The Differentiation and Assay of Vitamins D₂ and D₃ by Gas-Liquid Chromatography

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1. A method is described for the differentiation and determination of as little as 0.2 µg. of vitamins D₂ and D₃ by gas-liquid chromatography. 2. The vitamins are converted by treatment with antimony trichloride into isovitamins D₂ and D₃, which show single, separate peaks on gas-liquid chromatography, unlike the unmodified vitamins, which give twin peaks due to the formation of pyro and isopyro derivatives. 3. Since isovitamins D₂ and D₃ remain together in all steps of the procedure except during gas-liquid chromatography, one may be used as an internal standard for the other. 4. The use of an internal standard reduces the importance of loss during sample preparation and increases precision. 5. The application of the method to biological materials is demonstrated.

Many methods have been devised for the assay of vitamin D but none has been entirely satisfactory with regard to sensitivity, specificity, precision and ability to distinguish between vitamins D₂ and D₃. Gas chromatography, which has often provided the means of assay when other methods have failed, was first investigated as a method for vitamin D by Ziffer, Vanden Heuvel, Haahti & Horning (1960) and later applied to the assay of rat liver by Nair, Bucana, de Leon & Turner (1965). Vitamins D₂ and D₃ are converted, at the temperature of vaporization, to the 'pyro' and 'isopyro' forms and appear as twin peaks in gas chromatograms. Nair *et al.* (1965) demonstrated that these derivatives are formed in constant proportion and that they can be separated by gas chromatography. An examination of the relative retention volumes of the four derivatives indicates that the peaks fall near to each other and, were they present together, would be difficult to distinguish and measure. Cholesterol emerges between the pyro and isopyro forms and it is essential that it be eliminated before gas chromatography.

The present paper describes a sensitive and relatively simple method for the assay of vitamins D₂ and D₃ in which the vitamins are modified to give single, separate peaks during gas chromatography and in which one is used as an internal standard in the assay of the other. For preliminary communication, see Murray, Day & Kodicek (1965).

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EXPERIMENTAL

Materials. Crystalline vitamin D₂ (Glaxo Laboratories Ltd., Greenford, Middlesex) and vitamin D₃ (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) were dissolved in light petroleum (b.p. 40–60°) as required for standards or in arachis oil for animal dosing. The light petroleum was shaken twice with conc. H₂SO₄, washed with water until acid-free and redistilled. Ethanol was redistilled; other solvents and chemicals (analytical grade) were used as received.

Animal experiments. Weanling, hooded rats were given the rachitogenic diet 2965 of Steenbock & Black (1925) modified by the addition of 0.05% of lysine and the following B-vitamins (mg./kg.): thiamine 2, riboflavine 4, niacin 10, calcium pantothenate 4. Each rat received 7g. of fresh carrot/week. After at least 3 weeks they were dosed orally with 250 or 500 µg. of vitamin D₂ or D₃ in arachis oil and were decapitated at various time-intervals. Undosed rachitic rats were killed in the same way. Livers and kidneys were kept frozen until analysed.

Spectrophotometric studies. Spectra of the vitamin D derivatives were measured in a Unicam SP.700 spectrophotometer with ethanol as solvent.

Preparation of sample. The details given will be those suitable for the assay of liver and the minor changes appropriate for other tissues will be noted in the text.

Saponification. Since vitamins D₂ and D₃ could be measured separately, vitamin D₃ served as an internal standard if vitamin D₂ was to be determined and vice versa. Thus the vitamin D standard was added before saponification in an amount similar to that expected in the sample. The liver was chopped and saponified for 30 min. with 50 ml. of ethanol and 5 ml. of saturated KOH. Water (50 ml.) was added and the mixture extracted with three portions of diethyl ether (100 ml. and 2 × 50 ml.). The

ether was washed free of alkali, passed through anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure.

Precipitation of sterols. The unsaponifiable matter was dissolved in 5 ml. of absolute methanol and sterols were precipitated by the addition of 5 ml. of 3% (w/v) digitonin solution in 95% methanol and of 0.85 ml. of water. After standing overnight, the mixture was cooled to -4° and extracted three times with 5 ml. of light petroleum.

Conversion of vitamin A into anhydrovitamin A. The light petroleum was evaporated off in a stream of N_2 and the residue dissolved in 10 ml. of ethanolic 33mN-HCl and left in the dark for 30 min. according to the method of Barua & Rao (1964). After 30 min. 10 ml. of water and sufficient NaHCO_3 to neutralize the HCl were added and the solution was extracted three times with 20 ml. of diethyl ether. The ether was washed three times with water and evaporated to dryness under N_2 or reduced pressure. This treatment was not necessary when the total amount of vitamin A was low, as, for example, in kidney and serum.

First thin-layer chromatography. The residue from the ether extracts was dissolved in a small volume of light petroleum and applied in a strip to a plate coated with Kieselgel G (E. Merck A.-G., Darmstadt, Germany) with a marker of vitamin D at one side of the plate. The chromatogram was developed with chloroform, after which the edge bearing the marker was sprayed with SbCl_3 in chloroform. The adsorbent opposite the marker was scraped off the plate into a small sintered-glass funnel and eluted with ethanol and diethyl ether. The eluate was evaporated to dryness under a stream of N_2 . At this stage a generous width of adsorbent was removed, a little above and below that indicated by the marker spot. Vitamins D_2 and D_3 did not clearly separate in this system but stratified within the spot with vitamin D_2 forming the leading edge.

Treatment with antimony trichloride. The residue from the eluate was dissolved in 0.2 ml. of chloroform and 2 ml. of a 20% (w/v) SbCl_3 solution in chloroform was added with shaking. Small amounts of vitamin A were usually present and gave a blue colour but did not interfere with the assay. After 1 min. at room temperature the reaction was stopped by the addition of 3 ml. of 40% (w/v) tartaric acid in water and the suspension was shaken until two clear phases were obtained. Prolongation of the reaction time beyond 1 min. resulted in deterioration of the desired product. The mixture was extracted twice with 5 ml. of light petroleum and the combined extract washed three times with an equal volume of water and finally evaporated to a small volume under a stream of N_2 .

Second thin-layer chromatography. The combined extract was applied to a thin-layer plate and developed as before except that the marker was vitamin D_2 or D_3 which had been treated with SbCl_3 as described above. The position of the vitamin was found by spraying the whole plate with a 0.001% solution of rhodamine in water and examining under u.v. light (Hanovia, model 16). The vitamin appeared as a dark area and this was carefully marked, removed from the plate and eluted as before. This method of detecting vitamin D was not useful for the unaltered vitamin but after treatment with SbCl_3 as little as 0.2 $\mu\text{g.}$ could be detected in this way.

Gas-liquid chromatography. The residue from the eluate was dissolved in a volume of light petroleum so that not more than 2.5 $\mu\text{g.}$ or less than 0.2 $\mu\text{g.}$ could be conveniently applied to a small stainless-steel gauze pad with a Microcap

disposable micropipette (Shandon Scientific Co. Ltd., London, S.W.7). After 30 sec. at room temperature the pad was applied to a Pye Argon Chromatograph under the following conditions: silicon-treated glass column (4 ft. long; 4 mm. internal diam.), packed with silicone-treated Celite with a 3% coating of silicone oil; temperature 230° ; voltage 1750 v; gas flow 100 ml./min.

Vitamins D_2 and D_3 emerged as separate peaks and, from peak areas, the unknown was estimated by simple proportion with reference to the internal standard.

RESULTS

Ultraviolet spectra. The absorption spectra of vitamin D_2 (10 $\mu\text{g./ml.}$ of ethanol) before and after treatment with antimony trichloride are shown in Fig. 1. The extinction is more than doubled after treatment with antimony trichloride. It has been reported (Thibaudet, 1945) that vitamin D is converted, by treatment with antimony trichloride, into tachysterol, but the extinction curve more closely resembles that of isovitamin D as reported by Inhoffen, Brückner, Gründel & Quinkert (1954) (see Table 1). In the absence of further information the reaction product will be referred to hereafter as isovitamin D. No differences in absorption maxima or in extinction values were noted between isovitamins D_2 and D_3 prepared by treatment with antimony trichloride.

Thin-layer chromatography. The main purpose

Fig. 1. Ultraviolet-absorption spectra in ethanol, and structural formulae, of vitamin D_2 (a) and isovitamin D_2 (b), the product formed by treating vitamin D with SbCl_3 .

Table 1. Spectral characteristics of isovitamin D₂ and the product formed by treating vitamin D₂ with antimony trichloride

	$\lambda_{\max.}(m\mu)$			Ratios of extinctions	
	A	B	C	E_B/E_A	E_B/E_C
Isovitamin D ₂ *	276	287	300	1.31	1.37
Vitamin D ₂ -SbCl ₃	278	288	301	1.25	1.36

* Data from Inhoffen *et al.* (1954).

Table 2. Relative retention data for vitamins D, isovitamins D and cholesterol on 3% silicone oil

Other details of gas-liquid chromatography are given in the text.

	Relative retention volumes
Cholestane	1.0
Vitamin D ₃	1.67*
	1.89†
Vitamin D ₂	1.89*
	2.15†
Cholesterol	1.96
Isovitamin D ₃	2.84
Isovitamin D ₂	3.24

* Pyrovitamin.
† Isopyrovitamin.

Fig. 2. Gas-liquid chromatogram of a mixture of cholestane (1), cholesterol (2), isovitamin D₃ (3) and isovitamin D₂ (4), 1 μ g. each. Experimental details are given in the text.

of the first thin-layer chromatography is the removal of vitamin A as anhydrovitamin A, which moves near the solvent front. Some cholesterol is usually carried over with the vitamin D and is separated on the second thin-layer chromatographing

Table 3. Estimation of vitamin D₂ in the presence or absence of deficient rat liver

Tissue	No. of estimations	Vitamin D ₂ added (μ g.)	Vitamin D ₂ estimated (μ g.)
—	14	25	26.7 $\pm$ 0.3
Liver	10	25	25.2 $\pm$ 0.5

after treatment with antimony trichloride. The *R_r* of cholesterol (0.28) is not changed by the treatment with antimony trichloride for 1min., whereas that of vitamin D (0.36) is increased to 0.47 for isovitamin D.

Gas-liquid chromatography. Retention volumes, relative to cholestane, of vitamins D₂ and D₃, isovitamins D₂ and D₃ and cholesterol are shown in Table 2 and a typical chromatogram of cholestane, cholesterol, isovitamins D₂ and D₃, 1 μ g. each, is shown in Fig. 2. As little as 0.2 μ g. could be measured.

Application of the method. The use of the internal-standard method of calculation was tested by assaying standards alone and when added to livers of rachitic rats. In the experiments recorded in Table 3 vitamin D₂ was estimated and vitamin D₃ served as the internal standard. Good agreement was obtained between the amount estimated and that added both in the presence and absence of deficient rat liver.

Table 4 gives the results of assay of liver and kidney of rachitic rats dosed with vitamin D₂ or D₃. The disappearance of vitamin D₂ from the liver over a period of 10 days after an oral dose of 250 μ g. is shown by the following values (μ g. of vitamin D₂/liver): day 1 after dosing, 27.4; day 2, 13.0; day 3, 5.8; day 8, 2.6; day 10, 2.5.

DISCUSSION

Treatment of vitamin D with antimony trichloride has several valuable consequences. When a gas chromatograph is not available the ultraviolet-absorption curve of isovitamin D may be useful in the determination of vitamin D. The *E*_{1cm.}^{1%} value is more than double that of the unaltered vitamin and the curve is such that an indication of purity may be had by calculating the extinction ratios of the maxima (Table 1). As little as 1 μ g./ml. may be measured in pure solutions but vitamins D₂ and D₃ cannot be estimated separately and, of course, the internal-standard method cannot be used.

The second advantage of treatment with antimony trichloride is that any vitamin A not eliminated earlier in the procedure is converted into anhydrovitamin A, which runs near the leading edge of the developing solvent (chloroform) in the

Table 4. *Assay of vitamins D₂ and D₃ in liver and kidney of dosed rats*

Dose ($\mu\text{g.}$)	No. of estimations	Storage 24 hr. after dosing					
		Liver			Kidney		
		% of dose		Range	% of dose		Range
		As D ₂	As D ₃		As D ₂	As D ₃	
Vitamin D ₂ (500)	3	9.7	—	8.2–12.5	0.71	—	0.68–0.75
Vitamin D ₂ (250)	6	13.1	—	8.4–20.5			
Vitamin D ₃ (250)	4	—	6.4	5.1–7.2			

second thin-layer chromatographic step, well separated from isovitamin D. The differentiation of cholesterol and vitamin D is also facilitated by treatment with antimony trichloride as the R_f of cholesterol in chloroform is not affected by the treatment for 1 min. whereas that of vitamin D is appreciably increased. Consequently the sample applied to the gas chromatograph should not contain cholesterol.

Thirdly the isovitamins D₂ and D₃ produce only one peak each during gas chromatography and these are sufficiently separated from each other to permit an accurate measurement of each. Since the vitamins remain together in every step preceding gas chromatography, one may be used as an internal standard for the other. This reduces the importance of loss during sample preparation, obviates the necessity of running an additional sample with added standard and increases accuracy. Loss during assay has always been one of the great weaknesses of vitamin D determinations and has been recently discussed in detail by Chen, Terepka, Lane & Marsh (1965). The amount of internal standard and unknown should be nearly equal and this sometimes requires a preliminary run.

The assay of tissues of rats dosed with vitamins D₂ and D₃ gave results in reasonable agreement with those obtained by other methods (Kodicek & Ashby, 1960; Kodicek, 1958, 1963). Two high values, namely 15.2 and 20.5%, raised the amount of vitamin D₂ found in liver 24 hr. after dosing with 250 $\mu\text{g.}$ of vitamin D₂ to a value somewhat higher than expected, but the disappearance of the vitamin over the first days after dosing is so rapid (see Results) that it is not surprising to find considerable variation at any given time.

It has been reported (Bosmann & Chen, 1964) that the livers of rats dosed with vitamin D₂ contained more antirachitic activity for chicks than could be accounted for by the vitamin D₂ content. One explanation could be the conversion by the rat of vitamin D₂ into D₃, but judging by the gas-liquid chromatography results this does not happen. When vitamin D₂ is administered vitamin D₂ is

stored; similarly vitamin D₃ gives rise to vitamin D₃ (see Table 4).

The disappearance of vitamin D from the liver in the first 3 days after an oral dose was very rapid, the amount being halved on each of days 2 and 3. Thereafter the loss slowed so that in the next 7 days only 3.3 $\mu\text{g.}$ disappeared, which is the lowest amount that could be estimated with any accuracy in liver. This is similar to the results of Cruickshank & Kodicek (1953), except that the vitamin D content of their animals stabilized at about 10 $\mu\text{g.}$ /liver and reached a plateau at 2 days after dosing.

The method described has also been successfully applied to sera of rachitic rats to which vitamin D₂ was added in a concentration of 0.5 $\mu\text{g.}$ /ml. of serum. However, it was found that rat faeces and chick liver contain interfering material not removed by the purification procedure described above.

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