine. The low absorbance obtained at 490 nm after the addition of hydrogen peroxide (<0.08 as compared to 0.02–0.04 for reference standard ergocalciferol) shows that minimal interferences are eluted and are accounted for by the use of differential spectroscopy.

Procedure A, carried out on placebo tablets, verified that no significant interference was encountered. Quantitative recoveries within experimental error (±2%) of reference standard ergocalciferol added to placebo tablets showed that there was no loss through the assay procedure. The addition of sodium ascorbate or hydroquinone as an antioxidant before extraction did not improve the yield and sometimes interfered with the colorimetry.

Table I shows the results obtained with Procedure A for different samples compared with results obtained by the biological rat assay (17). The proposed method was also applied to commercially available tablets containing low potency amounts of ergocalciferol.

Variation in the results for the different lots of Product A (86–106% of the theoretical amounts) was expected due to the differences in the amounts of ergocalciferol in the lots of stabilized powder used in the tablets. In addition, low assay values could be attributed to the complex effect of heat, pressure, and long exposure to air and light during the manufacturing procedure of granulation, drying, compression, subbing, and sugar coating. Product C contained a comparatively large amount of salicylamide per tablet, which affected the accuracy of the differential spectroscopy analysis. The main bulk of the salicylamide was eliminated by concentration of an aliquot from the eluate, precipitation by addition of hexane, and filtration through cotton.

Although Procedure A takes 4–5 hr for completion, four assays can be carried out within 8 hr. The main advantages of the method are that the inconvenience of liquid–liquid partition extraction is avoided and the effect of spectral interferences can be accounted for. The differential spectroscopy method is currently under investigation for use in preparations containing vitamin A.

Ergocalciferol could be obtained commercially in the dry state, suitable for the preparation of granulations, as a stabilized form containing >2.5 mg ergocalciferol/g of powder. This stabilized powder contains mainly dicalcium phosphate, acacia, coconut oil, and preservatives as excipients. Procedure B was developed for quality control analysis of the powder as a raw material. The powder is mixed with water, which is necessary to dissolve the acacia and release the vitamin. A suitable amount of ethanol is added to dissolve the vitamin without precipitating the acacia, which would occlude some of the vitamin and thus cause a loss. Addition of the silicic acid is used to adsorb the water and also to distribute the vitamin on a large surface area and thus facilitate its extraction.

Chloroform quantitatively elutes ergocalciferol from the column. The trifluoroacetic acid colorimetric method recommended by Clements *et al.* (15) was chosen due to its simplicity and obvious advantages over the antimony trichloride method.

Procedure B was also applied to commercially available tablets containing milligram quantities of ergocalciferol. The results obtained (Table II) were compared with those obtained for samples from the rat bioassay (17) and the USP XVIII chemical method (7). Recoveries of reference standard ergocalciferol added to the stabilized powder in quantities of 0.3 and 3.0 mg for each gram of powder were 99 and 97%, respectively, which are within experimental error of the assay.

The results of Procedure B (Table II) are mostly slightly higher than those of the USP chemical method. A loss through the long procedure of saponification, solvent–solvent extraction, and column chromatography in the latter method is a possibility. The biological assay gave higher results than Procedure B for most samples. Procedure B (average deviation ≤2.6%) takes only 1.5 hr for completion; for quality control purposes, it is a suitable assay for high potency ergocalciferol preparations.

SUMMARY

Ergocalciferol could be assayed when present in microgram quantities in tablets by refluxing followed by column chromatography and differential spectroscopy using trifluoroacetic acid and hydrogen peroxide. The simplicity and accuracy of Procedure A make it adequate for quality control analysis. Preparations containing milligram quantities of ergocalciferol could be assayed for their ergocalciferol content (Procedure B) by partition/extraction chromatography followed by spectrophotometry using trifluoroacetic acid.

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[x] To whom inquiries should be directed.

Quantitative Determination of Phenol in Phenolated Calamine Lotion USP

V. DAS GUPTA [x] and KATHRYN A. BOMER

Abstract □ A method for the quantitative analysis of phenol in phenolated calamine lotion USP is described. The method is based on spectrophotometrically measuring the color produced by reacting phenol with either ferric chloride or ferric nitrate. Beer's law is followed. The effect of ferric-ion concentration on the sensitivity of the assay method is reported.

Keyphrases □ Calamine lotion, phenolated—colorimetric analysis of phenol □ Phenolated calamine lotion—colorimetric analysis of phenol

Phenolated calamine lotion is an official USP (1) product. Although phenol is a potent therapeutic agent, the USP does not require its quantitative analysis in phenolated calamine lotion.

Kiposki and Allen (2) reported a bromometric method for the analysis of phenol. Stanko and DeKay (3) recommended a complicated colorimetric method for the quantitative analysis of phenol in phenolated calamine lotion based on reaction with copper sulfate. This report describes a simple colorimetric assay method for the analysis of phenol in phenolated calamine lotion USP. The method is based on an identification test with ferric chloride as described for phenol USP (4).

EXPERIMENTAL

Reagents and Chemicals—All chemicals and reagents used were USP, NF, or ACS grade. $FeCl_3 \cdot 6H_2O$[1], $Fe(NO_3)_3 \cdot 9H_2O$[2], and phenol loose crystals[3] were purchased and used without further purification.

[1] Mallinckrodt Chemical Works.
[2] J. T. Baker Chemical Co.
[3] Matheson, Coleman and Bell.