Determination of Vitamin D in the Presence of Vitamin A by Cylindrical Thin-Layer Chromatography

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Vitamin A alcohol in vitamin D_2 preparations is converted to retinene by passage through a column of manganese dioxide, inactivated with acetone containing water, and eluted with the mixed solvent of acetone and petroleum ether (1:4). This eluate is used for cylindrical thin-layer chromatography (CTLC). The sample is applied to the thin layer under a stream of nitrogen gas, and the chromatogram is developed with chloroform. With this proposed procedure, no decomposition of vitamin D_2 took place and the recovery of vitamin D_2 from a vitamin A–D_2 preparation was about 97–102 per cent compared to untreated vitamin D_2.

THE FOLLOWING three points seem to be unresolved in the determination of vitamin D in pharmaceutical preparations using thin-layer chromatography (TLC).

(*a*) With commonly used adsorbents and developing solvents in TLC, vitamin A interferes with the isolation of vitamin D, due to the similarity of the R_f values of both vitamins. It is necessary, therefore, to convert vitamin A to substances that have R_f values different from that of vitamin D_2. In their earlier studies Barua and Rao (1) converted vitamin A to anhydrovitamin A with 0.033 *N* ethanolic hydrogen chloride. Later the same authors (2) reported its conversion to retinene on a column of precipitated manganese dioxide.

(*b*) Partial decomposition of vitamin D_2 occurs within a few minutes on a dry silica gel layer, but it is stable as long as the solvent is present. Therefore, if the ordinary thin-layer plate is used, it should be absolutely essential in the quantitative assay to place the plate in the developing chamber immediately after applying the sample solution—before the solvent (*e.g.*, petroleum ether) evaporates (3). However, this seems to be impossible in practice, since the solvent will evaporate during successive application on a single plate.

(*c*) To minimize errors and avoid concentration of sample extracts, it is very desirable that a large amount of sample solution be applied to the thin layer.

In the present paper, the conversion of vitamin A to retinene by a column of manganese dioxide according to Barua's method was studied in detail. Satisfactory results were obtained with the use of a new method of inactivating manganese dioxide so that the conversion of vitamin A to retinene was complete without alteration or adsorption of vitamin D_2.

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To apply a large amount (more than 1 ml.) of the sample solution and to prevent any decomposition of vitamin D_2 while applying the sample solution to the thin layer, a new apparatus was used for quantitative TLC. It was found that by using this apparatus and introducing nitrogen gas while applying the sample solution, no decomposition of vitamin D_2 took place.

After developing with chloroform, the vitamin D_2 zone is located between two visible yellow bands of vitamin A oxidation products. The use of an ultraviolet light is unnecessary.

The adsorbent between these two yellowish bands is scraped off while still damp with solvent (chloroform), and immediately eluted with ethyl ether. After evaporation of the ether, the residue is dissolved in ethylene dichloride for color development with antimony trichloride reagent (U.S.P. XVII).

EXPERIMENTAL

Apparatus—The cylindrical thin-layer chromatographic apparatus was previously described (4).

Reagents—Manganese dioxide, JIS 1st grade or extra pure; silica gel, Wakogel B-5 for TLC or Kieselgel G for TLC; calciferol, U.S.P. grade; antimony trichloride reagent, U.S.P. XVII vitamin D assay color reagent.

Other reagents (petroleum ether, acetone, chloroform, methanol, ethyl ether, ethylene dichloride, anhydrous sodium sulfate) were JIS special grade or guaranteed reagent.

Method—*Extraction*—To a convenient weight of the sample,[1] at least 0.1 Gm., equivalent to not less than 0.5 mg. (20,000 I.U.) of vitamin D_2, 20–30 ml. of water is added, and the fat-soluble materials (vitamin D_2 and A also included) are extracted with three 30-ml. portions of petroleum ether. If there is emulsifying agent in the sample, addition of acetone may be helpful for separation of the two phases.

The combined extracts are washed with water and dried over anhydrous sodium sulfate. The dry extract is evaporated under reduced pressure to

[1] This sample should contain enough vitamin A to provide the two yellowish pilot bands; add about 1000 I.U. of vitamin A alcohol, if necessary.

approximately 20 ml., transferred to a 25-ml. volumetric flask, and brought to volume with acetone.

Oxidation—*Preparation of the Column*—A column (1 cm. inside diameter) 3 cm. in height with manganese dioxide is prepared by mixing two different mesh sizes (equal parts by weight below 105 μ and 105–210 μ). The column is washed with 2 ml. of acetone containing 3% water, and then with two 1-ml. portions of acetone–petroleum ether (1:4).

Oxidation—Ten milliliters of the above dried extracted solution is transferred to the column and allowed to pass through dropwise. When all the solution has passed through, the column is washed with five 3-ml. portions of acetone–petroleum ether (1:4). During this procedure it is necessary to keep the solvent on the column. Collect the eluates in the same receiver and bring to 25 ml. with petroleum ether. This is the sample solution.

For samples of low vitamin D content (50–250 mcg.), the entire dried extract is evaporated almost to dryness and the residue transferred with acetone–petroleum ether (1:4) to the oxidation column. The eluate is evaporated under reduced pressure until free of solvent, the last few milliliters being removed in a current of nitrogen. The residue is redissolved accurately for cylindrical thin-layer chromatography.

Cylindrical Thin-Layer Chromatography (CTLC)—A volume (V_p ml.) of sample solution of at least 1.00 ml., equivalent to not less than 50 mcg., and preferably more than 2.00 ml., equivalent to a maximum of 100 mcg. of vitamin D_2, is pipeted into the thin-layer cylinder and allowed to exude to the outer thin layer. (Fig. 1.)

During the course of this procedure, nitrogen gas is introduced from the inlet (Fig. 2, a). In this experiment, "partial volume method" (see Fig. 2) is adopted for applying the sample solution.

Extraction from the Adsorbent—Immediately after development with chloroform, the colorless portion of the thin layer between the two yellowish ring bands (oxidation products of vitamin A alcohol), is scraped off with a flat spatula while rotating the thin-layer cylinder into a small flask containing a small amount of ethyl ether.[2]

The suspensions are vigorously shaken and rapidly filtered through a dry filter or centrifuged. This procedure is repeated using four or five 5-ml. portions of ethyl ether. Each filtrate or supernatant is collected in a 25-ml. volumetric flask and brought to volume with ether.

Determination of Vitamin D_2—An aliquot of the ether extract is pipeted into the test tube and the solvent perfectly evaporated to dryness *in vacuo* and with the aid of stream of nitrogen gas. The residue is redissolved in 3.0 ml. of ethylene dichloride, and the color is developed by mixing 3 ml. of antimony trichloride reagent. The absorbance is measured (A_{T500}) at 500 mμ 90 sec. after adding the reagent, and A_{T550} at 550 mμ after 120 sec.

RESULTS AND DISCUSSION

Stability of Vitamin D_2 Coloration with $SbCl_3$—In general, the absorbance at 500 and 550 mμ is measured 45 and 90 sec., respectively, after adding the reagent. But in this experiment the variation of absorbance with the time lapse from 45 to 120 sec. was very small (Fig. 3). Therefore, the A_{500} and A_{550} were measured after 90 and 120 sec., respectively, using the Zero method spectrophotometer.

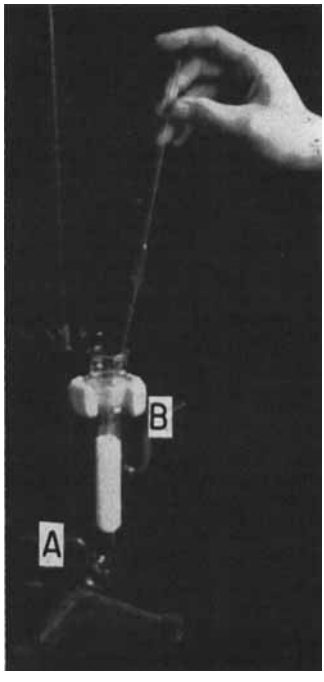

Fig. 1—Application of the sample solution to thin-layer cylinder.

Oxidation by Manganese Dioxide—The oxidation of vitamin A alcohol to retinene by manganese dioxide has been reported by Ball *et al.* (5) and Henbest *et al.* (6). These reports do not refer to the mesh size of the MnO_2. In the report of Barua *et al.*, precipitated MnO_2 was used, but the preparation and activation method was not described in detail.

In this experiment, investigation of mesh size and the inactivation method of MnO_2 are described.

Recovery Experiments with Pure Vitamin D_2 from the MnO_2 Column—A solution of vitamin D_2 in petroleum ether (equivalent to about 0.1 mg.) was pipeted into the prepared columns which contain various mesh sizes and heights of MnO_2 and was eluted with 20 ml. of each eluting solvent. In this procedure it is essential that the column never run dry; the moment the surface of the eluting solvent has dropped to the top of the column, fresh eluting solvent must be added. The eluate was collected in a 25-ml. volumetric flask and brought to volume with eluting solvent.

An aliquot of this solution equivalent to about 15 mcg. of vitamin D_2 was pipeted into the test tube and the vitamin D_2 determined as described above to obtain A_{T500} and A_{T550}. The A_{S500} and A_{S550} are determined for the D_2 solution (in petroleum ether) without passing through the MnO_2 column.

The recovery of vitamin D_2 from the MnO_2 was calculated from the following equation:

$$\text{recovery } (\%) = \frac{A_{T500} - A_{T550}}{A_{S500} - A_{S550}} \times 100$$

The results are shown in Table I.

[2] The most suitable solvents for extraction according to the literature are petroleum ether, ethyl ether, and chloroform. In this experiment ethyl ether was used.

The recovery experiments show (Table I) that vitamin D_2 was adsorbed on the column and cannot be eluted with *n*-pentane or petroleum ether as suggested in the literature (2).

Using the same procedure (1.5-cm. column of small mesh MnO_2), the inactivation of MnO_2 with water and eluting solvent was studied. The results are shown in Table II.

The recovery experiment shows (Table II) that vitamin D_2 is adsorbed firmly on the column of MnO_2 small mesh size, that the inactivation process with acetone containing water is necessary, and that the mixed solvent of acetone–petroleum ether (1:4) is superior to petroleum ether alone.

In the case of MnO_2 large mesh size, using the same procedure, the results are shown in Tables III and IV. The data in Table III are from untreated MnO_2, and the data in Table IV are those from inactivated MnO_2.

From the comparison of data in Tables III and IV, it is necessary to inactivate MnO_2. Inactivation method *A* is no different from *B* (*cf.* $\bar{A}$ = 99.13; $\bar{B}$ = 99.27%). Washing method *A* is little different from *B* (*cf.* $\bar{A}$ = 99.47; $\bar{B}$ = 98.93%). The results show that a little petroleum ether, used for washing the remaining acetone in the column, is superior to the excess; therefore, acetone–petroleum ether (1:4) should be used as the washing solvent.

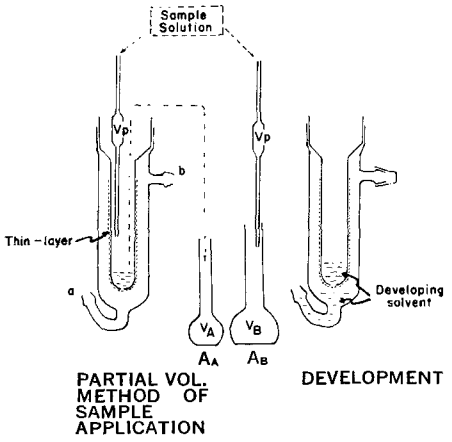

Fig. 2—Cylindrical thin-layer chromatography. The outside of inner cylinder (like a test tube), the bottom of which has a small hole (about 1 mm. in diameter) used for exuding the sample solution and developing solvent from the inside to outside, is covered with a thin layer of adsorbent. The sample solution is pipeted into the thin-layer cylinder and it exudes to the outer thin layer automatically. Thus, the sample makes the ring band around the bottom. The size of the ring band is regulated with the stream of introducing gas. In this case, if the gas flows very rapidly, the hole becomes plugged but if the gas flow is suitably regulated, the sample does not plug. The optional amount of sample solution (in this case the whole of solution exuding is total volume method) has exuded to the outer thin layer, then the remaining solution is removed, quantitatively diluted to a volume, and a color developed (or directly measured at suitable wavelength) for both solutions (original and diluted). The volume of the sample solution exuded to the outer thin layer is then calculated by difference.

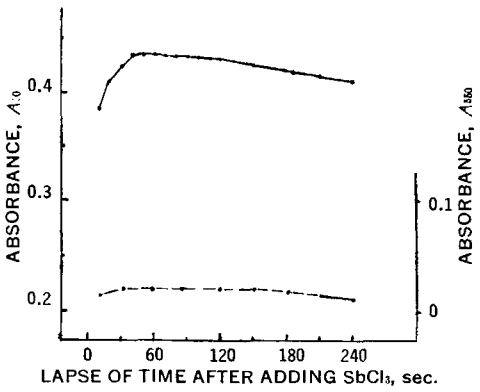

Fig. 3—Stabilities of vitamin D_2 color developed with $SbCl_3$. Key: top curve, A_{500}; bottom curve, A_{550}.

TABLE I—RECOVERY OF VITAMIN D_2 FROM MnO_2 COLUMN

MnO_2 Mfgr.	MnO_2 Mesh Size, μ	MnO_2 Ht., cm.	Eluting Solvent	Recovery, %
A	Below 105	1.5	Pet. ether	13.1
A	Below 105	1.5	*n*-Pentane	8.8
B	Below 105; 105–210 (1:1)	1.5	Pet. ether	14.8
B	Below 105; 105–210 (1:1)	1.5	*n*-Pentane	16.5
B	Below 105; 105–210 (1:1)	3.0	Pet. ether	0

TABLE II—RECOVERY OF VITAMIN D_2 FROM INACTIVATED MnO_2 (Below 105 μ) COLUMN (1.5 cm. HEIGHT) USING VARIOUS ELUTING SOLVENTS

Inactivation of MnO_2	Eluting Solvent	Recovery, %
1% water[a]	Pet. ether	0
5% water[a]	Pet. ether	30.3
No water	EtOH–pet. ether (1:9)	10.2
No water	EtOH–pet. ether (1:4)	10.7
No water	Acetone–pet. ether (1:9)	15.2
No water	Acetone–pet. ether (1:4)	44.1
No water	Acetone–pet. ether (3:7)	18.5
Acetone, 1 ml.	Pet. ether	17.5
Acetone, 1% water[b], 1 ml.	Acetone–pet. ether (1:4)	56.5
Acetone, 3% water, 1 ml.	Acetone–pet. ether (1:4)	59.5
Acetone, 5% water, 1 ml.	Acetone–pet. ether (1:4)	74.9
...	Ethyl ether	2.5

[a] Inactivation method with water: to 10 Gm. of MnO_2 in 30 ml. of petroleum ether, add 0.1 ml. or 0.5 ml. of water and vigorously shake for about 30 min. [b] Inactivation method with acetone containing 1–5% water: pour 1 ml. of acetone containing 1, 3, or 5% water into the MnO_2 columns soaked in petroleum ether.

In Tables I–IV, vitamin D_2 in petroleum ether is used. Results for vitamin D_2 in acetone–petroleum ether (1:4) using the column of below 105 μ: 105–210 μ (1:1) MnO_2, inactivation method *A*, and washing with two 1-ml. portions of acetone–petroleum ether (1:4), are shown in Table V. The only difference here is that the vitamin D was placed on the column in the acetone–petroleum ether (1:4) solution. The results are no different from those in Table IV.

Stability of Vitamin D_2 in Eluting Solvent—To vitamin D_2 in petroleum ether was added the eluting solvent which had passed through the column of MnO_2. This mixed solution was allowed to stand for various periods of time and the color developed by using the same procedure described above. The results are shown in Table VI.

After allowing to stand for 3 hr., an aliquot of each of the vitamin D_2 solutions *1–6* was evaporated to about 0.3 ml. and analyzed by TLC. Spots other than vitamin D_2 were not detected on the plate. The results show that the vitamin D_2 was not altered in these eluting solvents.

Oxidation of Vitamin A Alcohol by MnO_2—Vitamin A alcohol (about 5000–10,000 I.U.) in petroleum ether was poured into the column of MnO_2 with or without inactivation, and the eluate was evaporated to about 1 ml. under nitrogen gas. This concentrated solution was used for micro TLC[3] to determine the completeness of vitamin A alcohol oxidation. The results are shown in Table VII.

The results show that under the conditions for good recovery of vitamin D_2, almost all vitamin A alcohol is converted to retinene so that little vitamin A alcohol is detected on the thin layer.

The chromatogram of vitamin A and D_2 passing through the column of MnO_2 inactivated with 2 ml. of acetone containing 3% water is shown in Fig. 4. (Table VIII.)

Determination of Vitamin D_2 in the Presence of Vitamin A by CTLC—*CTLC of Vitamin D_2 Alone*—The vitamin D_2 in petroleum ether solution (about 50 mcg./ml.) (V_p ml.) was pipeted into the thin-layer cylinder and the vitamin D_2 was isolated according to the described procedure. The absorbance A_c was obtained (when dilution was V_c).

For an evaluation of the volume (V) of vitamin D_2 solution exuding through a hole to the outer thin layer, aliquots of V_A and V_B solutions (see Fig. 2) were evaluated with antimony trichloride and A_A, A_B were obtained.

Recovery (R) of vitamin D_2 from the thin layer against no treatment of vitamin D_2 was calculated by:

$$R(\%) = \frac{A_C \times V_C \times V_p}{A_B \times V_B \times V} \times 100 \quad \text{(Eq. 1)}$$

Equation 2 (volume determination) was combined with Eq. 1 to obtain Eq. 3

$$V(\text{ml.}) = \left(1 - \frac{A_A \times V_A}{A_B \times V_B}\right) \times V_p \quad \text{(Eq. 2)}$$

$$R(\%) = \frac{A_C \times V_C}{A_B \times V_B - A_A \times V_A} \times 100 \quad \text{(Eq. 3)}$$

The results are shown in Table IX.

The results show that if nitrogen gas was introduced using CTLC, decomposition of vitamin D_2 did not occur during the application of the sample solution to the thin layer.

Determination of Vitamin D_2 in Vitamin A and D_2 Emulsion by CTLC—To vitamin A emulsion containing gum arabic, vitamin D_2 in petroleum ether was added. Water and acetone were added and the vitamins A and D_2 extracted according to the method described.

For evaluation of the volume (V) of the sample extract solution exuding through a hole to the outer thin layer, absorbances of V_A and V_B solutions were measured spectrophotometrically at 328 mμ[4] and

TABLE III—RECOVERY OF VITAMIN D_2 FROM UNTREATED MnO_2[a]

Eluting Solvent	Recovery Av., % (n = 2)	Range, %
1, Ethyl ether	82.2	3.1
2, Acetone–pet. ether (1:4)	83.8	3.2
3, Acetone–*n*-pentane (1:4)	82.6	4.3
4, Acetone–ethyl ether (1:4)	80.9	4.5
5, MEK[b]–pet. ether (1:4)	67.7	4.0
6, MEK–ethyl ether (1:4)	74.0	7.9

[a] Equal part by weight below 105 and 105–210 μ. [b] MEK, methyl ethyl ketone.

TABLE IV—RECOVERY OF VITAMIN D_2 FROM INACTIVATED MnO_2 COLUMN USING ACETONE–PETROLEUM ETHER (1:4) AS ELUTING SOLVENT

	MnO_2		Conditioning of MnO_2		
	Mesh Size (Below 105 μ: 105–210 μ)	Ht., cm.	Inactivation Method[a]	Washing Method[b]	Recovery, %
1,	1:1	1.5	*A*	*A*	98.6
2,	1:1	1.5	*B*	*B*	98.7
3,	2:3	2.0	*A*	*B*	98.7
4,	2:3	2.0	*B*	*A*	100.9
5,	1:2	3.0	*A*	*A*	99.0
6,	1:2	3.0	*B*	*B*	98.1
7,	1:3	3.0	*A*	*B*	100.2
8,	1:3	1.3	*B*	*A*	99.4

[a] Method *A*: 2 ml. of acetone containing 3% water pass through the column. Method *B*: 1 ml. of acetone containing 5% water pass through the column. [b] Method *A*: two 1-ml. portions of pet. ether pass through the column. Method *B*: two 3-ml. portions of pet. ether after inactivation.

TABLE V—RECOVERY OF VITAMIN D_2 IN ACETONE–PETROLEUM ETHER (1:4) SOLUTION

	Eluting Solvent Acetone–Pet. Ether	Recovery, % Av.	Range, % n = 2
1,	1:9	99.2	1.2
2,	1:4	100.3	0.8
3,	3:7	98.9	1.0
4,[a]	1:4	98.0	2.1

[a] Without inactivation of MnO_2.

[3] Adsorbent, Silica Gel G (Wakogel B-5); developing solvent, chloroform; method, microchromatoplate according to Peifer (7); detecting method, U.V. light (365 mμ), $SbCl_3$ T.S., and concentrated H_2SO_4.

[4] The wavelength 328 mμ is the maximum of vitamin A palmitate. In this experiment, to avoid isomerization of vitamin D_2, the hydrolysis was not adopted for extraction of vitamin D_2 from the sample. Therefore, only vitamin A alcohol which existed in vitamin A palmitate was converted to retinene by MnO_2 and almost all vitamin A ester remained unchanged.

TABLE VI—STABILITY OF VITAMIN D_2 IN ELUTING SOLVENTS[a]

	Eluting Solvent Added	T_1 Initial	T_2 3 hr.	T_3 24 hr.	$\frac{T_1 - T_2}{T_1}$ (%) % Loss (3 hr.)	$\frac{T_1 - T_3}{T_1}$ (%) % Loss (24 hr.)
1	Acetone	0.4255	0.4255	0.419	0	1.5
2	Acetone, 1 ml. acetone–pet. ether (1:4)	0.4275	...	0.426	...	0.35
3	Acetone, 2 ml. acetone–pet. ether (1:4)	0.424	0.422	0.418	0.47	1.4
4	Acetone–pet. ether (1:9)	0.4275	...	0.422	...	1.3
5	Acetone–pet. ether (1:4)	0.426	0.425	0.427	0.23	−0.23
6	Acetone–pet. ether (3:7)	0.426	...	0.4245	...	0.35
S_T	Pet. ether	0.427	0.4265	0.4265	0.12	0.12
S_T	Acetone	0.4265	0.4250	0.421	0.35	1.3

[a] The data are represented as the absorbances ($A_{500} - A_{550}$). T_1, T_2, T_3: color developed immediately, 3 hr., and 24 hr., respectively, after mixing vitamin D_2 solution and eluted solvent.

TABLE VII—OXIDATION OF VITAMIN A ALCOHOL BY MnO_2

	MnO_2 Col. Ht., cm.	MnO_2 Col. Inactivation	Treated Vit. A Alc., I.U.	Eluting Solvent	Detection of Vit. A Alc.
1,	1.5	...	5×10^3	Pet. ether	−
2,	1.5	...	5×10^3	Acetone–pet. ether (1:4)	−
3,	1.5	...	10×10^3	Acetone–pet. ether (1:4)	−
4,	1.5	Acetone (1–3 ml.)	10×10^3	Acetone–pet. ether (1:4)	−
5,	1.5	Acetone, 3% water, 2 ml.	10×10^3	Acetone	±
6,	3.0	Acetone, 3% water, 2 ml.	10×10^3	Acetone	−
7,	1.5	Acetone, 3% water, 2 ml.	10×10^3	Acetone–pet. ether (1:4)	±
8,	3.0	Acetone, 3% water, 2 ml.	10×10^3	Acetone–pet. ether (1:4)	−

A_A and A_B were obtained, respectively. The volume (V) of the sample extracting solution exuded was calculated from Eq. 2.

The recoveries of vitamin D_2 from vitamin A and D_2 emulsion against untreated vitamin D_2 (standard) are shown in Table X.

These experiments show that the recoveries of vitamin D_2 from vitamin A–D_2 emulsion are excellent with the proposed procedure.

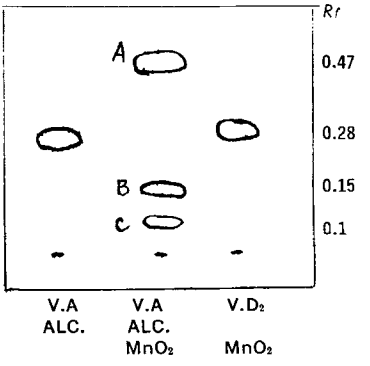

Fig. 4—Thin-layer chromatogram of oxidation products of vitamin A alcohol by MnO_2.

SUMMARY

It was found that vitamin D_2 was adsorbed on untreated MnO_2 and was not eluted with petroleum ether or pentane. Good recovery of the vitamin D_2 was obtained from water–acetone inactivated MnO_2 with an eluant of acetone in petroleum ether (1:4). Thus results were satisfactory.

Vitamin A alcohol having an R_f value similar to vitamin D_2 (R_f 0.28) was oxidized with inactivated MnO_2 on TLC into three yellowish bands which have R_f values of 0.47, 0.15, and 0.10.

Vitamin D_2 was located between the two yellowish bands which have R_f values of 0.47 and 0.15. It is unnecessary to use U.V. light to locate the bands.

By means of a cylindrical thin-layer chromatograph (4) with the introduction of nitrogen gas during the application of sample solution, the partial decomposition of vitamin D_2 was prevented.

With this technique it was found that the application of more than 1 ml. of sample solution, thus minimizing this source of error, was achieved without damaging the thin layer.

TABLE VIII—DETECTION OF OXIDATION PRODUCTS OF VITAMIN A ALCOHOL BY MnO_2 ON TLC PLATE

Spot	Daylight	U.V. Light	$SbCl_3$ T.S.	Conc. H_2SO_4
A	Yellow	Brown absorption	Blue	Red violet
B	Yellow	Brown absorption	Red	Red
C	Yellow	Brown absorption	Orange red	Blue
Vitamin A alc.	...	Yellow fluorescence	Blue	Blue

TABLE IX—RECOVERY OF VITAMIN D_2 FROM THE THIN LAYER

	Day of Expt. 1			2		
Repetition →	1	2	3	1	2	3
No. 1[a]		0.2495			0.280	
V_B - - - A_B No. 2[a]		0.320			0.350	
No. 3[a]		0.432			0.462	
V_A - - - A_A	0.356	0.210	0.3275	0.365	0.327	0.420
V (ml.)	0.8875	1.3438	0.9766	0.9571	1.0657	0.8000
V_C - - - A_C	0.280	0.425	0.310	0.332	0.378	0.281
Recovery, %	98.8	99.0	99.4	99.1	101.3	100.4
Av. %		99.07			100.27	
Range		0.6			2.2	

[a] Vitamin D_2 concentration of developed color solutions No. 1, 2, and 3 was 2, 2.5, and 3.3 mcg./ml., respectively. Absorbance data were represented as the value of $(A_{600} - A_{560})$.

TABLE X—RECOVERIES OF VITAMIN D_2 FROM A-D_2 EMULSION

	Day of Expt. 1			2		
Repetition→	1	2	3	1	2	3
Std. No. 1[a]		0.187			0.183	
A_S No. 2[a]		0.321			0.242	
No. 3[a]		0.461			0.326	
A_C	0.234	0.362	0.362	0.1745	0.236	0.333
V_A - - - A_A	0.332	0.2735	0.325	0.319	0.3125	0.292
V_B - - - A_B	0.318	0.312	0.3055	0.3115	0.3145	0.307
V (ml.)	0.9560	1.1234	0.9362	0.9759	1.0064	1.0489
Recovery, %	101.7	100.4	96.4	97.5	96.9	97.5
Av.		99.50			97.30	
Range		5.3			0.6	

[a] In the first day of experiment, vitamin D_2 concentration of developed color solution No. 1, 2, and 3 was 1.5, 2.5, and 3.5 mcg./ml., respectively, and in the second day, that of No. 1, 2, and 3 was 1.5, 2.0, and 2.5 mcg./ml., respectively.

REFERENCES

(1) Barua, R. K., and Rao, M. V. K., *Analyst*, **89**, 534 (1964).
(2) *Ibid.*, **90**, 571(1965).
(3) Bolliger, H. R., and König, A., *Z. Anal. Chem.*, **214**, 1(1965).
(4) Hirayama, K., *J. Chromatog.*, to be published.
(5) Ball, B., Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **42**, 516(1948).
(6) Henbest, H. B., Jones, E. R. H., and Owen, T. C., *J. Chem. Soc.*, **1957**, 4909.
(7) Peifer, J. J., *Mikrochim. Acta*, **1962/3**, 529.

Method for the Evaluation of Antihidrotic Substances in the Anesthetized Cat

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An improved method using newly designed equipment is described for the evaluation of antihidrotic substances in the anesthetized cat. The substances to be tested were applied locally on the foot pads. The effect on the sweating induced by the administration of a pharmacological agent such as pilocarpine was then observed by recording changes in the moisture content of air passed at a constant flow over the foot pads. The method as described gave consistent and reproducible results with a substance known to produce antihidrotic effects.

FROM A SEARCH of the literature, there appear to be few methods available for investigating the pharmacological activity of substances on sweating in laboratory animals. Kuno (1) was among the first to use the moisture produced by the sweat glands as a measurement of sweating in man. The method had limitations since changes in sweat secretion could only be observed for a period of 5 min. Adams *et al.* (2) have

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