Thin-Layer Chromatographic Assay of Vitamin D in High-Potency Preparations

Analysis of Fat-Soluble Vitamins IX

By K. H. HANEWALD, F. J. MULDER, and K. J. KEUNING

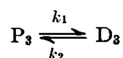
Attention is called to the reversible thermal isomerization of calciferols (D) to precalciferols (P) as a factor interfering with vitamin D assay. Distinction is made between potential vitamin D content (D + P) and actual vitamin D content (D only). The potential D content can be determined by the well-known Nield reaction. For the determination of the actual D content the Bolliger TLC method was modified to make it suitable for irradiation products: tachysterol is eliminated, the protection against oxidation was improved, P and D are isolated and may be determined separately. Applying this method, the isomerization rates of vitamins D_2 and D_3 were proved to be equal; the effects of boiling in solutions, saponification, and Soxhlet extraction on the ratio of P and D were examined. "Glassy" vitamin D resins appear to isomerize very slowly; "pasty" resins isomerize at the same rate as dilute solutions.

DISCREPANT RESULTS of vitamin D assays are frequent. Though admittedly the analytical techniques of the determination of ergocalciferol (D_2) and cholecalciferol (D_3) are not simple, a very real source of error is the neglect of the slow reversible isomerization, calciferol $\rightleftharpoons$ precalciferol (P) (1). It induced this laboratory to investigate the influence of the isomerization reaction on the results of vitamin D assay methods.

Potential and Actual Vitamin D Content—In vitamin D analysis the first thing necessary is to define what is meant by "vitamin D content." Distinction must be made between the *actual* vitamin D content, that is the amount of calciferol alone, and the *potential* vitamin D content, meaning the sum of calciferol and precalciferol, which is independent of the ratio of both components. The authors posit that the actual vitamin D content alone does not give any useful information and that only the potential vitamin D content is suitable for the evaluation of preparations. Simultaneous determination of both components D and P separately gives additional information, if wanted.

Isomerization of the Calciferols—Only in solutions do the calciferols isomerize reversibly to the corresponding precalciferols, forming an equilibrium mixture (2–4).

Hanewald (5) determined by ultraviolet absorptiometry the first-order rate constants for



$$P_3 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D_3$$

and their dependence on temperature:

$$\log k_1 = \frac{-4200}{T} + 10.29 \quad \text{and}$$

$$\log k_2 = \frac{-5180}{T} + 12.53$$

(k in min.$^{-1}$; T in °K.).

From these rate constants the fractions P/(P + D) and D/(P + D) can be calculated at a given temperature, as can the time needed to approach the equilibrium (1).

From the literature it is not clear if the isomerization rates of $P_2 \rightleftharpoons D_2$ and $P_3 \rightleftharpoons D_3$ are equal. Using the analytical methods described below this equality was affirmed. Furthermore, the isomerization rates in the crude irradiation products of 7-dehydrocholesterol were investigated, which after evaporation to dryness have a resin-like consistency. Besides the commercial importance of these resins, it is of interest to compare the rates in the resins with those in solutions which seem to be temperature dependent only and with those in the solid state (crystalline state is temperature independent).

Determinations of Potential and Actual Vitamin D Content—Since too little attention has been given to the isomerization of the calciferols, few methods of vitamin D assay give reliable values either of the potential or of the actual vitamin D content. Most methods lead to results between these two values (1).

The *potential* vitamin D content can be determined directly by absorptiometry, at 500 mμ, of the pink color obtained with antimony trichloride (Nield reagent). The result is identical for D, P, and tachysterol (6). Vitamins A and E and many other polyenes interfere and are eliminated beforehand (7).

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The *actual* vitamin D content of solutions containing only D and P can be determined by an ultraviolet-absorptiometric two-component analysis. It is calculated from the absorbance at 265 mμ and the potential vitamin D content known from Nield reaction or other sources (5, 6). The P content is obtained simultaneously. Separation is not required. In solutions of irradiated ergosterol or 7-dehydrocholesterol, by-products such as lumisterols and tachysterols interfere.

Biological assay can give the potential vitamin D content, provided that the necessary precautions for equilibration of sample and standard are observed; the actual vitamin D content cannot be determined by biological assay (1).

A reliable and not too vulnerable determination of the actual vitamin D content requires the separation of D and P. Presently this can be done only by thin-layer chromatography (TLC), or in a much longer time by paper chromatography (8). Bolliger and König (9) published a useful TLC method for vitamin D assay in 1965; for previous work, most of it qualitative or semiquantitative, the authors refer to the paper (9) and to Bolliger's chapter in Stahl's handbook on TLC (10). Bolliger succeeds in separating calciferol and precalciferol; unfortunately, in an attempt to correct for its biological activity, only part of the precalciferol is included in the determination which results in values between the potential and actual vitamin D content. Isomerization of samples and standard solutions before and during the analysis is not taken into account, nor is the contamination with tachysterol, often present in vitamin D preparations, which is not separated from calciferol by TLC (11). Tachysterol gives the same absorptivity with Nield reagent but can be eliminated in the analysis of irradiation products by the addition of maleic anhydride (MA) at 75° (6).

As no practical method for the determination of the actual vitamin D content and of previtamin D was available, the Bolliger method was modified for this purpose (*a*) by collecting the P and D spots separately for quantitative analysis, (*b*) by preventing oxidation more thoroughly, (*c*) by elimination of tachysterol (TA) from the D + TA mixture isolated by TLC.

EXPERIMENTAL[1]

Chromatography—On silica plates as described below the R_f values were measured for the calciferols (D), the precalciferols (P), lumisterol (L), and tachysterol (TA), using the solvent media: cyclohexane + ether (1 + 1) (9):0.32 (D), 0.42(P), 0.37 (L), 0.32 (TA); benzene + ether (3 + 2): 0.37 (D), 0.48 (P), 0.43 (L), 0.37 (TA); benzene + ether (4 + 1) (solvent III):0.29 (D), 0.42 (P), 0.34 (L), 0.29 (TA). D and TA are not separated.

BHT (0.01%) in the solvent medium suffices to prevent oxidation during development; squalane is necessary for protection during the application. The protection is demonstrated easily with ^{14}C-labeled cholecalciferol. Using both squalane and BHT no tailing occurs, but with only BHT tailing by deterioration is evident. The developed and dried plate is stable for 1 day.

Elimination of Tachysterol—In the manufacture of vitamins D by irradiation P is formed, which is isomerized to D. The resins are obtained by evaporation of the solvent. Of the by-products, only the tachysterols interfere with the Nield reaction. Tachysterols, calciferols, and precalciferols are converted by addition of maleic anhydride into adducts indifferent to antimony trichloride, but the addition rate of TA is much larger, therefore TA can be eliminated. In the chemical assay this has been done at 75° (6). To avoid side reactions like oxidation and to simplify the analysis, this temperature was lowered to 25°, but the rate was restored by a 10-fold MA concentration. One milligram of tachysterol was dissolved in 1 ml. of 10% MA solution in benzene; 15, 30, 60, and 120 min. after dissolving, the solution was diluted with benzene and the residual tachysterol content determined with Nield's reagent.

No color was observed in any of the four experiments, thus at least 98% of the tachysterol had been eliminated within 15 min. The chosen reaction time for addition at room temperature was 0.5 hr. The same experiments with 1 mg. of pure cholecalciferol gave calciferol recoveries of 98.1, 98.6, 99.7, 99.0%. Recovery experiments with mixtures of tachysterol and calciferol gave the same results (recovery TA = 0%, recovery D = 99%).

Thin-Layer Chromatographic Determination of Actual Vitamin D and Previtamin D in the Presence of Tachysterol

Reagents and Chemicals—Benzene, thiophene-free; ether, free from peroxide and acids; dichloromethane, distilled; ethanol, 96% v/v, free from aldehyde and acids. Solvents: (I) 0.01% BHT in benzene; (II) 1% squalane and 0.01% BHT in benzene; (III) benzene + ether (4 + 1 vol.) + 0.01% BHT. Maleic anhydride (MA), freshly distilled, 10% w/v solution in benzene. Calciferol standard solution, 10 mcg./ml. crystalline cholecalciferol in solvent I (stable for at least 3 months in an amber-colored bottle in the dark, control occasionally by comparing with a fresh standard solution). Nield reagent, 20% $SbCl_3$ and 2% acetylchloride in chloroform; for preparation the authors refer to *Reference 12.*

Apparatus—Chromatoplates, 200 × 100 × 4 mm. with a thin layer (0.25 mm.) of Soxhlet extracted (24 hr. with methanol and 24 hr. with dichloromethane) Silica Gel GF 254 (Merck) and dried at 110°; vacuum collectors with porous-glass disk and ground joints; spectrophotometer, *e.g.*, Coleman Junior with 19 × 105-mm. cells; rapid-delivery pipet, 5 ml.

Procedure—Dissolve *w* g. of sample (containing approximately 50 mg. of D) in 50.0 ml. of solvent I

[1] With the collaboration of Mrs. M. Damen-van't Sant.

(for oils) or solvent II (for resins and for ethanolic solutions after evaporation to dryness *in vacuo* at room temperature with 3 × 10 ml. of benzene). Pipet 0.5 ml. of the solution on a 8-cm. line at 15 mm. from the lower edge of a chromatoplate. Develop with solvent III until the front is 17 cm. past the origin and dry the plate. Mark in ultraviolet light (254 mμ) the P region (R_f = 0.42) and the D + TA region (R_f = 0.29) and remove the light. Scratch (9) or suck (13) the silica from between the marks quantitatively into two dry collectors. Clean both open spaces on the plate with cotton wool moistened slightly with ethanol and put each plug into the corresponding collector. Extract P from its collector with 2 × 25 ml. of dichloromethane (nitrogen pressure). Evaporate extract to dryness *in vacuo* (cold), release vacuum with nitrogen, and dissolve P residue in 5.00 ml. of benzene. [According to USP XVII (14) 1,2-dichloroethane can be used instead.] Extract D + TA similarly but collect and evaporate the extract in a 50-ml. volumetric flask. Dissolve D residue in 1.00 ml. of MA solution. Keep at room temperature for 30 min. Then make up to volume with benzene.

Measurement—Pipet 1 ml. of the final benzene solution (P or D, respectively) into a cell. Add 5 ml. of Nield reagent from rapid-delivery pipet, swirl, and 60 sec. (stopwatch) after addition, measure the absorbance at 500 mμ against air (= A). Reagent blank: treat 1 ml. of benzene accordingly (absorbance = A_b). Standard: treat 1 ml. of standard solution accordingly (absorbance = A_s). The cholecalciferol (ergocalciferol) and the precholecalciferol (preergocalciferol) contents of the sample are:

$$\frac{C_s}{C} \times \frac{A - A_b}{A_s - A_b} \text{ mcg./g.}$$

C_s = concentration of standard solution in mcg./ml. (= 10); C = sample in the final benzene solution in g./ml. (= $w/500$ for P, and $w/5000$ for D).

Remarks—This modified determination of actual D and P simultaneously by TLC is suitable for samples containing irradiation products and vitamin D resins. However, in the determination of the actual D content the conditions starting from the moment of sampling should be such that isomerization virtually cannot occur, *i.e.*, by handling at low temperature.

By this method the actual D content in oily concentrates can only be determined in samples containing at least 6 mg. (240,000 I.U.) of vitamin D per g. of oil. At lower concentrations the oil must be removed, for which Bolliger and König (9) made suggestions. The problem is to maintain unchanged the ratio of P and D. Generally, high-potency vitamin D preparations do not contain vitamin A. Vitamin A, thus far not separated from P and D and thus contaminating the isolated spots, should be eliminated before the Nield reaction is carried out, *e.g.*, by chromatography on Florex (7, 9). The modified TLC method also gives the potential D content by adding the contents found for D and P. If only the potential D is desired both removed regions can be combined and thereafter treated as a single sample of D + TA.

APPLICATIONS AND RESULTS

In the following experiments determinations were done by the TLC method described above, by absorptiometric two-component analysis, and by Nield reaction.

Absorptiometry—The absorptivity $a(= A^{1\%}_{1\text{ cm.}})$ is independent of the solvents used here; this applies to each of the compounds D_2 (475), D_3 (485), P_2 (208), and P_3 (218). All absorbances were measured at 265 mμ, the absorption maximum of D_2 and D_3, though the absorption maximum of P_2 and P_3 is 260 mμ. The fractions of P and D can be calculated from the difference of the absorptivities (a = sample, a_D = pure D; a_P = pure P):

$$[P] = \frac{a_D - a}{a_D - a_P} \cdot 100\% \quad \text{and} \quad [D] = \frac{a - a_P}{a_D - a_P} \cdot 100\%.$$

Nield Reactions—All were performed in the same way: aliquot parts of the solutions to be analyzed were evaporated to dryness *in vacuo* at room temperature and redissolved in a measured volume of benzene to obtain a concentration near 10 mcg./ml. These solutions were treated as described in the TLC procedure, starting at "Measurement."

Isomerization by Boiling for 0.5 Hour in Various Solvents—Ergocalciferol and cholecalciferol were dissolved in cold methanol, ethanol, and isooctane and refluxed for 0.5 hr. in a nitrogen atmosphere. The absorbances of the cold solutions were measured at 265 mμ, immediately after preparation and again after refluxing. The TLC and the absorptiometric determinations were done in the same solutions. In Table I the fractions of P and D are given in % of potential D content (P + D) found. The recovery is the potential D found in % of submitted calciferol.

Saponification—Crystalline cholecalciferol was dissolved in peanut oil at low temperature. The potential as well as the actual D content = 47.5 mg./g.

Two grams of the oily solution was saponified by heating for 0.5 hr. under nitrogen with 5 ml. of 2 *N* KOH in methanol, in ethanol, or in glycolmonomethylether (purified) at 60, 80, and 100°, respectively. After cooling (nitrogen), 8 ml. of methanol and 20 ml. of water were added. The liquids were extracted with 4 × 40 ml. of ether and washed with 2 × 50 ml. of 0.5 *N* aqueous KOH and 4 × 50 ml. of water.

In all samples the following determinations were done: (*a*) D + P with Nield's reagent (potential D content). (*b*) The apparent vitamin D content, by absorptiometry at 265 mμ (actual D + part of P). (*c*) D and P separately by TLC (actual D content). The effect of the saponification temperature on actual and potential vitamin D contents is seen in Table II.

Soxhlet Extraction—The samples consisted of 1 g. of cotton seed oil, containing 48.8 mg. of cholecalciferol, adsorbed on 5 g. of cellulose powder. These were extracted during 5 hr. under nitrogen, with ether, cis-dichloroethene, *n*-hexane, cyclohexane, or isooctane, each containing 0.01% of BHT, at 35, 60, 68, 80, and 100°, respectively, and the extracts analyzed (see Table III): (*a*) P + D with Nield reagent (potential D content), (*b*)

TABLE I—P AND D CONTENT AFTER BOILING CALCIFEROLS FOR 0.5 HR.

Solvents and B.p., °C.	Ergocalciferol: P_2 + D_2 Recovery, %	By TLC Fractions, % of Potential D: P_2	D_2	Absorptiometric: a at 265 mμ	Fractions, % of Potential D: P_2	D_2	Cholecalciferol: P_3 + D_3 Recovery, %	By TLC Fractions, % of Potential D: P_3	D_3	Absorptiometric: a at 265 mμ	Fractions, % of Potential D: P_3	D_3	Calcd. from k_1, k_2: P_3
Methanol, 65	101.8	4.8	95.2	465	3.7	96.3	100.2	4.2	95.8	473	4.5	95.5	4.3
Ethanol, 78	101.4	13.5	86.5	440	13.1	86.9	101.0	12.2	87.8	452	12.4	87.6	12.2
Isooctane, 100	101.2	26.6	73.4	407	25.5	74.5	101.7	28.8	71.2	408	28.6	71.4	28.3

TABLE II—EFFECT OF SAPONIFICATION AT DIFFERENT TEMPERATURES

Solvent with 2 *N* KOH	Heated for 30 min., °C.	By Nield Reaction Potential D mg./g.	By TLC: Fractions, % of Potential D: P	D	Potential D (P + D), mg./g.	Actual D, mg./g.	By Absorptiometry Apparent Vitamin D, mg./g.	Calcd. P Fractions, % of Potential D: After 0.5 hr.	Equilibrium
Methanol	60	47.7	4.5	95.5	46.2	44.2	46.5	2.6	16.1
Ethanol	80	47.7	13.6	86.4	47.0	40.7	44.2	13.9	22.0
Methyl cellosolve	100	48.0	20.2	79.8	48.0	38.2	42.3	28.3	28.5

TABLE III—EFFECT OF SOXHLET EXTRACTION: CONTENTS OF EXTRACTS

5 Hr. Extraction with	B.p., °C.	TLC: Fraction, % of Potential D: P	D	P + D, mg./g.	Nield Reaction Potential D, mg./g.	Calcd. Equilibrium P Fraction, % of Potential D
Ether	35	8.5	91.5	48.2	47.8	10.0
cis-$C_2H_2Cl_2$	60	12.5	87.5	48.8	48.8	16.1
n-Hexane	68	18.9	81.1	48.8	48.2	18.4
Cyclohexane	80	21.8	78.2	47.4	47.4	22.0
Isooctane	100	27.7	72.3	49.2	48.8	28.5

D (actual D content) and P separately by TLC.

Storage and Analysis of Resins—In the experiments of Table IV samples of resins were stored at different temperatures during times in which diluted calciferol solutions are equilibrated completely. In Table V stored resins are compared with solutions of the same in oil; in this case the storage time is insufficient to reach the equilibrium for solutions.

P + D + TA was determined by Nield reaction absorptiometry in this solution. P and D + TA were isolated by TLC of 0.5 ml. of the solution (containing approximately 0.5 mg. of D) and P determined by Nield reaction. From D + TA the TA was eliminated by MA addition and the remaining D determined by Nield reaction. The values for P + D in Table IV were obtained by adding those for P and for D. The values for TA

TABLE IV—STORAGE OF A SOLID (GLASSY) VITAMIN D_3 RESIN IN NITROGEN DURING TIMES NEEDED BY DISSOLVED CALCIFEROL TO REACH P-D EQUILIBRIUM

Determined (Storage Time and Temp. →)	% w/w: After 1 Month, 20°	After 1 month, 20° and: 11 Days, 30°	3.8 Days, 40°	1.7 Days, 50°
P + D + TA	68.5	67.3	69.6	69.9
P + D (= potential D)	62.6	59.5	63.0	63.5
D (= actual D)	54.8	51.3	53.9	53.6
P	7.8	8.2	9.1	9.9
TA	5.9	7.8	6.6	6.4
	P and D Fractions, % of Potential D[a]			
D	87.5 (93)	86.3 (91.1)	85.5 (89.9)	84.4 (86.5)
P	12.5 (7)	13.7 (8.9)	14.5 (11.1)	15.6 (13.5)

[a] The numbers in parentheses are the equilibrium fractions in solutions.

TABLE V—COMPARISON OF THE P-D RATIO AFTER STORAGE OF NONCOOLED (LIQUID OR PASTY) VITAMIN D_3 RESIN AND OF THE SAME RESIN DISSOLVED IN OIL

Compd.	P and D Fractions, % of Potential D_3: Start Resin	Start Oil	19 Days at 20°C. Resin	19 Days at 20°C. Oil	6 Days at 30°C. Resin	6 Days at 30°C. Oil	2 Days at 40°C. Resin	2 Days at 40°C. Oil
P	25	23	10	12	9.5	9.5	11	13
D	75	77	90	88	90.5	90.5	89	87
Potential D, % w/w	60.7	5.4	61.5	5.9	59.2	6.0	60.0	5.9

were obtained by subtracting P + D from P + D + TA. Determination of TA or D + TA by Nield reaction after TLC is wrong as TA decomposes during TLC.

The samples of Table IV were obtained by weighing 500 mg. of briefly heated (100°) vitamin D_3 resin into volumetric flasks, sealing the flasks under nitrogen, and storing them for 5 hr. at −25° at which time the resin had solidified and taken its glassy appearance. These samples were stored for the times mentioned in Table IV, then dissolved in solvent III at room temperature and promptly analyzed.

In Table V, vitamin D_3 resin was heated for 2 hr. at 85°. The 500-mg. portions were sealed in volumetric flasks under nitrogen and stored immediately at the temperatures of the storage tests. Ten grams of the same vitamin D_3 resin was heated for 2 hr. at 85° in 90 g. of peanut oil. The solution was stored in completely filled, stoppered 5-ml. flasks.

DISCUSSION

Recovery—The recoveries of the sum of P and D found by the described TLC method (Table I) and the comparison with potential D content found by Nield reaction before chromatography (Tables II and III) are satisfactory.

Isomerization, Equilibrium, and Rate—In the experiment of Table I the equilibrium is reached within 30 min. at 100°, as can be calculated from the rate constants (1); at 65 and 78°, 8 and 3 hr. would be needed. Accordingly at 100°, the P fractions are near the equilibrium value of 28.5%; at 65 and 78° they are much lower than the equilibrium value (4.5 instead of 17.6% and 13 instead of 21.4%, respectively). It is evident that the P and D fractions, even those measured *during* the conversion, are virtually equal for ergocalciferol and cholecalciferol. This means that the equilibrium and the isomerization rates of vitamin D_2 and vitamin D_3 differ only very slightly.

Saponification—From Table II it is clear that the actual D content decreases considerably during saponification and progressively so with increasing temperature; the potential D content remains unchanged. In a comparison of Tables I and II the P and D fractions found after saponifying 0.5 hr. at 60 and 80° agree reasonably with the values found without KOH at 65 and 78° after 0.5 hr., experimentally and calculated. At 100° there is a certain discrepancy. The conclusion is that the rate, and thus the equilibrium P $\rightleftharpoons$ D is not altered by the presence of KOH if the temperature does not exceed 80°, but that at 100° KOH causes other effects. *Without trying to explain the effect of* KOH, the authors advise saponification only at temperatures of 80° or less.

Extraction (Table III)—After extraction at 68, 80, and 100° the P and D fractions are in fair agreement with the equilibrium fractions. Below 68° the agreement fails because 5 hr. is too short a time to reach equilibrium.

Resins—The effect of temperature on glassy resins appears to be very small, which emphasizes their solid character (Table IV). Unlike the glassy vitamin D_3 resin the viscous noncooled vitamin D_3 resin of Table V isomerizes at the same rate as its solution.

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Keyphrases

Vitamin D—analysis
Tachysterol—elimination
Isomerization—equilibrium, rate
TLC—separation
UV spectrophotometry—analysis