Assay for Vitamins D_2 and D_3 in the Presence of Tocopherols (Vitamin E) and Vitamin A

D. K. FEETER, M. F. JACOBS, and H. W. RAWLINGS

Abstract ☐ A GLC procedure for determination of vitamins D_2 and D_3 is described. By pretreatment consisting of saponification and subsequent esterification of all free hydroxyl groups to their respective propionates, vitamins D_2 and D_3 can be isolated from the interference usually encountered from other oil-soluble vitamins during GLC analysis. The procedure described, including the "internal standard" technique, supplies precise data rapidly, with accuracy comparable to that obtained by the vitamin D assay method given in USP XVII.

Keyphrases ☐ Vitamins D_2, D_3, determination, differentiation—dosage forms ☐ Tocopherols, vitamin A in dosage forms—vitamins D_2, D_3 determination ☐ Trioctanoin—internal standard ☐ GLC—analysis

Much work has been done to develop a satisfactory physicochemical method for assaying vitamin D. Although biological methods for assaying vitamins D_2 and D_3 give answers of good accuracy, they are imprecise, time consuming, and expensive. The biological methods are thus impractical for use in trade negotiations or in manufacturing control.

An excellent review of the extensive work done in developing an instrumental procedure was given by Richter (1). All this work finally culminated in the adoption by USP XVII (2) of a procedure closely based on that described by Theivagt and Campbell (3). In the USP procedure, the Nield *et al.* (4) antimony trichloride reagent is used.

Although this USP procedure serves industry as the "official assay" to date, serious shortcomings still exist. Its basis is an elaborate work-up, including two column chromatographic steps to eliminate interference due mainly to vitamin A. Mixtures of vitamins A and D can be assayed with fair success up to a maximum A:D ratio of about 20:1. Serious interference has been noted when vitamin E and certain sterols (notably tachysterol) are also present. Osadca and De Ritter (5) and Mulder *et al.* (6) described additional steps recommended for overcoming these difficulties. Furthermore, this assay procedure requires a minimum of 6 hr.

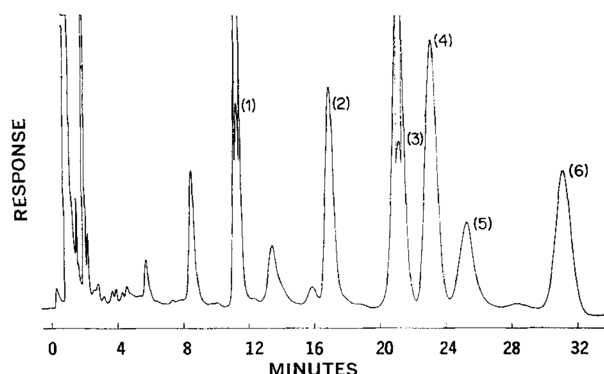

Figure 1—*Vitamin D_2-tocopherol resolution (for propionate esters). Key: (1), trioctanoin; (2), δ-tocopherol; (3), γ-tocopherol; (4) pyrovitamin D_2; (5), α-tocopherol; and (6), isopyrovitamin D_2.*

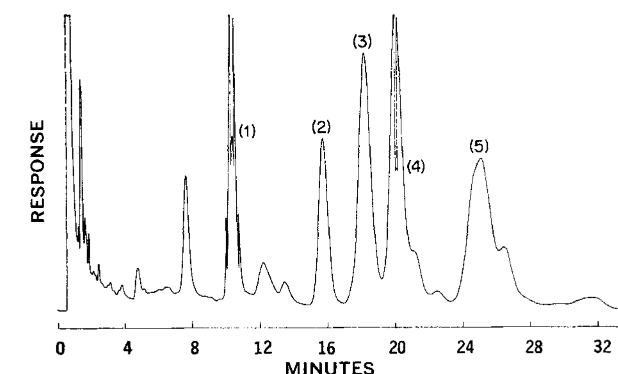

Figure 2—*Vitamin D_3-tocopherol resolution (for propionate esters). Key: (1), trioctanoin; (2), δ-tocopherol; (3), pyrovitamin D_3; (4), γ-tocopherol; and (5) isopyrovitamin D_3 + α-tocopherol.*

An analyst must be experienced in the art of preparing the critically important chromatographic columns. Under the best conditions, intralaboratory assay precision is in the range of $CV \pm 5\%$, but interlaboratory discrepancies of 10–25% are not uncommon.

The interference of vitamin A with the determination of vitamin D by GLC is widely recognized (7–9). Removal of vitamin A generally necessitates lengthy pre-GLC work-up. No mention is made in the literature of the interference of tocopherols which have the same retention times as the D vitamins when the natural vitamins or their trimethylsilyl ether derivatives are chromatographed.

To overcome these disadvantages, an improved assay procedure was developed based on GLC. The procedure consists of preliminary saponification and extraction steps to remove some interfering materials, and then isolation and concentration of vitamin D, vitamin E, and vitamin A in their alcohol forms. The mixture so obtained is treated with propionic anhydride to esterify all the alcohols to their corresponding propionates.

By using trioctanoin as an internal standard and GLC columns of SE-52 (for vitamin D_2, see Fig. 1) and

Table I—Relative Retention Data of Pertinent Propionate Esters to Trioctanoin

Compound	SE-52 Column	SE-30 Column
Trioctanoin	1.00	1.00
Propionate ester of:		
α-Tocopherol	2.38	2.38
β-Tocopherol	1.96	1.96
γ-Tocopherol	1.96	1.96
δ-Tocopherol	1.56	1.54
Pyrovitamin D_2	2.17	2.04
Isopyrovitamin D_2	2.94	2.86
Pyrovitamin D_3	1.96	1.82
Isopyrovitamin D_3	2.63	2.50

Table II—Replicate Vitamin D_2 Assays of Six Randomly Picked Commercial Blends of Vitamins A, D_2, and E

	Label Potency, Vitamin D_2 Units/g.					
	50,000	50,000	50,000	105,000	400,000	150,000
Assay 1	60,000	65,600	63,500	131,200	408,900	169,000
Assay 2	61,500	64,600	61,000	132,400	406,100	163,700
Assay 3	62,200	66,000	63,300	132,700	401,100	168,100
Assay 4	61,300	63,200	62,500	133,600	413,000	169,000
Average	61,250	64,850	62,580	132,500	407,200	168,000

SE-30 (for vitamin D_3, see Fig. 2), vitamin D and the tocopherols are separated effectively, while vitamin A pyrolysis products elute in the "solvent front."

EXPERIMENTAL

Materials—The following were used: (*a*) reference standards of activated 7-dehydrocholesterol USP and ergocalciferol USP; (*b*) vitamin D_2 for preparing known blends—ergocalciferol; (*c*) vitamin D_3 for preparing known blends—activated 7-dehydrocholesterol[1]; (*d*) trioctanoin for internal standard[2]; (*e*) Diatoport-S solid support, 80/100 mesh[3]; (*f*) silicone liquid phases SE-30 and SE-52[4]; (*g*) chloroform, B&A reagent grade; (*h*) propionic anhydride[5]; (*i*) pyridine[6]; (*j*) toluene, B&A reagent grade; (*k*) diethyl ether, B&A reagent grade; and (*l*) butylated hydroxytoluene[7].

Pretreatment of Liquid Samples—One gram of sample and 0.05 g. of butylated hydroxytoluene were weighed into a 250-ml. saponification flask. To this mixture, 6 ml. of 50% aqueous potassium hydroxide solution and 34 ml. of denatured (3A) alcohol were added. An air condenser was attached, and the mixture was placed on a hot plate to reflux for 30 min. The flask was then cooled under cold water, 100 ml. (pipet) of toluene was added, and the flask was stoppered and shaken vigorously for 1 min. The toluene solution was then transferred to a 250-ml. separator and washed once with 40 ml. of 1 *N* aqueous potassium hydroxide solution, once with 40 ml. of 0.5 *N* aqueous potassium hydroxide solution, and then with 40-ml. portions of distilled water until the water layer was neutral to phenolphthalein. The last water wash was drawn off, and the toluene solution was dried by shaking vigorously with about 20 g. of anhydrous sodium sulfate.

An aliquot of the toluene solution, containing at least 100 mcg. of vitamin D, was transferred to a suitable small flask; 5 ml. of a chloroform solution of purified trioctanoin was added (trioctanoin should not exceed 10 times the estimated weight of vitamin D).

The solvents were removed under vacuum on a 40° water bath. To the residue were added 1 ml. of pyridine and 2 ml. of propionic anhydride, and the mixture was heated for 15 min. at 80° on a hot plate. The reagents were then removed at 50-70° under a stream of nitrogen, and the residue was immediately dissolved in an appropriate volume of chloroform.

Pretreatment of Tablet Samples—A number of tablets (equivalent to at least 25 mcg. of vitamin D) and 0.050 g. of butylated hydroxytoluene were finely ground in a 250-ml. saponification flask. To this powder, 5 ml. of distilled water and 6 ml. of 50% aqueous potassium hydroxide solution were added. The mixture was warmed to make a slurry, 34 ml. of denatured alcohol was added, and the sample was saponified as with liquid samples. The flask was then cooled under cold water, and its content was transferred quantitatively to a 500-ml. separator with 150 ml. of diethyl ether. Fifty milliliters of distilled water was added to the saponification flask, which was warmed and swirled to disperse the residue. The resultant dispersion was cooled and added to the separator. The flask was then rinsed with 20-30 ml. of ether, which was also added to the separator. The water layer was extracted twice with ether, and the combined ether extracts were washed with 50-ml. portions of distilled water until the water layer was neutral to phenolphthalein.

To the ether solution, a 5-ml. chloroform solution of purified trioctanoin (not to exceed 10 times the estimated weight of vitamin D) was added. The solution was transferred to a 500-ml. conical flask and the solvents were removed under nitrogen. The esters were then prepared in a suitable small flask.

Crystalline and resin samples received no preesterification treatment.

Low-actinic glassware was used throughout the preparation of samples.

Column Preparation—Prior to coating, 15 g. of solid support was added to 90 ml. of propionic anhydride and heated at 80° for 2 hr. The bulk of the anhydride was removed by filtration through a büchner funnel, and final traces were removed in a 75° vacuum oven at 10 mm. Hg.

The vitamin D_2 column packing was prepared by adding 15 g. of propionated support to 90 ml. of toluene, containing 2.0% (w/v) of SE-52 liquid phase, and allowing the mixture to stand at room temperature for 60 min. with occasional gentle stirring. The coated support was filtered on a büchner funnel until dry. A 2.44-m. × 0.63-cm. (8-ft. × 0.25-in.) glass column was packed by applying suction and tapping gently.

The vitamin D_3 column was prepared by adding 15 g. of the propionated support to 90 ml. of chloroform, in which 0.60 g. of

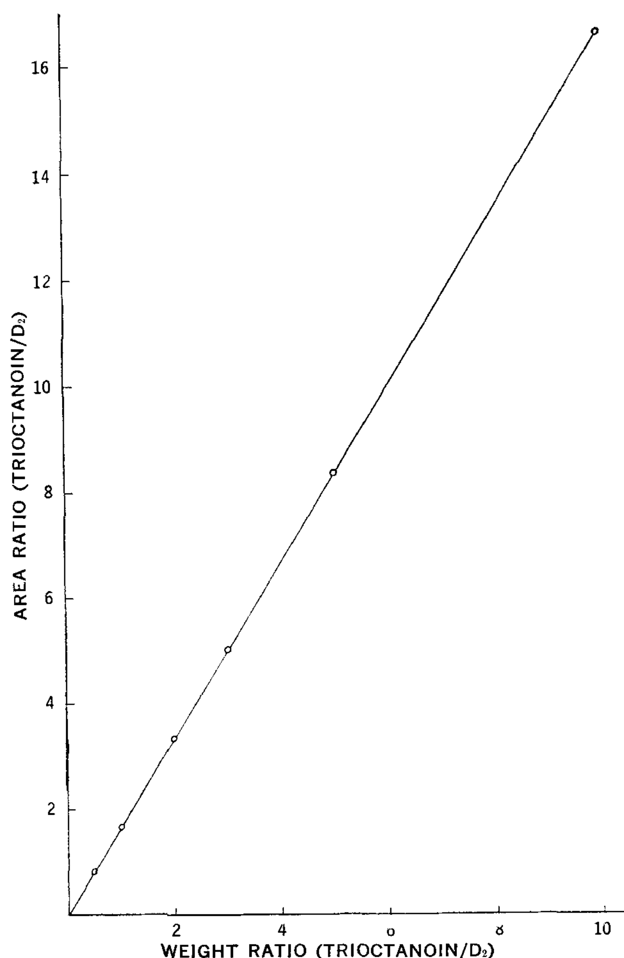

Figure 3—*Relative weight response of vitamin D_2 propionate* versus *trioctanoin:* (a) *peak areas are average of three determinations made over a period of 2 days; and* (b) *1.6 mcg. trioctanoin injected each time.*

[1] Eastman Organic Chemical 8090.
[2] Eastman Organic Chemical 2097, redistilled in laboratory.
[3] Hewlett-Packard, Avondale, Pa.
[4] Supelco Inc., Bellefonte, Pa.
[5] Eastman Organic Chemical 1291.
[6] Eastman Organic Chemical H-214.
[7] Tenox, Tennesse Eastman Co., Kingsport, Tenn.

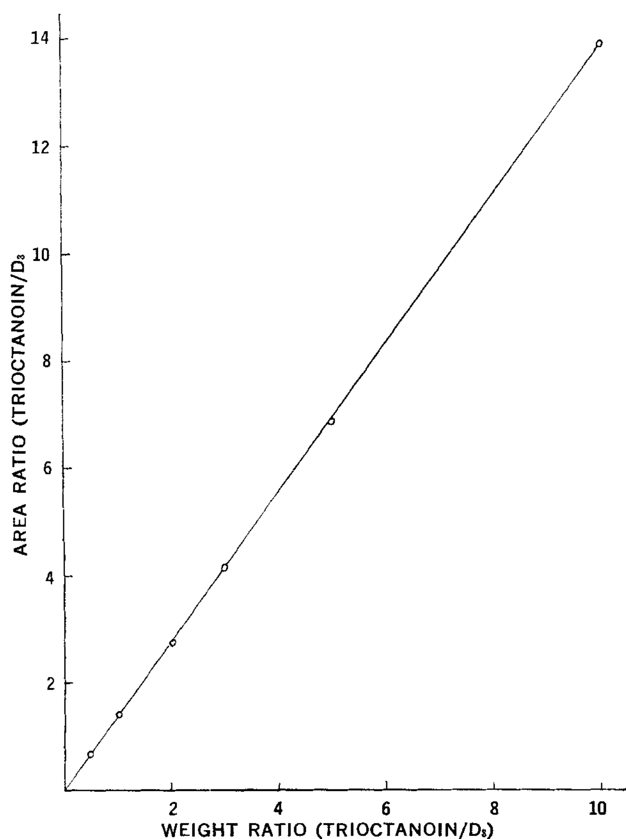

Figure 4—*Relative weight response of vitamin D_3 propionate* versus *trioctanoin:* (a) *peak areas are averages of three determinations made over a period of 3 days; and* (b) *1.6 mcg. trioctanoin injected each time.*

SE-30 liquid phase was dissolved, and allowing the mixture to stand at room temperature for 18 min. with occasional gentle stirring. The coated support was filtered, dried, and packed into a 2.44-m. × 0.63-cm. (8-ft. × 0.25-in.) column.

Columns were conditioned with the column attached to the oven inlet port only. Air was purged from the column with helium; then the helium flow was shut off. The oven temperature was programmed from ambient to 300° at 1°/min. and held for 64 hr. The oven temperature was then lowered to 275°, and the helium flow was adjusted to 75 ml./min. These conditions were maintained for 2 hr., after which the column was ready for use.

Analysis—An F&M model 810 gas chromatograph equipped with a hydrogen flame-ionization detector was used. The injection port temperature was set at 280°; the detector block temperature at 300°; and the column oven temperature at 270° for vitamin D_2 or at 240–250° for vitamin D_3. Helium flow rate was 75 ml./min.; hydrogen flow rate, 60 ml./min.; and air flow rate, 500 ml./min.

For each determination, a volume of sample solution containing approximately 1.6 mcg. of internal standard was injected at an attenuation of 10 × 8. Following elution of the internal standard, the attenuation was adjusted to give a satisfactory vitamin D peak.

Standard relative weight responses were obtained from chromatograms of the USP reference standards *versus* trioctanoin. These solutions were prepared by propionating (as before) a mixture of 30 mg. USP standard and 15 mg. trioctanoin, removing reagents, and diluting the residue to 10 ml. with chloroform.

The vitamin D potency in units per gram is given by the equation:

$$\text{potency (u./g.)} = \frac{Ax}{Wx} \times \frac{Ws}{As} \times F \times (40 \times 10^6) \quad \text{(Eq. 1)}$$

where Ax = area of the sample pyrovitamin peak, Wx = weight of sample, As = area of internal standard peak, Ws = weight of internal standard, $F = (As/Ws) \times (WR/AR)$ = relative weight response factor, WR = weight of *reference* standard, AR = area of reference pyrovitamin peak, and (40×10^6) = potency as units per gram of pure vitamin D. Areas of all peaks were determined by peak height times width at one-half peak height.

Table III—Vitamin D_3 Assays of Laboratory-Prepared Blends of 7-Dehydrocholesterol, Vitamin A Palmitate, Mixed Tocopherols, and Vegetable Oil

Blend[a]	Ratio A:D	Vitamin D Potency, Units/g. Calculated	Found	
M	2.2:1	686,000	690,000	680,000
A	2.2:1	344,000	339,000	340,000
B	2.2:1	172,900	169,600	170,000
C	2.2:1	70,700	70,000	69,200
D	2.2:1	35,800	34,700	35,800

[a] Each blend contained a 1.3:1 ratio of γ-tocopherol to vitamin D_3.

Table IV—Vitamin D_2 Assays of Laboratory-Prepared Blends of Ergocalciferol, Vitamin A Palmitate, and Mixed Tocopherols

Blend[a]	A:D Ratio	Vitamin D Potency, Units/g. Calculated	Found
M	1.5:1	643,000	640,000
A	3.2:1	319,000	320,000
B	6.5:1	160,400	157,400
C	16.0:1	65,800	66,100
D	32.0:1	33,200	33,400

[a] Each blend contained a 1.5:1 ratio of γ-tocopherol to vitamin D_2.

RESULTS AND DISCUSSION

The treatment of the solid support and the column-conditioning procedure were critical factors for eliminating on-column degradation of the vitamin D esters. The results obtained were satisfactorily reproducible; the columns so prepared exhibited efficiencies of 300–400 plates/ft. [$16\,(tR/\Delta t)^2$], where tR is the uncorrected retention time and Δt is the peak width at the base.

Optimum conditions for esterification of vitamin D were determined by monitoring the rate of ester formation with GLC and by comparing the weight response of various levels of vitamin reacted. The corresponding pyro and isopyro thermal rearrangement isomers (10, 11) were observed.

The retention times of pertinent propionate esters relative to trioctanoin are given in Table I.

As noted previously (11), thermal rearrangement is only slightly dependent upon injection port temperature. For the propionate esters, the ratio of pyro to isopyro forms was constant for port temperatures between 230 and 300°. Walle *et al.* (12) reported a

Table V—Comparison of GLC and USP XVII Procedures for Various Multivitamin Preparations

Sample	Potency by USP XVII	Potency by GLC	Label Potency
Vitamin D_3 resin, units/g.	23.5×10^6	23.4×10^6	25×10^6
	22.7×10^6	25.7×10^6	26×10^6
Vitamin D_3 oil, units/g.	33,000	47,000	40,000
Vitamin D_2 oil, units/g.	63,400	61,800	50,000
	60,100	63,200	50,000
	48,900	50,300	50,000
	1.14×10^6	1.19×10^6	1.0×10^6
	60,900	61,200	62,500
	37,000	62,000	50,000
	54,600	52,100	50,000
	45,200	60,000	50,000
Vitamin D_2 crystal, units/g.	14.9×10^6	15.2×10^6	—
Vitamin D_2 beaded, units/g.	169,600	168,000	150,000
	62,600	62,600	50,000
	420,000	407,200	400,000
	128,800	135,000	100,000
Vitamin D_2 tablet, units/tablet	16	12.5	10–15
	13	14.6	10–15
	12	11.6	10–15
	13	11.5	10–15

contribution of vitamin A to the degree of rearrangement of the trimethylsilane derivatives. No such effect was noted in this study using the propionate esters. However, some difficulty was encountered with high proportions of vitamin A causing a large "solvent front" in the region where the internal standard was eluting. This was readily overcome by using a higher level of internal standard which allowed a higher attenuation in the region of interest and a consequent reduction of the "front" magnitude.

The response of vitamin D relative to trioctanoin was determined over the range of weight ratios used by plotting the mean ratios of the peak areas against the weight ratios and calculating the equations of the lines by the method of least squares (Figs. 3 and 4). The lines passed through the origin and had standard deviations of 0.011 and 0.013 for vitamin D_2 and vitamin D_3, respectively.

Column overloading was observed when injections of the internal standard exceeded 4 mcg.

At the start of each day's run, the chromatogram of the first injection was discarded because its value was sometimes erroneous.

The precision attained by this method is indicated by the data in Tables II and III.

Blends for accuracy analysis (Tables III and IV) contained sufficient γ-tocopherol to produce a peak height equivalent to pyrovitamin D and vitamin A in excess of the amount normally encountered in multivitamin preparations.

For comparison, assays of a variety of samples which had recently been assayed by the procedure given in USP XVII are given in Table V. The "oil," "beaded," and "tablet" samples are multivitamin preparations of various potencies.

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X-Ray Diffraction Analysis for Identification of Kaolin NF and Bentonite USP

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Abstract ☐ The identities of kaolin NF and bentonite USP were tested by using the identification test stated in the NF XIII monograph on kaolin. The results were inconclusive because both clays gave positive results to the test. Since kaolin NF and bentonite USP are crystalline in nature, X-ray diffraction analysis could be used for their identification. Because of its accuracy, X-ray diffraction analysis is recommended to be included in the NF and USP monographs on kaolin and bentonite, respectively, as an alternative test.

Keyphrases ☐ Kaolin—identification ☐ Bentonite—identification ☐ X-ray diffractometry—identification, kaolin, bentonite

Kaolin was first introduced into the USP VIII in 1905 as a constituent of Cataplasma Kaolini. Later, it was deleted from the USP IX and was introduced into the NF IV in 1916. Bentonite was first introduced into the USP XII in 1942. The NF XIII monograph on kaolin includes a specific identification test, which is merely a test for the presence of aluminum. On the other hand, the USP XVIII monograph on bentonite does not include a specific identification test but relies on tests such as gel formation and swelling power.

Since both clays are chemically similar and contain aluminum, it was obvious that methods other than the chemical analysis for aluminum needed to be included in the NF monograph on kaolin in order to distinguish between kaolin NF and bentonite USP. Furthermore, it was felt that a specific identification test should be included in the USP monograph on bentonite. The objective of this study was to show the importance of the inclusion of X-ray diffraction analysis in the NF and USP monographs on kaolin and bentonite, respectively. Since both clays are crystalline in nature (1), it was possible to utilize X-ray diffraction analysis to measure the angles of diffraction of the X-ray from the atomic planes of the crystal.

EXPERIMENTAL

NF XIII Identification Test for Kaolin—The NF XIII identification test for kaolin was conducted on kaolin NF and bentonite USP. Both clays responded positively to the test for aluminum, *i.e.*, a gelatinous, white precipitate was produced.

X-Ray Diffraction Analysis—Two types of samples were prepared. An unoriented powder sample was prepared by packing the powder in an aluminum planchet. The powder was smoothed by