THE CHEMICAL ESTIMATION OF CALCIFEROL IN PHARMACOPŒIAL PREPARATIONS

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The chemical estimation of calciferol (ergocalciferol) presents many problems, but a considerable number of methods including colorimetric[1–7] and spectrophotometric[8,9], as well as methods based on volumetric estimations have been published. Among the latter are the iodine trichloride method of Green[10] and the *p*-benzoquinone method of Tamayo[11], but both suffer from the major disadvantage that very large quantities of calciferol are required, and their usefulness is therefore chiefly confined to the determination of calciferol in high concentrations such as in irradiation products. Among the colorimetric methods, reagents based on antimony trichloride-acetyl chloride have found the widest acceptance[9,12–19], and although the reagent is corrosive and unpleasant to handle the sensitivity is high. Results obtained with such reagents on natural oils have been found to be in good agreement with those obtained by biological assays[9,16,19,20], but so far there has been no proof of the validity of the results obtained in the assay of pharmaceutical preparations which might contain decomposition products.

The biological assay method is a long and expensive procedure and it is desirable that it should be replaced by chemical assay where possible. The problems are considerably simplified if vitamin A is absent, the general composition of the preparations is known, and comparatively large quantities of calciferol are present. The aim of this communication is to recommend simple and rapid methods which give results in good agreement with biological assays and which could therefore be acceptable as official methods.

It was hoped that by suitable extraction procedures followed by chromatography where necessary, the antimony trichloride–acetyl chloride reagent of Nield, Russell and Zimmerli[21], would give satisfactory results with Tablets of Calciferol B.P. and Solution of Calciferol B.P. This reagent, which has been in use in our laboratories for a number of years, was chosen because solutions of antimony trichloride in alcohol-free chloroform are commercially available as Carr-Price reagent, and chloroform is more easily available in a pure state than ethylene dichloride, the alternative solvent recommended by De Witt and Sullivan[16] for the reagent. The reagent chosen, when correctly prepared, has the same sensitivity and stability as that of De Witt and Sullivan and the latter therefore offers no advantage.

The following considerations were borne in mind during the investigation. (1) To confirm the optimum conditions for colour formation and to make a thorough investigation of the technique. (2) To show that only calciferol, and not decomposition products, were measured. (3) To

show that materials likely to be present in tablets do not interfere with the estimations. (4) To investigate interference by substances present in oils likely to be used in the preparation of Solution of Calciferol B.P. and to develop a suitable method of purification. (5) To obtain confirmation of the results by biological assay and by other available methods.

General Procedures Used in the Investigation

Colorimetric Estimation Using Antimony Trichloride-Acetyl Chloride Reagent

The method finally adopted was substantially that described by Nield, Russell and Zimmerli[21]. When one volume of a solution, containing about 25 μg. per ml. of calciferol, is mixed with nine volumes of a 21 to 23 per cent. w/v solution of antimony trichloride containing approximately 2·5 per cent. of acetyl chloride, an orange colour develops, which is maximal 1·5 to 2 minutes after addition of the reagent. The absorption spectrum of the brown-orange colour has a maximum extinction at 500 mμ and an almost zero extinction at 550 mμ. In the absence of interference the extinction at 500 mμ is proportional to the quantity of calciferol present. The quantity of acetyl chloride is not critical and from 1·5 to 5 per cent. may be used. Satisfactory colour development can also be obtained if each volume of calciferol solution is mixed with as little as 5 volumes of reagent.

As it is convenient to use undiluted reagent as the blank, and as a partial correction for any general absorption in the sample, the extinction at 550 mμ is always subtracted from the extinction at 500 mμ. The extinction at 550 mμ should be low and certainly not greater than 0·015. The blank solution may become cloudy after a time, and also the cuvette may become misty. If readings at 550 mμ are always taken immediately after readings at 500 mμ, these possible errors are always detected.

The reagent is prepared by dissolving 22 g. of antimony trichloride, preferably vacuum distilled[23], in dry alcohol-free chloroform (sp.gr. not less than 1·499 at 15·5° C.), diluting to 100 ml. with the chloroform and adding 2·5 ml. acetyl chloride A.R. The reagent must be allowed to stand for at least 30 minutes before use and is stable for several weeks if kept in the dark.

Method

To 1 ml. of the chloroform solution under test (containing about 25 μg. of calciferol) in a stoppered tube, add 9 ml. of the antimony trichloride-acetyl chloride reagent from a rapid delivery burette or pipette, stopper the tube and shake. Transfer to a stoppered 1 cm. cuvette and measure the extinction at 500 mμ and 550 mμ in a suitable spectrophotometer from one and a half to two minutes after addition of the reagent. If no spectrophotometer is available a filter instrument can be used. An interference filter, Wratten filter No. 65 A, or less satisfactorily, a Chance OB2, is suitable. A typical calibration curve obtained by using the Wratten filter is shown in Figure 1.

Determine $E_{1\text{ cm.}}$ (500 mμ minus 550 mμ) and by comparing the figure so obtained with that obtained at the same time with a sample of calciferol B.P. calculate the amount of calciferol present in the sample. $E^{1\text{ per cent.}}_{1\text{ cm.}}$ (500 mμ minus 550 mμ) for calciferol will be found to vary slightly from one batch of reagent to another and from day to day, but generally lies between 1810 and 1900.

Precautions: The reagent is volatile and corrosive and should be kept away from the skin and from metal parts of the instrument. Apparatus must be thoroughly dry as even traces of moisture cause cloudiness. The glass apparatus can usually be cleaned by washing with acetone, but concentrated hydrochloric acid may be necessary if moisture has been allowed to come in contact with the reagent. After completion of the measurements the cell compartment should always be freed from corrosive fumes by blowing in clean compressed air or by vacuum. If a filter instrument is used it is advisable to prepare a fresh calibration curve daily.

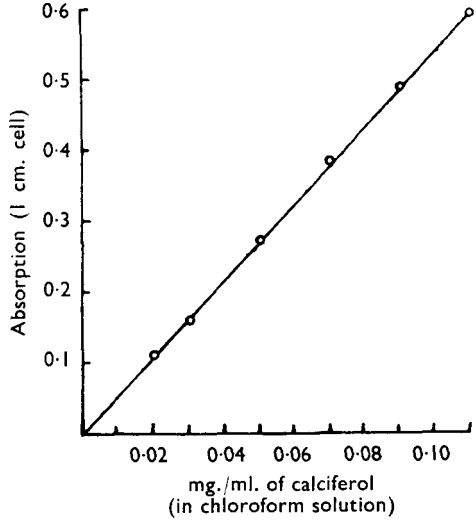

FIG. 1. Typical calibration curve (Wratten filter No. 65A).

Ultra-violet Absorption

The ultra-violet absorption spectrum of calciferol has a characteristic peak at 265 mμ, and this was used as the basis of another assay method. Decomposition products no longer show this selective absorption because of the disruption of the conjugated triene structure, and their absorption at 265 mμ is small compared with that of calciferol and is approximately linear over the range 254 mμ to 272 mμ. The specificity of the method can therefore be improved by the application of a geometric three point correction similar to that described by Morton and Stubbs[24] for vitamin A. It is undesirable to lay down a general formula for this purpose based on data obtained from a limited number of instruments and it is advisable that each laboratory should work out its own formula using pure calciferol for calibration. The formula in use in our laboratories is:—

$$E\ 264\ \text{m}\mu\ \text{(corrected)} = 10{\cdot}50\ \text{B} - 4{\cdot}666\ \text{A} - 5{\cdot}834\ \text{C}$$

(where A, B, and C are the readings at 254 mμ, 264 mμ and 272 mμ respectively using *n*-hexane as solvent) and is based on the ratio:

$$\frac{E\ 254\ \text{m}\mu}{E\ 264\ \text{m}\mu} = \frac{E\ 272\ \text{m}\mu}{E\ 264\ \text{m}\mu} = 0{\cdot}905$$

Since the absorption peak of calciferol is rather flat the correction is of limited value, but the procedure can be used for the assay of high potency tablets and oils, particularly in control laboratories where blank readings are available.

Method

Prepare a solution in spectroscopically pure *n*-hexane containing approximately 10 μg./ml. of the calciferol under test. Measure the extinction in a 1 cm. cuvette over the range 245 mμ to 280 mμ, paying particular attention to 254, 264 and 272 mμ. Calculate the amount of calciferol present using the formula given above or a similar formula. $E^{1\text{ per cent.}}_{1\text{ cm.}}$ 264 mμ for calciferol = 470.

Chromatography

Calciferol can be separated from its decomposition products by chromatography on an alumina column using mixtures of *n*-hexane as basic solvent, with ether and ethanol to increase its polarity for elution purposes. Separation is also effected from phytosterols but is not always complete. It is difficult to lay down standard procedures owing to variation in the alumina and to such factors as rate of flow, but a typical method which has been found effective in our hands is as follows.

Reagents. Alumina: standardised according to the method of Brockman[25] and deactivated by water if necessary to give activity intermediate between grades 1 and 2. *n*-Hexane: This must be of spectroscopic purity, and such that when 100 ml. are evaporated to dryness and redissolved in 10 ml., the extinction (using the original *n*-hexane as the blank) is not greater than 0·03 in the range 255 to 275 mμ. Ether: anæsthetic B.P. washed thoroughly with water, dried with anhydrous sodium sulphate and redistilled from anhydrous sodium sulphate and anhydrous ferrous sulphate. Ethanol: absolute, A.R.

Method. Prepare a chromatographic column of alumina in amber glass 15 to 20 cm. long by 1 cm. diameter by pouring in a slurry of alumina in *n*-hexane. Tamp the alumina down to give a flow rate of about 1 to 2 ml. per minute under slight nitrogen pressure. Wash the column with spectroscopic *n*-hexane until the washings have an absorption at 255 mμ no higher than the original hexane. Transfer the sample containing about 1 mg. (as little as 50 μg. can be used) to the top of the column using the minimum quantity of *n*-hexane, wash with 50 ml. of 10 per cent. ether in *n*-hexane calling the eluate fraction I. Wash further with 50 ml. 20 per cent. ether in *n*-hexane—fraction II, elute the calciferol with 100 ml. 1 per cent. ethanol in a mixture of 1 : 1 ether in *n*-hexane—fraction III, further wash with 10 per cent. ethanol in 1 : 1 ether, *n*-hexane—fraction IV. Evaporate each fraction to dryness, in a stream of nitrogen at a temperature not exceeding 30° C., dissolve in a suitable amount of *n*-hexane and examine for calciferol using the spectrophotometric method given above (it may be found more convenient to dilute fraction III to 100 ml. in a volumetric flask and measure the absorption directly), evaporate an aliquot to dryness, redissolve in chloroform and examine by the colorimetric

method. 95 to 100 per cent. of the calciferol will normally be found in fraction III, but fraction IV should always be examined.

Using this technique recoveries of pure calciferol of 98 to 102 per cent. have regularly been obtained by either method of determination.

To confirm that fraction III really contains calciferol and not an artefact or decomposition product, an experiment was run on a larger scale. 100 mg. of a naturally decomposed sample of calciferol, shown to contain 70 per cent. calciferol by the above colorimetric and spectrophotometric methods, was subjected to the above chromatographic procedure. Fraction III evaporated to dryness weighed 70·4 mg. Table I gives the physical characteristics of the solid material obtained, compared with those of calciferol B.P.

TABLE I

PHYSICAL CONSTANTS OF CALCIFEROL OBTAINED FROM CHROMATOGRAPHIC FRACTION III

	Sample	Calciferol B.P.
m.pt.*	117° C.	114° to 117° C.
Mixed m.pt.	117° C.	,,
$[\alpha]_D^{20}$	+102·9°	+102·5° to 107·5°
m.pt. dinitrobenzoate ..	144° C. rext. 147° C.	About 148° C.
$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 264 mμ	466	460
$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 500 mμ minus 550 mμ ..	1830	1850
Infra-red	Normal	—

* m.pt. of original material = 101° to 107° C.

Infra-red Absorption

The infra-red absorption curves of calciferol have been published[26,27] and these have been confirmed. For diagnostic purposes three peaks with wave numbers between 1600 and 1650 are important and are thought to be due to the conjugated triene structure. In a Nujol mull an examination of both pure calciferol and of partially decomposed materials showed that this absorption band disappears as decomposition proceeds and a very broad band due to C=O bonding appears centred at about 1700 wave numbers. At this wavelength calciferol shows no absorption; see Figure 2.

For quantitative purposes solution spectra are required. Two per cent. solutions of the same pure and decomposed samples of calciferol in carbon disulphide were run over the range 700 to 1400 wave numbers and a peak at about 1050 wave numbers found to be suitable for assay purposes, since it is produced only by calciferol, and suffers no interference from decomposition products, Figure 3.

Method

Prepare a two per cent. solution of the sample in carbon disulphide and fill into a 1 mm. cuvette. Record its absorption between 950 and 1150 wave numbers, calculate the extinction of the peak at about 1050 wave numbers using a base line technique, and relate this to a standard curve obtained by using pure calciferol.

Interfering Materials

In the assay of pharmaceutical preparations containing calciferol, interference may be due to decomposition products of calciferol or the vehicle used in the preparation.

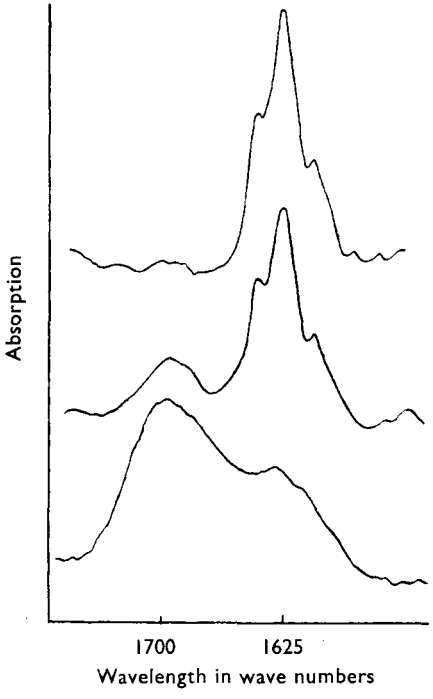

Fig. 2. Nujol mull spectra of calciferols.

Decomposition Products

Four samples of calciferol which had been allowed to decompose naturally to varying degrees were examined by all the techniques outlined above (see Table II). The figures quoted in this table for spectrophotometric and colorimetric determination after chromatography, were obtained from fraction III in the chromatographic method outlined above.

As a further check in an experiment on sample 2 the column was stripped as free as possible by increasing the solvent polarity stepwise up to two per cent. acetic acid in 2 : 1 : 1 ethanol : ether : *n*-hexane mixture.

TABLE II

Examination of decomposed calciferol samples

Sample	Per cent. calciferol				
	Method				
	Colorimetric		Spectrophotometric		Infra-red
	Direct	After chromatography	Direct	After chromatography	
1	83·0	81·0	85·0	82·0	88·5
	84·0	—	82·0	83·0	
2	40·0	39·0	no results possible	39·5	40·0
	36·0	—		39·0	
3	6·0	4·2	no results possible	4·5	2·85
	7·0	5·5			
4	1·0	0·9	no results possible	1·0	0·80
	0·9	1·1		1·2	

All fractions were examined by both spectrophotometric and colorimetric procedures. The results are summarised in Table III, which emphasises that the direct determination using the colorimetric method will give the correct result on a decomposed sample.

Vegetable Oils

The B.P. directs that in the preparation of tablets crystalline calciferol (Calciferol B.P.) shall be dissolved in a suitable vegetable oil and tablets prepared using the normal excipients. Solution of Calciferol B.P. is a solution of crystalline calciferol in a suitable vegetable oil. It is well known that phytosterols present in vegetable oils interfere with the estimation of calciferol by both colorimetric and spectrophotometric methods[15,19]. Table IV gives E (500 mμ minus 550 mμ) figures given by some vegetable oils (without saponification) after colour development with antimony trichloride-acetyl chloride reagent.

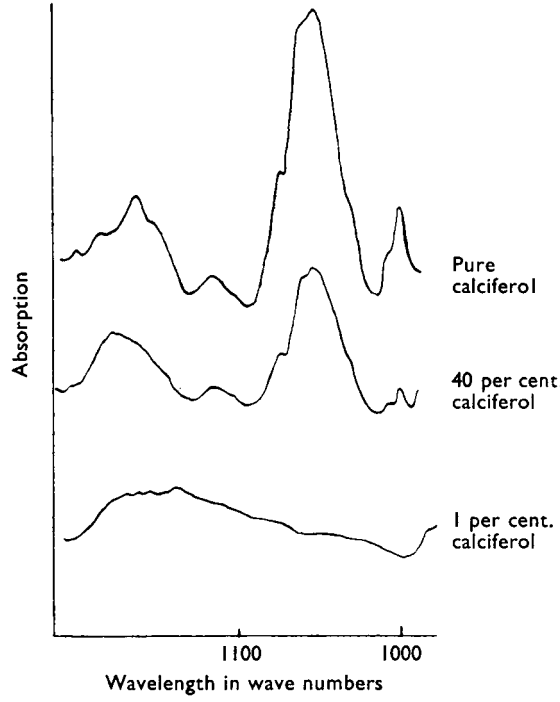

FIG. 3. Infra-red spectra of 2 per cent. solutions of pure and decomposed calciferols in carbon disulphide solution.

A number of tablets made by different manufacturers were found to contain between 2·5 and 5 per cent. vegetable oil. Assuming a 50,000 I.U. tablet to weigh 5 grains uncoated and to contain say 10 per cent. of oil the interference due to a hypothetical oil with an $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ (500 mμ minus 550 mμ) value of 0·2 will be only 0·2 per cent.

In Solution of Calciferol B.P. the interference of the vegetable oil is a major problem[22]. The chromatographic method outlined above, applied to the unsaponifiable fraction of the oil followed by the colorimetric procedure, has given satisfactory results on oil solutions made in olive oil, arachis oil, cotton seed oil and tea seed oil. One batch of arachis oil gave a chromatographic fraction III which contained interfering material amounting to 5 per cent. of the calciferol. This interference was reduced to less than 2 per cent. by modifying the chromatographic procedure. The column was eluted with 15 to 20 per cent. ether in *n*-hexane, 25 ml.

TABLE III

SUMMATION OF THE ABSORPTION OF CHROMATOGRAPHIC FRACTIONS OF SAMPLE 2

Fraction	Volume (ml.)	Solvent	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 265 mμ	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ (500 minus 550 mμ)
1	90	10 per cent. ether in *n*-hexane ..	1·04	3·1
2	20	20 per cent. ether in *n*-hexane ..	zero	0·2
3	50	0·25 per cent. ethanol in 1 : 1 ether : *n*-hexane	184·9	725
4	20	1 per cent. ethanol in 1 : 1 ether : *n*-hexane	4·5	5·1
5	25	5 per cent. ethanol in 1 : 1 ether : *n*-hexane	4·5	3·6
6	25	10 per cent. ethanol in 1 : 1 ether : *n*-hexane	6·1	1·5
7	25	20 per cent. ethanol in 1 : 1 ether : *n*-hexane	zero	zero
8	30	50 per cent. ethanol in 1 : 1 ether : *n*-hexane	zero	zero
9	50	50 per cent. ethanol in 1 : 1 ether : *n*-hexane + 2 per cent. acetic acid	19·5	0·6
		Total	220·5	739·1
		Original before chromatography ..	244·9	743
		Per cent. calciferol from fraction 3 ..	39·5	39·0
		Per cent. calciferol on total unchromatographed material	—	40·0

fractions were collected and examined separately. One interesting feature arising from this work was that although the interfering materials, present in the calciferol fraction, when examined by the extrapolation method of Rogers[22], gave zero colour at zero time, those present in the other fractions did not.

TABLE IV

COLOUR DEVELOPMENT GIVEN BY VEGETABLE OILS WITH ANTIMONY TRICHLORIDE-ACETYL CHLORIDE REAGENT

Oil	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ 500 minus 550 mμ*
Arachis	0·1
Olive	0·1
Cotton Seed	0·08
Theobroma	0·07

* Readings taken after 2 minutes.

Antioxidants

It seems likely that vegetable oils used in the future may contain small quantities of antioxidants, the addition of 0·01 per cent. propyl or octyl gallate and 0·02 per cent. of butylated hydroxy anisole to vegetable oils being permitted in the United States. These and similar compounds even in considerably greater quantities do not interfere with the determination of calciferol either in tablets or in oily solutions.

Tablet Excipients

Excipients commonly used in calciferol tablets include lactose, dextrose, starch, cocoa powder, calcium phosphate, sucrose, and acacia. The coating consists of sugar and varnish. None of these materials have been found to interfere with the direct colorimetric estimation.

Conclusions

From the results obtained by the various methods and a detailed study of the ultra-violet and infra-red absorption curves, it was concluded that although the absorption curves of the calciferol-containing fraction

obtained after chromatography are generally identical to those of calciferol, a three point correction is sometimes necessary. This is particularly so when samples are being analysed in which a very high proportion (60 per cent. or more) of the calciferol originally present has undergone decomposition. Such a three point correction is always a source of error, and only very rarely corrects for all the irrelevant absorption. With tablets made by one manufacturer, the spectrophotometric method, even after chromatography, always gave very much too high results. In the analysis of comparatively low potency oils, saponification, followed by chromatography of the unsaponifiable matter does not remove everything which interferes with the spectrophotometric assay, and such an assay, even if a correction procedure is used, will give very high results. On the other hand, decomposition products of calciferol appear to give practically no colour with the antimony trichloride-acetyl chloride reagent, and the colorimetric method applied direct to a chloroformic extract of a decomposed sample of calciferol, or of ground tablet material, gives the same result before and after chromatography. It was therefore concluded that a chromatographic step is unnecessary in the colorimetric analysis of Tablets of Calciferol. In the case of the comparatively low potency oily solutions of calciferol where the phytosterols present in the oil give colours which interfere, chromatography is necessary. This then separates the calciferol from the bulk of the phytosterols and from any decomposition products. The colorimetric procedure is then applied, as before, to the purified calciferol.

Recommended Methods

Tablets of Calciferol B.P.

Method. Weigh 20 tablets, crush and grind to a very fine powder. Weigh accurately the equivalent of about 5 tablets and shake mechanically during one hour in the dark with 20 ml. of *n*-hexane. The shaking must be vigorous and should be continuous for one hour because decomposition products of calciferol, which may be present, are rather insoluble in *n*-hexane and prevent the calciferol from dissolving.

Allow to settle, centrifuge if necessary, and evaporate 2 ml. to dryness under nitrogen keeping the temperature below 30° C. Dissolve the residue in chloroform and make up to 20 ml. Transfer 1 ml. to a stoppered glass tube and add 9 ml. of antimony trichloride-acetyl chloride reagent from a fast flowing pipette or burette. Shake, transfer a portion to a stoppered 1 cm. cuvette and measure the absorption at 500 mμ and 550 mμ from $1\frac{1}{2}$ minutes to 2 minutes after the addition of the reagent.

Prepare a chloroform solution containing a known quantity (about 30 μg./ml.) of a standard sample of calciferol, and treat 1 ml. exactly as above, using the same reagents.

From the $E_{1\,\text{cm.}}$ (500 mμ minus 550 mμ) figures obtained calculate the calciferol content in a tablet of average weight.

Solution of Calciferol B.P. To 1·5 g. sample add 0·1 g. hydroquinone and 25 ml. 0·5N ethanolic potassium hydroxide solution. Boil under reflux during 20 minutes, cool, add 50 ml. of water and transfer to a

TABLE V

SUMMARISED RESULTS ON TABLETS

No.	Sample	Labelled potency	Age	Method 1†	2†	3†	4†	5†	6†	7‡	Limits of error p = 0·95
1	Tablets non-chocolate basis ..	50,000	5 years	58,600	36,600	37,800	36,900			34,000	(29,000–39,000)
2	"	50,000	5 years		43,200		41,700				
3	"	50,000	4½ years	65,000	36,700	37,800	36,500			43,000 43,000	(35,000–53,000) (37,000–51,000)
4	"	50,000	3 years	47,700	37,700	38,400	37,300			39,000	(30,000–51,000)
5	"	50,000	2 years		48,500		46,600				
6		50,000	2 years		44,600						
7		50,000	2 years		44,600						
8	"	50,000	2 years		50,800						
9	"	50,000	2 years		52,800						
10	"	50,000	1 year	85,500	54,600 54,400	53,800	53,800				
11	Tablets chocolate basis	50,000	1 year	58,800	55,600	54,100	54,900				
12	Tablets non-chocolate basis ..	50,000	less than ½ year	72,400	51,500	53,200	50,500			60,000	(35,000–72,000)
13	Tablets chocolate basis (dragées)	50,000	unknown	36,650	39,400 33,800*	36,250 31,500*	36,200 33,600*	35,800	38,800	25,000*	(21,000–30,000)
14	Non-chocolate basis	50,000	"	95,250 100,700	47,500 47,500	90,000 62,000	46,000 44,400	47,000	46,750	44,000	(37,000–53,000)
15	"	50,000	"	28,250 36,200	26,250 27,300	28,250 27,700	25,500 27,000			31,000	(26,000–38,000)
Tablets Calc. & Vit. D. B.P.C. 1949		500 500 500 500 500 500 500					400 250 290 360 370 480 280			400 280 330 420 350 350 290	(300–530) (140–590) (220–500) (330–550) (240–520) (250–470) (210–420)

* 6 months after first assay. † Calciferol converted to units. ‡ vitamin D units.

KEY TO TABLE V

Methods:—
(1) Spectrophotometric—direct, correction applied.
(2) Colorimetric—direct (recommended method for tablets).
(3) Spectrophotometric—on chromatographic fraction III, correction applied.
(4) Colorimetric—on chromatographic fraction III.
(5) Spectrophotometric—after saponification, correction applied.
(6) Colorimetric—after saponification.
(7) Biological.

separator. Extract with 3 × 30 ml. portions of anæsthetic ether. Wash the combined ethereal layers with 20 ml. of water followed by 10 ml. 0·5N aqueous potassium hydroxide and then with 20 ml. portions of water until the washings are no longer alkaline to phenolphthalein.

Dry the ethereal solution with anhydrous sodium sulphate, wash the latter with two 10 ml. portions of ether and evaporate the combined solution and washings to dryness under nitrogen at a temperature not exceeding 30° C.

Dissolve the residue in about 10 ml. of *n*-hexane and transfer to an alumina column 20 cm. long and 1 cm. diameter; using a flow rate of 1 to 2 ml. per minute with nitrogen pressure if necessary, elute continuously with 15 to 20 per cent. ether in *n*-hexane collecting that fraction which contains the calciferol Its position is determined previously for the alumina and solvents actually used.

TABLE VI

SUMMARISED RESULTS ON OILY SOLUTIONS OF CALCIFEROL

No.	Sample	Comments	Theory	Found (recommended method) (Calciferol converted to vitamin D units)
1	Soln. B.P. in arachis oil	Freshly prepared	3100	3050 3130
2	,, ...	Freshly prepared	3450	3450 3280
3	,, ...	Freshly prepared. Production batch	3200	2920 3230
4	Soln. in arachis oil		1000	1090
5	,, ...		1000	900
6	Soln. B.P. in olive oil	Freshly prepared	2800	2980
7	Soln. B.P. in cotton seed oil	Freshly prepared	3270	3060
8	Soln. in arachis oil	Production batch	51,500	51,700
9	,, ...	Production batch	51,500	50,800
10	Soln. B.P. in arachis oil	2½ years old	3070	3070
11	,, ...	1½ years old	3070	3100

Evaporate the solvent under nitrogen at a temperature not exceeding 30° C., and dissolve the residue in 5 ml. chloroform. Transfer 1 ml. to a glass stoppered tube and add quickly 9 ml. of antimony trichloride-acetyl chloride reagent. Measure the absorption at 500 mμ and 550 mμ. A high 550 mμ value is an indication that separation from interfering materials is not complete, because the alumina is too inactive or the concentration of ether in the *n*-hexane used for elution too high, or both.

Determine *E* (500 mμ minus 550 mμ) for the sample and for calciferol, and calculate the amount of calciferol in the sample. All work in both of the above methods must be performed in non-actinic glassware.

Biological assays have been carried out on a sufficient number of samples of tablets to confirm the recommended methods. In addition some results obtained by biological and chemical assays on tablets of Calcium

and Vitamin D B.P.C., are given as further evidence of the good agreement obtained by the two methods. The various results on tablets are summarised in Table V. The results obtained by the recommended method on various solutions of calciferol in oil are summarised in Table VI.

SUMMARY

1. The antimony trichloride-acetyl chloride reagent of Nield, Russell, and Zimmerli has been applied to the assay of calciferol in a number of preparations.

2. Results obtained by this method have been compared with results obtained by spectrophotometric and infra-red methods.

3. A chromatographic procedure of purification of decomposed calciferol has been described.

4. Assay procedures have been recommended for calciferol in Tablets of Calciferol B.P. and Solution of Calciferol B.P.

5. The results obtained by chemical assays have been found to be in agreement with results obtained by biological assays.

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DISCUSSION

The papers were presented by MR. A. R. ROGERS and MR. P. S. STROSS respectively.

DR. F. WOKES (Kings Langley) said that there were other colour tests, including that of Schaltegger (*Helv. chim. Acta*, 1946, **29**, 285) which had shown reasonable agreement with biological tests but was not mentioned in the papers. Because of the fading of the colour there was only time in the antimony trichloride test to take readings at one extinction. That did not characterise the absorption band, and as the test was not specific it would be helpful if methods could be devised for taking readings on either side of 500 mμ. Nearly thirty years ago he had shown that the antimony trichloride reagent could give colours with many sterol derivatives but paper chromatography might overcome this difficulty. The spectrophotometric method should be more specific than any colour test, provided the peak could be adequately characterised by taking readings around the maximum. That had been done by the authors of the second paper and he agreed with their suggestion to use the Morton-Stubbs correction. Their extinction readings had been taken rather close to maximum so that at 254 to 264 there was a ratio of 0·905 which, in his view, was not as accurate as if the readings had been taken lower down from the peak. The nearer the ratio came to 1 the less accurate it was. He questioned the statement by Stross and Brealey that "Decomposition products no longer show this selective absorption because of the disruption of the conjugated triene structure, and their absorption at 265 mμ is small compared with that of calciferol and is approximately linear over the range 254 mμ to 272 mμ." His own experience had been that when calciferol was exposed to light an initial decomposition product had a peak at 275 to 280 mμ. There was another decomposition product which appeared later with a peak round about 250, and it was only when calciferol continued to be exposed for long periods that the peaks disappeared.

MR. W. H. C. SHAW (London) observed that Mr. Rogers used ethylene dichloride as solvent whilst Messrs. Stross and Brealey preferred chloroform. Some time ago he reached the conclusion that ethylene dichloride offered no advantages over chloroform, particularly as it was difficult to obtain in a satisfactorily pure condition. In Table IV Mr. Rogers included some direct spectrophotometric results with ethylene dichloride. It should be remembered that a solution of calciferol could readily be polarized, particularly in a chlorinated solvent, with the formation of the compound known as *iso*tachysterol. This had a much higher extinction value than calciferol. In a brief investigation carried out some time ago on the atmospheric oxidation of calciferol at 37° C. it was found that when there was no detectable biological activity left, a small reaction with antimony trichloride was still obtained. He agreed that simple chromatography was usually effective in eliminating oxidation products which were undoubtedly a very complex mixture. It should be remembered that the Morton-Stubbs correction was only an approximation. In his view

the antimony trichloride method was preferable to that employing the Morton-Stubbs correction.

DR. G. E. FOSTER (Dartford) said that in his experience the chemical and spectrophotometric methods were usually applicable to high potency preparations of calciferol. Difficulties were encountered with low potency products. That was illustrated to some extent by the present work, particularly in the Tables showing the assays on the B.P. tablets of calcium with vitamin D which only contained 500 units. The authors of the second paper stated that their results were supported by the results of biological tests, but in Table V there were no limits of error stated for the biological figures. In his experience the biological estimation for vitamin D was subject to a very large experimental error, and for this reason there would be some difficulty in stating categorically that the colorimetric method agreed with the biological activity.

DR. F. HARTLEY (London) said that the errors of the biological assay were quite considerable even in one laboratory with one worker and if an attempt were made to obtain correlation in a combined test in several laboratories the range of error would be widened. The colorimetric method was recommended, and he suggested that in fact the validity had indeed been established by taking the potencies as set out in Table V of the second paper. If necessary a further column could be added setting out in detail the limits of error. He wished to avoid the situation where calciferol and its preparations continued to be determined only by biological assay. Since the second paper was prepared for publication biological results were completed on some of the solutions, and it was to be hoped that Mr. Stross would outline those results in his reply. The object of the work could be said to be a move from the biological assay for vitamin D to a chemical or physical method.

DR. D. C. GARRATT (Nottingham) said that in considering the determination of biologically active material which was liable to decomposition it was necessary to correlate the method used with the biological assay. Mr. Roger's paper was incomplete. It was no use getting correlation between chemical and physical assays unless they could be correlated with the potency of the material being examined. The potencies of various manufacturers' products had been estimated, but that did not necessarily mean that they were correct.

MR. H. E. BROOKES (Nottingham) said he understood that the term "error" was very different in bioassay work from that used in analytical chemistry. When one talked of error in analytical chemistry it was rather due to some fault of the person. The limits of error of the bioassayist were rather the limits of probability than limits of error, and it was surprising in the case of calciferol that a series of chemical tests and a series of biological results correlated so well.

MR. K. L. SMITH (Nottingham), speaking as one of those who supplied some of the biological figures, said the estimates in the column referred to were made to $\pm$ 25 per cent., and if the authors had stated this, precise limits of error would not be necessary. The only biological assay carried out on a chocolate-base substance was the assay of tablet 13. The result,

for which he was responsible, was significantly different from the chemical assay result.

MR. A. R. ROGERS, in reply, said that no adsorbent was used in his elution method of extraction, the eluent being added directly to the powdered tablets in the column. The batches of ethylene dichloride used had always been found to be free from interfering impurities, including hydrochloric acid. The main objection to the use of chloroform was the formation of carbonyl chloride on storage. He would like to ask the other authors if it had been established that carbonyl chloride did not interfere with the colour reaction. The limitations of the Morton-Stubbs correction formula must be stressed. It was unusual to find linear irrelevant absorption, and errors were large. He also asked Mr. Stoss if he and his co-author had established by recovery experiments that no decomposition of calciferol occurred during evaporation. Interfering materials which did not show zero colour at zero time with the reagent were reported to be present in some samples of vegetable oils, and he enquired whether they were present in amounts sufficient to invalidate the extrapolation procedure. He also asked for information regarding the chief factors causing deterioration of calciferol preparations on storage. MR. P. S. STROSS, in reply, said that recovery experiments had shown that there was no loss of calciferol during evaporation when below 30° C. if the solution were protected from light and oxygen-free nitrogen was used. The solution used in the B.P. identification test is too strong and the specificity could be increased by measuring the extinction at 450, 500 and 550 mμ. Alcohol-free chloroform need be used only for the preparation of the reagent and not as solvent for the sample, and no trouble has been encountered with the formation of phosgene. The absence of air and light and the use of peroxide-free oils is essential for the stability of calciferol preparations, which should be stored in a cool place. *n*-Hexane was chosen for the chromatographic and spectrophotometric work as chlorinated hydrocarbons should be avoided as pointed out by Mr. Shaw. The low results obtained by Mr. Rogers using hexane (or light petroleum) for this extractions were probably due to decomposition products and to insufficient time being allowed for the extractions.

Results which were more than 25 per cent. high were sometimes obtained using Mr. Roger's extrapolation technique for the assay of Solution of Calciferol B.P., and he considered that unless blanks are available chromatography is necessary for low potency oils. In reply to Dr. Wokes, he said that they had realised that the antimony trichloride-acetyl chloride reagent was not specific for calciferol. He considered that when analysing calciferol preparations whose general composition is known, high specificity though very desirable is not absolutely essential. One must, however, be certain that the concentrations of decomposition products and other constituents are not sufficient to interfere. The selection of wavelengths for the three point correction was a compromise. Whilst decomposition products of calciferol formed by the action of light, particularly ultra-violet light are well known not to have linear absorption

over the range 254 to 272 mμ, the absorption of oxidation products and decomposition products formed in the dark seems to be linear over this range. In reply to Mr. Shaw, he said that they had avoided using chlorinated hydrocarbons for extraction, and only allowed the calciferol to be in contact with chloroform for the minimum possible time. He agreed that some colour is given by decomposition products as seen from Table III, but is so small as to be almost negligible. On the points raised by Dr. Foster, Mr. Stross said that the limits of error of the various biological assays were now available and that it may be possible to include these in the paper. It was realised when this work was started that the limits of error would be rather wide, and every effort was made to confirm the results by other methods as well as by the biological tests. In answer to other points raised, biological figures of 3100 units per g. and 3700 units per g. had since been found for oils Nos. 10 and 11 (Table VI). As could be seen from Table V, they had attempted to obtain a large number of biological results rather than a single result with a narrow limit of error. The interference of chocolate base and other excipients was assessed by blank and recovery experiments, but for brevity the description of these had been omitted.