THE SPECTROPHOTOMETRIC DETERMINATION OF VITAMIN D IN PHARMACEUTICAL PREPARATIONS

PART II. TABLETS OF CALCIFEROL B.P.

BY A. R. ROGERS*

From the Analytical Department, Allen and Hanburys, Ltd.

Received June 13, 1955

INTRODUCTION

TABLETS of calciferol are the subject of monographs in the National Formulary 1949 and the British Pharmaceutical Codex 1949 under the title, Strong Tablets of Calciferol, and in the British Pharmacopœia 1953 as Tablets of Calciferol. The various monographs relate to substantially the same preparation, namely coated tablets which, unless otherwise stated, each contain 50,000 units of antirachitic activity. The preparation which appeared in the British Pharmaceutical Codex 1949 and 1954 under the title Tablets of Calcium with Vitamin D will not be discussed in this paper.

Very little has been published about the assay of tablets containing vitamin D. The British Pharmacopœia 1953 describes a procedure involving shaking the powdered tablets with light petroleum followed by evaporation of the solvent and subsequent biological assay. The extraction of vitamin A from tablets is a similar problem, and several methods are described in the literature[1–6], including continuous extraction in a Soxhlet apparatus. Another possibility is elution of the powder with a suitable solvent using the technique of adsorption or partition chromatography[7].

The vitamin D, extracted from the tablets and brought into solution by one or other of these methods, may then be assayed biologically[8], microbiologically[9], by direct ultra-violet spectrophotometry (using the absorption maximum at ca. 265 mμ), or colorimetrically[10]. Infra-red methods are probably unsuitable because of the large amounts of vitamin D that would be needed[11].

APPARATUS, MATERIAL AND REAGENTS

Spectrophotometry. The work in this paper was carried out in part on a Beckman Model DU spectrophotometer and in part on a Unicam SP 500 spectrophotometer. Matched 1 cm. glass stoppered cells with a tungsten lamp as light source were used for colorimetry in the visible region of the spectrum and matched 1 cm. fused silica cells with a hydrogen lamp for the ultra-violet measurements.

Extraction apparatus. For the elution method, a chromatography-type tube of internal dimensions 30 cm. × 1 cm. fitted at the bottom with a cotton-wool plug on which the powder to be extracted was supported. The rate of flow of the eluant was of the order of 3 ml. per minute.

* Present address: School of Pharmacy, Brighton Technical College, Brighton, Sussex.

A small Soxhlet apparatus attached by ground-glass joints to a 150 ml. flat-bottomed flask and an efficient double-surface condenser. Heat was supplied by an electric hot-plate. The size of extraction thimble used was 2·2 cm. × 8·0 cm.

Antimony trichloride. Antimony trichloride, reagent grade. *Calcium phosphate.* Calcium phosphate, B.P. *Calciferol.* Crystalline calciferol, B.P. *Chocolate basis.* A mixture of non-alkalised cocoa powder 15 parts, sucrose B.P. 15 parts and lactose B.P. 70 parts. *Lactose.* Lactose, B.P. *Starch.* Maize starch, B.P. *Acetyl chloride.* Acetyl chloride, reagent grade. *Carbon tetrachloride.* Carbon tetrachloride dried over anhydrous calcium chloride, and distilled, the first and last 10 per cent. portions being rejected. *Chloroform.* Chloroform B.P., containing 1 to 2 per cent. of ethanol, shaken successively with three equal volumes of water, dried over anhydrous calcium chloride, and distilled, the first and last 10 per cent. portions being rejected. Used within 24 hours of preparation. cyclo*Hexane.* *cyclo*Hexane dried over anhydrous potassium carbonate, passed through a column of activated silica gel[12,13], and distilled, the first and last 10 per cent. portions being rejected. The transmission, compared with water in 1 cm. cells, exceeded 90 per cent. at all wavelengths down to 245 mμ, and exceeded 95 per cent. at 265 mμ. *Ethanol.* Dehydrated ethanol refluxed for two hours with 2 per cent. of potassium hydroxide pellets and 2 per cent. of zinc dust, and distilled, the first and last 10 per cent. portions being rejected. The transmission, compared with water in 1 cm. cells, exceeded 90 per cent. at all wavelengths down to 235 mμ, and exceeded 95 per cent. at 265 mμ. *Ethylene dichloride.* 1:2-dichloroethane dried over anhydrous calcium chloride, and fractionally distilled, the portion of boiling range 82° to 84° C. being collected. *Light petroleum.* "Aromatic-free" light petroleum fractionated, the portion of boiling range 40° to 50° C. being collected. This was preferred to the 50°/60° material used in the pharmacopœial assay, since it is more suitable as a spectroscopic solvent in the ultra-violet region. The transmission, compared with water in 1 cm. cells, exceeded 90 per cent. at all wavelengths down to 225 mμ, and exceeded 95 per cent. at 265 mμ.

Antimony trichloride reagent. For the B.P. identification test, a solution of 20 per cent. of antimony trichloride and 1 per cent. of acetyl chloride in chloroform. For the assay, a solution of 20 per cent. of antimony trichloride in ethylene dichloride with the addition of 2 per cent. of acetyl chloride, prepared according to the method of de Witt and Sullivan[14].

Methods of Extraction

Shaking. Weigh and powder 20 tablets. To an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol, add 50 ml. of solvent, shake for 5 minutes, adjust the volume to 100 ml. with solvent, shake for 2 minutes, and centrifuge or allow to stand until the insoluble matter has settled. Determine the calciferol in the supernatant liquid by the colorimetric method.

Elution. Weigh and powder 20 tablets. Elute an accurately weighed

quantity of the powder, equivalent to about 1·5 mg. of calciferol, with solvent, collecting 100 ml. of eluate. Mix by shaking, and determine the calciferol in the solution spectrophotometrically or colorimetrically.

Soxhlet extraction. Weigh and powder 20 tablets. Extract an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol with 90 ml. of solvent for 60 minutes. Cool the solution, and adjust the volume to 100 ml. with solvent. Mix by shaking, and determine the calciferol in the solution colorimetrically.

Methods of Determination

Spectrophotometric. Compare the extinction of the solution with that of pure solvent at the wavelength of maximum absorption, 265 mμ (or 267 mμ in the case of ethylene dichloride). The value of $E_{1\,\text{cm.}}^{1\,\text{per cent.}}$ (265 mμ) for calciferol in *cyclo*hexane, ethanol or light petroleum is 470; the value of $E_{1\,\text{cm.}}^{1\,\text{per cent.}}$ (267 mμ) for calciferol in ethylene dichloride is 420. Calculate the weight of calciferol in each tablet of average weight.

Colorimetric. To 0·4 ml. of solution add 2·2 ml. of antimony trichloride reagent, and compare the extinctions at 500 mμ at ½-minute intervals for 3 minutes with those of 0·4 ml. of solvent + 2·2 ml. of reagent in the blank cell. The colour reaches a maximum in about one minute, then slowly fades; record the maximum extinction. The value of $E_{1\,\text{cm.}}^{1\,\text{per cent.}}$ (500 mμ) for the colour given by pure calciferol in these conditions is about 2000; it varies slightly from batch to batch of reagent, and should be determined afresh for each batch which is prepared. Calculate the weight of calciferol in each tablet of average weight.

This is essentially the method described in a previous paper[10]. It has been found with a few batches that the reagent blank was rather high, and the use of a mixture of reagent and solvent in the blank cell instead of solvent only has been adopted to correct this source of error. Also, recent batches of reagent indicate a value of $E_{1\,\text{cm.}}^{1\,\text{per cent.}}$ (500 mμ) for calciferol nearer to 2000 than 1900 which was the figure previously reported.

Other Tests

B.P. test of identification. Powder one tablet, shake with 5 ml. of chloroform, filter, and to 1 ml. of filtrate add 9 ml. of antimony trichloride reagent. A brownish-red colour should be produced.

B.P. test of disintegration. Carry out the official test in the 1955 addendum.

Commercial Tablets

Samples of calciferol tablets, labelled as Tab. Calciferol. B.P., or Tab. Calciferol.Fort. B.P.C. 1949, or Tab. Calciferol.Fort. N.F. 1949, or otherwise stated to contain 50,000 units of antirachitic activity (vitamin D) per tablet, were purchased from fourteen different manufacturers, A to N. All samples were analysed within 2 months of purchase. Sample E_2 was obtained from the manufacturers of sample E_1 when the results of the analysis of E_1 were known.

The calciferol in the tablets was extracted by three methods with a

number of solvents and determined spectrophotometrically or colorimetrically, and the B.P. tests of identity and of disintegration were applied. The results are recorded in Tables I to V. Although physico-chemical and not biological methods were used, the estimates of calciferol are stated in units of vitamin D, on the assumption that calciferol contains in 1 mg. 40,000 units of antirachitic activity.

Discussion

Ethylene dichloride appears to extract the most calciferol from the tablets (see Table II), although chloroform and carbon tetrachloride are nearly as good. The use of ethylene dichloride simplifies the experimental work since it is the solvent used in the preparation of the antimony trichloride reagent.

The Soxhlet technique appears to be the least satisfactory of the three methods of extraction studied. This may be due to partial destruction of the calciferol by heat; extension of the length of time of extraction to three hours gave lower results. The elution technique has the advantage of presenting fresh solvent to the partially exhausted tablet powder, but extraction may be incomplete because of channelling effects, and Table I shows that the shaking technique gives slightly higher results in most instances.

The higher results obtained spectrophotometrically compared with colorimetrically (Tables II and IV) are attributed to the ultra-violet light absorption of material extracted from the tablet excipients and coating material. The recovery experiments (Table VI) show that significant blanks are obtained by the spectrophotometric method on commonly used tablet excipients. While the spectrophotometric method of determination may be regarded as satisfactory for the routine control of production batches of tablets by the manufacturer, when the precise composition of the ingredients of the tablets is known and allowance can be made for interference, it cannot be recommended for adoption as an official method of assay.

Those samples which contained more than 50,000 units of vitamin D per tablet all gave an orange-brown colour in the B.P. identity test; this presumably corresponds to the official description "brownish-red." Those which assayed 10,000 to 40,000 units per tablet gave a light brown or light orange-brown colour; the samples with less than 1000 units per tablet gave none or at the most a pale straw-yellow colour.

The samples from ten manufacturers complied with the official disintegration test. Of the four which did not comply, one sample made no labelled claim to be of B.P. quality.

Proposed Methods of Assay

As a result of the work described above, the following alternative methods are recommended for the assay of tablets of calciferol.

Method 1. Weigh and powder 20 tablets. To an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol, add 50 ml. of ethylene dichloride, shake for 5 minutes, adjust the volume to

TABLE I

COLORIMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL BY VARIOUS METHODS. CALCIFEROL CONVERTED TO VITAMIN D UNITS

Manufacturer	Calciferol "units" per tablet			
	Shaking with ethylene dichloride	Elution with ethylene dichloride	Soxhlet extraction with ethylene dichloride	Shaking with light petroleum
A	—	56,900	56,600	49,000
B	27,600	26,500	26,500	26,200
C	28,400	27,300	23,100	23,600
D	50,600	52,200	51,600	47,300
E_1	0	0	0	0
E_2	1200	900	900	0
F	27,400	26,800	28,500	24,400
G	31,100	29,900	30,000	29,400
H	62,400	63,400	62,500	59,500
I	12,300	11,300	10,900	10,800
J	50,500	51,000	52,000	50,000
K	51,300	51,400	51,000	49,200
L	38,500	38,500	37,800	36,800
M	21,300	20,100	20,900	19,600
N	33,300	33,000	33,200	34,400

TABLE II

COLORIMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL BY ELUTION WITH VARIOUS SOLVENTS. CALCIFEROL CONVERTED TO VITAMIN D UNITS

Manufacturer	Calciferol "units" per tablet				
	Carbon tetrachloride	Chloroform	*cyclo*Hexane	Ethylene dichloride	Light petroleum
A	56,100	54,200	51,100	56,900	51,100
C	25,300	28,300	25,000	27,300	24,300
D	48,100	—	45,800	52,200	45,000
G	30,200	30,000	29,300	29,900	27,400
I	11,400	7600	10,900	11,300	10,500
J	51,800	48,500	49,600	51,000	51,000

TABLE III

COLORIMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL USING SOXHLET APPARATUS WITH VARIOUS SOLVENTS

Manufacturer	Calciferol "units" per tablet		
	*cyclo*Hexane	Ethylene dichloride	Light petroleum
A	56,300	56,600	52,000
C	28,100	23,100	23,300
D	57,600	51,600	52,600
I	11,100	10,900	10,600
J	43,900	52,000	43,600

TABLE IV

SPECTROPHOTOMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL BY ELUTION WITH VARIOUS SOLVENTS

Manufacturer	Calciferol "units" per tablet			
	*cyclo*Hexane	Ethanol	Ethylene dichloride	Light petroleum
A	67,200	—	79,400	69,800
C	28,300	34,400	31,200	25,500
D	47,600	57,200	57,500	52,200
E_2	5200	10,800	9100	3700
G	30,700	45,000	36,700	30,400
I	13,700	19,900	19,100	14,800
J	55,200	100,000	90,900	53,400

TABLE V

B.P. TESTS OF IDENTITY AND DISINTEGRATION APPLIED TO COMMERCIAL TABLETS OF CALCIFEROL

Manufacturer	Test of identity	Disintegration time (minutes)
A	Orange-brown	31
B	Light orange-brown	17
C	Light brown	More than 150
D	Orange-brown	19
E_1	Colourless	79
E_2	Straw-yellow	—
F	Light brown	More than 150
G	Light brown	11
H	Orange-brown	More than 150
I	Light brown	21
J	Orange-brown	32
K	Orange-brown	31
L	Light orange-brown	27
M	Light orange-brown	10
N	Light orange-brown	29

TABLE VI

RECOVERY OF CALCIFEROL FROM VARIOUS TABLET EXCIPIENTS USING THE COLORIMETRIC METHOD OF DETERMINATION

	Calciferol "units" per g.						
	Blank				Calciferol found		
Diluent	Method 1	Method 2	Method 2*a**	Calciferol added	Method 1	Method 2	Method 2*a**
Calcium phosphate	1000	2000	20,000	206,000	203,000	207,000	234,000
Chocolate basis ..	1000	1000	83,000	193,000	192,000	193,000	266,000
Lactose	0	0	15,000	197,000	195,000	196,000	217,000
Starch	0	0	29,000	201,000	202,000	201,000	233,000

* Method 2*a* is extraction by elution with ethylene dichloride followed by spectrophotometric determination.

100 ml. with ethylene dichloride, shake for 2 minutes, and centrifuge or allow to stand until the insoluble matter has settled. To 0·4 ml. of the supernatant liquid add 2·2 ml. of antimony trichloride reagent, and compare the extinctions at 500 mμ at ½-minute intervals for 3 minutes with those of 0·4 ml. of ethylene dichloride + 2·2 ml. of reagent. Record the maximum extinction. Calculate the weight of calciferol in each tablet of average weight.

Method 2. Weigh and powder 20 tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol, into a chromatography-type tube of internal dimension 30 cm. × 1 cm., and fitted at the bottom with a cotton-wool plug. Elute with ethylene dichloride, collecting 100 ml. of eluate. Mix by shaking. To 0·4 ml. of solution add 2·2 ml. of antimony trichloride reagent, and complete the assay as in method 1.

VALIDITY OF METHODS

The colorimetric and spectrophotometric methods of determination were applied to the solutions obtained by the elution and the shaking methods of extraction from calcium phosphate, chocolate basis, lactose and starch, and to mixtures of each of these excipients with calciferol.

The results are recorded in Table VI. The colorimetric method gave the correct concentration of calciferol in all cases, within ± 2 per cent. By the spectrophotometric method, however, there were positive "blanks" in the absence of calciferol, and the estimates of calciferol concentration were high by as much as 35 per cent.

Table I shows that the methods of extraction recommended in this paper gave results as high as those obtained by a method, involving shaking with light petroleum, almost identical with that specified in the official assay. Also, for method 2, preliminary work had shown that the powder was completely extracted by the first 80 ml. of ethylene dichloride.

Evers and Smith[15] state that neither colour tests nor the absorption band at 265 mμ can be used as evidence for the presence of vitamin D in pharmaceutical products such as tablets, since they may be given by the decomposition products of vitamin D. On the other hand, Mariani[16] has isolated a product of decomposition from calciferol which gave a negative reaction with antimony trichloride, and the data of Brockmann and Chen[17] and of Mueller[18] also indicate that other steroids are unlikely to produce a colour with antimony trichloride comparable in intensity with that given by calciferol in 2 to 3 minutes at 500 mμ.

Samples obtained from manufacturers A and B in May 1952 were assayed in 1955 when the tablets were at least three years old; the results were 33,100 and 13,300 units per tablet respectively. This is in accordance with expectation.

Biological assays made at the same time as the chemical determinations are of course necessary to establish complete validity, but the indications are that the methods proposed in this paper correctly assay fresh tablets, and give good estimates of the amount of calciferol in partly decomposed tablets.

Summary

1. Two methods are recommended for the colorimetric determination of calciferol in Tablets of Calciferol B.P.

2. The tablets of fourteen manufacturers have been examined by these and other methods, and the results are analysed.

The author thanks Mr. Wilfred Smith and Mr. R. V. Swann for helpful suggestions and criticisms.

References

1. Wodsak, *Die Pharmazie*, 1950, **5**, 520.
2. Feinstein, *J. biol. Chem.*, 1945, **159**, 569.
3. Vastagh, *Pharm. Zentralh.*, 1942, **83**, 481; through *Chem. Abstr.*, 1944, **38**, 3091.
4. Vastagh, *Arch. Pharm. Berl.*, 1942, **280**, 406.
5. Martinez, de Canesa and Oneto, *Ph.*, 1941, No. 2, 17; through *Chem. Abstr.*, 1942, **36**, 1440.
6. Tastaldi, *Anais faculdade farm, odontol., univ. S. Paulo (Brasil)*, 1944, **4**, 27; through *Chem. Abstr.*, 1947, **41**, 2107.
7. Banes, *J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 580.
8. Coward, *The Biological Standardization of the Vitamins*, 2nd edition, Baillière, Tindall and Cox, London, 1947.
9. Kodicek, *Biochem. J.*, 1950, **46**, xiv.
10. Rogers, *J. Pharm. Pharmacol.*, 1954, **6**, 780.

11. Pirlot, *Anal. Chim. Acta*, 1948, **2**, 744.
12. Graff, O'Connor and Skau, *Industr. Engng Chem.* (*Anal.*), 1944, **16**, 556.
13. Ashmore, *Analyst*, 1947, **72**, 206.
14. de Witt and Sullivan, *Industr. Engng Chem.* (*Anal.*), 1946, **18**, 117.
15. Evers and Smith, *The Analysis of Drugs and Chemicals*, Charles Griffin, London, 1955.
16. Mariani, *Ann. chim. appl. Roma*, 1951, **41**, 655.
17. Brockmann and Chen, *Z. physiol. Chem.*, 1936, **241**, 129.
18. Mueller, *J. Amer. chem. Soc.*, 1949, **71**, 924.