PREPARATION OF ^{3}H-VITAMIN D_3, USING COLUMN AND THIN-LAYER CHROMATOGRAPHY*

C. K. PAREKH AND R. H. WASSERMAN
Department of Physical Biology, New York State Veterinary College, Cornell University, Ithaca, N.Y. (U.S.A.)
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Vitamin D is essential for the optimal absorption of calcium in a number of species and influences the resorption and/or accretion of calcium in bone[1]; an effect on the kidney has also been described[2]. The mechanism of vitamin D action is unknown although various general theories have been proposed[3,4,5]. In order to understand more fully the manner by which this vitamin influences calcium metabolism, it would be of considerable value to determine the localization, distribution and metabolism of vitamin D itself. This type of study has been augmented by the preparation, isolation and purification of radioactive-labeled vitamin D.

Described herein are two procedures that have been used in the preparation of vitamin D_3 labeled with tritium. These were based on techniques previously reported by NORMAN AND DELUCA[6] and PENG[7].

METHOD

The tritium-labeled vitamin D_3, as mentioned above, was prepared by modifications of the procedures described by NORMAN AND DELUCA[6] and by PENG[7]; the first involved tritiation of 7-dehydrocholesterol by the Wilzbach procedure and subsequent conversion to vitamin D_3 and the second was done by tritiating vitamin D_3 directly at low temperatures. Each will be described in turn.

Tritiation of 7-dehydrocholesterol

Five hundred milligrams of 7-dehydrocholesterol were exposed to 3 C of ^{3}H gas at 0° and 255 mm Hg pressure for four days by the Wilzbach procedure[8]**. Following evacuation of excess ^{3}H gas, the product was dissolved in 20 ml of chloroform into which freely exchangeable ^{3}H was incorporated; the ^{3}H-contaminated solvent was then removed by vacuum distillation. The specific activity of the crude preparation was 0.015 mC/mg.

Since preliminary purification entailed losses, the tritiated 7-dehydrocholesterol in the crude preparation was converted directly to vitamin D_3 by ultraviolet irradiation by the following procedure: 20 mg of the crude ^{3}H-7-dehydrocholesterol was dissolved in 40 ml of anhydrous ether and placed in a quartz cuvette. Irradiation was carried out by the use of a sunlamp (LUXOR 20900-2 from Hanovia Lamp Division,

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** Tritiation procedure done by New England Nuclear Corporation, Boston, Mass.

Newark, N.J.) at a distance of 8" with a quartz lens interposed between the cuvette and the lamp. The mixture was constantly stirred with a magnetic stirrer during the process. An irradiation time of 30 min gave maximum vitamin D_3 production. The ether was evaporated under a stream of nitrogen at room temperature. The crude irradiated product was first chromatographed on a water-jacketed, 13 × 100 cm column packed with 35 g of silicic acid (Biorad) slurried in *n*-hexane and under 10 p.s.i. pressure of nitrogen as described by NORMAN AND DELUCA[6]. The sample to be chromatographed was applied to the column in 10% ether in *n*-hexane and eluted with the same solvent at —5°. The eluent was collected in 5 ml fractions delivered at 15 min intervals, and those fractions known to contain vitamin D_3 were immediately refrigerated.

The tritiated vitamin D_3 was identified by the use of thin-layer chromatography (TLC). An aliquot of 100 μl from the 5 ml fractions was spotted on the TLC plates that were coated with 250 μ thick silica gel G and previously activated by heating at 100° for 1 h. All plates used for identification were also spotted with pure 7-dehydrocholesterol and vitamin D_3 for reference purposes. The TLC plates were developed in acetone–*n*-hexane–MeOH (15:135:3) mixture for 50 min, and then air dried and sprayed with a saturated solution of $SbCl_3$ in chloroform for the identification of the steroids. The steroid spots appear at room temperature, but are intensified by blowing a stream of hot air over them. 7-Dehydrocholesterol appears as a green-violet spot changing to intense violet, whereas vitamin D_3 is initially yellow, changing subsequently to brown-orange. The colors are stable for 3 to 4 h but, with time, the spots fade and become hazy in outline.

The fractions now shown to contain vitamin D_3 were pooled and the solvent removed under a stream of nitrogen. The crude vitamin D_3-^3H was then dissolved in chloroform and spotted on the TLC plate to a maximum concentration of 100 μg per spot. Twenty μg of pure, commercial 7-dehydrocholesterol was applied on either side of the vitamin D_3-^3H spots and the plate was developed in the solvent system given above for 50 min. After developing, the plate was air dried and examined under U.V. light. The fluorescing 7-dehydrocholesterol spots were useful in locating the vitamin D_3-^3H regions, since it was previously determined that the vitamin D_3 zone begins immediately above the 7-dehydrocholesterol spot for about 10 to 13 mm. Another fluorescent spot (a degradation product of vitamin D_3) occasionally appears above the vitamin D_3 zone, which further aids in delineating the vitamin D_3 area. Once located, the vitamin D_3 zone was removed by scraping with a sharp spatula. The traces of silicic acid adhering to the glass plate were removed with a cotton swab soaked in chloroform. The vitamin D_3-^3H was then eluted from the silicic acid with chloroform. Recovery by this procedure was found to vary from 85 to 95 % as checked previously with known amounts of the vitamin D_3.

The concentration of vitamin D_3-^3H in the preparation was determined by its absorbency at 264 mμ in alcohol (λ = 264, ε = 18,200), using a Beckman DU spectrophotometer. The tritium activity was measured by counting in a liquid scintillation detector (Packard Tricarb) by the usual procedures[9].

The purification of vitamin D_3-^3H on a TLC plate was repeated until a constant specific activity product was obtained and the U.V. fluorescent by-products were removed. The U.V. and the infra-red spectra (Infracord, Perkin-Elmer) of the final purified tritiated product was the same as that for crystalline vitamin D_3.

Usually, a two-fold purification was sufficient, but occasionally it was necessary to re-purify three to four times. During these procedures, it was observed that temperature was an important factor in the stability of vitamin D_3-^{3}H; however, degradation products appear with time even when stored at —20° under nitrogen. This indicates that, for biological experimentation, vitamin D_3-^{3}H should be purified immediately before use. The purification by the present TLC method would be of advantage since the entire operation takes about 2 h.

Direct tritiation of vitamin D_3

Vitamin D_3 was directly tritiated, according to the method of PENG[7,*]. Fifty mg of vitamin D_3 was tritiated with 3 C of ^{3}H gas at —196° and 130 mm Hg pressure for 39 days. The tritium gas was evacuated and the vitamin D_3-^{3}H dissolved in 10 ml of methanol. The contaminated solvent was removed by vacuum distillation and this procedure was repeated. Ten mg samples of the crude vitamin D_3-^{3}H were column chromatographed and the pooled vitamin D_3-^{3}H fractions purified on TLC plates (as previously described) to a constant specific activity and until no by-products were detected by infrared and ultraviolet spectroscopy, or by inspection of the plates under U.V. light for fluorescent areas.

RESULTS AND DISCUSSION

Fig. 1 shows the elution pattern of some of the components of the crude tritiated preparation and also illustrates the patterns observed on the TLC plates. It is seen that a separation of the major products (tachysterol, vitamin D_3 and 7-dehydrocholesterol) was obtained on the column and that these could be reasonably separated by TLC. In addition, the separation on the TLC was shown to be facilitated by the addition of methanol to the acetone–hexane solvent; a comparison of the R_F values obtained with the acetone–hexane system with and without methanol is given in Table I. It is apparent that the methanol addition not only increased the

TABLE I

R_F VALUES FOR 7-DEHYDROCHOLESTEROL, VITAMIN D_3 AND THE BY-PRODUCT (PINK COLORED) WITH TWO SOLVENT SYSTEMS[a]

	R_F value	
	n-Hexane–acetone (135:15)	*n-Hexane–acetone–methanol (135:15:3)*
7-Dehydrocholesterol	0.20	0.46
Pink by-product (as colored by $SbCl_3$)	0.23	0.56
Vitamin D_3	0.26	0.58

[a] R_F values represent the mean of 6 runs.

degree of separation between 7-dehydrocholesterol and vitamin D_3, but also separated the former compound from other by-products so that it could be recovered and subsequently converted to vitamin D_3.

* Tritiation procedure done by New England Nuclear Corporation, Boston, Mass.

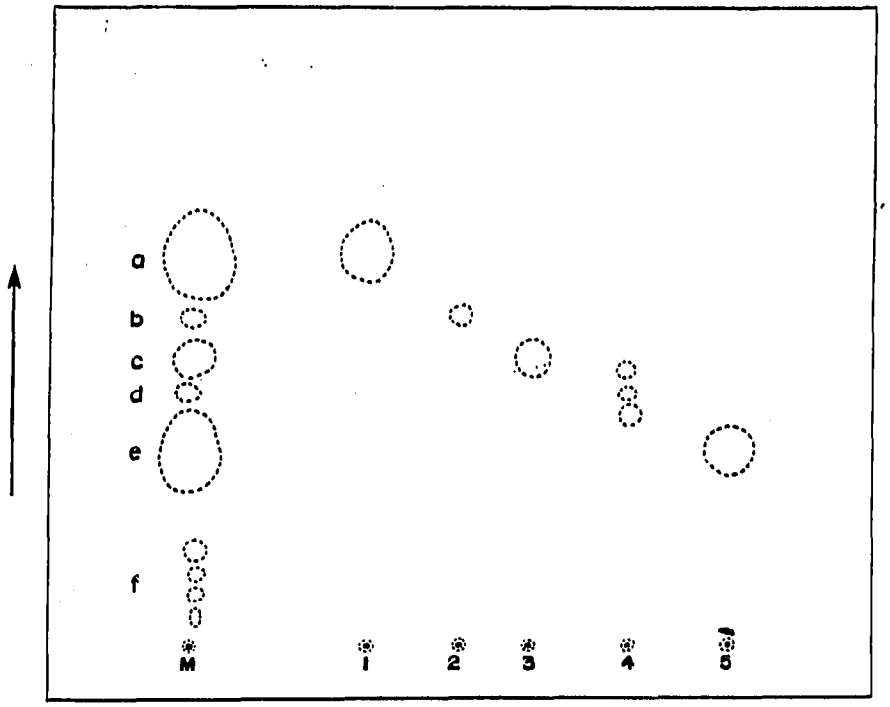

Fig. 1. Identification on TLC plates of compounds from crude U.V.-irradiated 7-dehydrocholesterol-^{3}H as collected from a silicic acid column. Eluting solvent on column: 10% ether in *n*-hexane, temp. —5°. Developing solvent on TLC: acetone–*n*-hexane–MeOH (15:135:3). Vertical spots were visualized by spraying with $SbCl_3$ in chloroform, these being: (a) tachysterol (yellow-brown); (b) unknown by product (blue-green); (c) vitamin D_3-^{3}H (brown); (d) 7-dehydrocholesterol by-product (pink); (e) 7-dehydrocholesterol (violet); (f) several by-products. On the horizontal axis, 100 μl of the following solutions were spotted at the origin: (M) crude material before column chromatography; (1) column fraction No. 40; (2) fraction No. 50; (3) fractions Nos. 56–60; (4) fraction No. 64; (5) fractions Nos. 68–90.

The specific activities and recoveries of vitamin D_3 prepared and purified by several methods are shown in Table II. It may be seen that tritiation of 7-dehydrocholesterol followed by ultraviolet irradiation gave a product of higher specific activity than obtained from direct tritiation of the vitamin D_3 molecule; the percentage recovery of tritiated vitamin D_3 tended to be greater than that for 7-dehydrocholesterol. However, NORMAN AND DELUCA[6], using a somewhat different method for purification, were able to recover approximately 6.6% of their product.

The major problem in the preparation of vitamin D_3-^{3}H was in the separation of a by-product from 7-dehydrocholesterol which appears exactly between the vitamin D_3 and 7-dehydrocholesterol. This shows up as a pink colored spot upon spraying with $SbCl_3$ reagent, but does not yield a colored product with $KMnO_4$ spray (0.2% in 1% Na_2CO_3 w/v) or with H_2SO_4 as used by NORMAN AND DELUCA[10]. Further, it takes about 1 to 2 h to appear on the plate after spraying. The area first appears as a violet spot similar to that of 7-dehydrocholesterol but, with time, turns pink. This by-product has a maximum absorption at 265 mμ in ethanol (similar to vitamin D_3), has no vitamin D_3 biological activity and is eluted from the column after vitamin D_3, as shown in Fig. 1.

Because of the presence of the pink by-product in the tritiated 7-dehydrocholesterol sample, purification could not be affected by thin layer chromatography alone. However, the crude product arising from the direct tritiation of vitamin D_3

TABLE II

SPECIFIC ACTIVITY AND AMOUNT OF VITAMIN D_3-^{3}H RECOVERED AFTER TRITIATION OF 7-DEHYDROCHOLESTEROL AND VITAMIN D_3

Substance tritiated	*Purification procedure*	*Amount of tritiated material purified (mg)*	*Amount of vitamin D_3 recovered (μg)*	*Per cent recovery*	*Specific activity (d.p.m./I.U.)*
7-Dehydrocholesterol*	Column chromatography followed by TLC	20	435	2.2	744
Vitamin D_3**	Column chromatography followed by TLC	10	758	7.6	234
Vitamin D_3**	TLC alone	10	555	5.6	226
Vitamin D_3**	TLC alone	10	378	3.8	279

* By the method of NORMAN AND DELUCA[6].
** By the method of PENG[7].

could be purified by using the TLC alone (Table II) because of the absence of this by-product.

Novel features of the present system are the use of a modified solvent system composed of acetone–*n*-hexane–methanol (15:135:3) that improves the separation of vitamin D_3 and 7-dehydrocholesterol on TLC plates, and the use of saturated $SbCl_3$ in chloroform which detects a contaminated by-product not detected by other procedures. It was also helpful to use reference spots of pure 7-dehydrocholesterol on the TLC plates so that the vitamin D_3 zones could be located under U.V. light without chemical treatment of the plate. During the course of these investigations, it also became apparent that vitamin D_3-^{3}H is highly labile even when stored at —20° under nitrogen. The consequence of this is that the vitamin D_3 products must be re-purified immediately prior to use.

SUMMARY

Vitamin D_3 was prepared by two different methods as reported by others, *i.e.*, (a) the tritiation of 7-dehydrocholesterol by the Wilzbach procedure with subsequent conversion to vitamin D_3 and (b) the direct tritiation of vitamin D_3 at —190° under nitrogen. A combination of column and thin-layer chromatographic procedures were necessary to purify the product resulting from procedure (a), primarily due to the presence of a pink by-product (after $SbCl_3$ treatment) arising from the degradation of 7-dehydrocholesterol. Purification of the product resulting from procedure (b) could be affected on thin-layer chromatographic plates alone. A solvent is described which increases the degree of separation of vitamin D_3 and 7-dehydrocholesterol on TLC plates.

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