THE SPECTROPHOTOMETRIC DETERMINATION OF VITAMIN D IN PHARMACEUTICAL PREPARATIONS

PART I. SOLUTION OF CALCIFEROL. B.P.

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INTRODUCTION

SEVERAL colorimetric methods have been proposed[1–8] for the quantitative determination of vitamin D. The reagents used by different workers include aluminium chloride in the presence of pyrogallol, glycerol dichlorohydrin, acid solutions of certain aromatic aldehydes (particularly furfural), iodine trichloride, and antimony trichloride, which is the most promising.

Brockmann and Chen[8] added a saturated solution of antimony trichloride in chloroform to the vitamin, and measured the extinction at 500 mμ of the orange-yellow colour produced. This reagent is unstable, its sensitivity changing with time, and it must be free from ethanol and moisture. Nield, Russell, and Zimmerli[9] have found that the addition of 2 per cent. of acetyl chloride improves the stability, and increases the sensitivity of the reagent. de Witt and Sullivan[10] recommend the use of ethylene dichloride instead of chloroform as solvent, thus avoiding the trouble of freeing the chloroform from the ethanol normally present as a preservative. Brüggemann, Krauss, and Tiews[11] state that it is the antimony pentachloride usually present as an impurity in commercial antimony trichloride, which is responsible for the colour developed. On the other hand, Zimmerli, Nield and Russell[12], use, with satisfactory results, a reagent incorporating metallic zinc, tin or antimony in order to reduce any quinquevalent antimony.

A solution of 20 per cent. of antimony trichloride in ethylene dichloride with 2 per cent. of acetyl chloride added, prepared according to the method of de Witt and Sullivan[10], has been used for all the work described in this paper.

Estimation of Calciferol in Inert Solvent

A solution of calciferol (vitamin D_2) in ethylene dichloride mixed with this reagent rapidly develops an orange-yellow colour, reaching maximal extinction at 500 mμ in about 1 minute, then slowly fading (see curve 1). The use of ethylene dichloride (boiling point 83° to 84° C.) as solvent is preferred to light petroleum, *cyclo*hexane or chloroform, which have been used, since there is less evaporation during manipulation, and fractional distillation of the commercial material is sufficient purification.

The colour intensity is measured using matched 1 cm. Corex stoppered cells in a Unicam SP500 spectrophotometer with a tungsten lamp as light source. The cells used in this work hold about 2·7 ml., and it has been

found convenient to introduce 0·1 ml. of calciferol solution (containing about 250 units), then add 2·5 ml. of reagent from a rapid-delivery pipette, simultaneously starting a stop-watch. No agitation is necessary to mix the two liquids. The blank cell is filled with 2·6 ml. of reagent. Extinction readings at 500 mμ are taken at ½-minute intervals for 6 minutes.

The maximal extinction over the range 0·3 to 0·6 is directly proportional to the concentration of calciferol in the solution in the cell. It is permissible to increase the amount of calciferol solution at the expense of reagent, keeping the total volume constant, as far as 0·6 ml. of solution + 2·0 ml. of reagent; beyond this there is apparently insufficient reagent to produce the colour quantitatively. The reagent blank is in any case so small that no compensating change in the composition of the liquid in the blank cell is necessary.

Effect of Vegetable Oil Base

Arachis oil, which is the usual base for this preparation, is known[13] to contain phytosterols, and these also produce a colour with the antimony trichloride reagent. They have sufficient absorption at 265 mμ to prevent estimation of the calciferol directly, without the necessity of using a colorimetric reagent. Saponification of the oil, followed by extraction of the unsaponifiable matter, reduces the bulk considerably, and concentrates the vitamin and other sterols. Cox[14] has shown that the composition of the unsaponifiable matter of different samples of arachis oil is approximately constant, so that empirical correction could be applied to correct for this effect, but it seems preferable, to determine the interference of the base from the internal evidence of the sample itself, rather than to assume identity of composition with other samples of arachis oil.

The extinction at 500 mμ of a mixture of an ethylene dichloride solution of the unsaponifiable matter of arachis oil with antimony trichloride reagent rises slowly for several hours, and a graph of extinction plotted against time is almost linear for the first 6 minutes (see Fig. 1, curve 2).

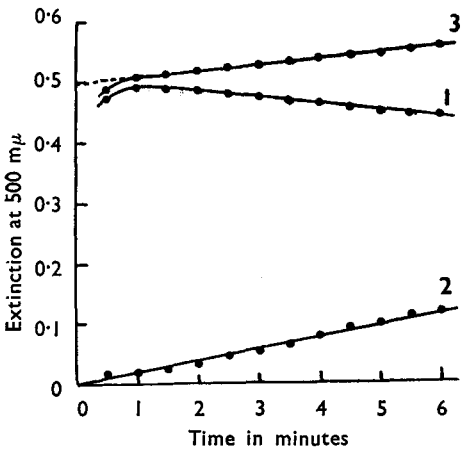

FIG. 1. The effect of time on the intensity of colour produced by mixing antimony trichloride reagent with: 1 calciferol, 2 unsaponifiable matter of arachis oil, 3 unsaponifiable matter of solution of calciferol B.P.

The graph of the extinction of a mixture of reagent with an ethylene dichloride solution of the unsaponifiable matter extracted from a sample of solution of calciferol in arachis oil plotted against time (curve 3) is identical with that obtained by the arithmetic summation of curves 1 and 2. Curve 3 is almost linear after the first minute, and can be extrapolated to zero time with ease. The value of the extinction at zero time obtained by extrapolation of curve 3 is equal to the maximal extinction in curve 1.

Curves similar to 2 and 3 are also obtained if olive oil is used instead of arachis oil.

Estimation of Calciferol in Vegetable Oil Base

Weigh 2·5 g. and reflux for 30 minutes with 25 ml. of freshly prepared 0·5N ethanolic potassium hydroxide. Dilute with 60 ml. water. Extract with 3 × 50 ml. quantities of anæsthetic ether. Wash the combined ether layers with two 20 ml. portions of water, 20 ml. of 0·5N aqueous potassium hydroxide, 20 ml. of water, 20 ml. of 0·5N aqueous potassium hydroxide, followed by 20 ml. quantities of water until the washings are neutral to phenolphthalein. Evaporate off the ether on a steam bath, and dry the residue by evaporation with successive 5 ml. quantities of acetone. Dissolve the residue in 15·0 ml. of ethylene dichloride. To 0·4 ml. of this solution in the spectrophotometer cell add 2·2 ml. of reagent. Record the extinction at 500 mμ every ½ minute for 6 minutes, and plot a graph of extinction against time. Extrapolate linearly to zero time, and calculate $E^{1\text{ per cent.}}_{1\text{ cm.}}$. The potency of the sample in units per g. is given by

$$\text{potency} = \frac{E^{1\text{ per cent.}}_{1\text{ cm.}}\ \text{sample} \times 40{,}000{,}000}{E^{1\text{ per cent.}}_{1\text{ cm.}}\ \text{calciferol}},$$

where $E^{1\text{ per cent.}}_{1\text{ cm.}}$ calciferol is the value obtained using a standard solution of calciferol in ethylene dichloride, determined as outlined earlier in this paper, using the maximal extinction (curve 1). It varies slightly from batch to batch of reagent, but is usually about 1900, and should be determined afresh for each batch of reagent prepared. We have not yet found a value as high as 2200, claimed by de Witt and Sullivan[10].

If a preliminary determination indicates that the strength of the sample deviates considerably from the official strength of the preparation (namely 3000 units/g.), a different aliquot of the ethylene dichloride solution should be used together with sufficient reagent to produce 2·6 ml., so that the extinctions are within the range 0·3 to 0·6. The development and measurement of the colour should be performed at least twice, and the curves plotted and extrapolated. The values of the extinction at zero time thus obtained should agree within 2 per cent. The mean value is taken for the calculation of the potency.

Recovery experiments over the range 2000 to 4500 units/g. have been performed, using both arachis oil and olive oil as base. The results are shown in the table. The estimated potency is in each case within ± 3 per cent. of the theoretical.

Vehicle used.	Calciferol added units/g.	Calciferol found units/g.	Recovery per cent.
Arachis Oil	2050	2050	100·0
Arachis Oil	2040	2040	100·0
Olive Oil	2230	2200	98·7
Arachis Oil	3200	3290	102·8
Arachis Oil	3200	3240	101·3
Olive Oil	3020	2960	98·0
Arachis Oil	4670	4790	102·6
Arachis Oil	4670	4530	97·0
Olive Oil	4400	4310	98·0

The test was carried out at intervals on a sample of Solution of Calciferol B.P. prepared with arachis oil and kept under ordinary conditions, with the following results.

Date	*Assay*
18.9.'53	3000 units/g
8.3.'54	2780 ,,
23.6.'54	2190 ,,
11.10.'54	2120 ,,

SUMMARY

1. A method is described for the colorimetric determination of calciferol in solution of calciferol B.P.

2. The effect of the absorption due to the solvent oil on that produced by calciferol is eliminated by a graphical extrapolation procedure.

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DISCUSSION

The paper was presented by MR. W. SMITH.

MR. K. A. PROCTOR (Nottingham) asked whether chromatographic separation of the phytosterols and calciferol had been attempted.

DR. F. HARTLEY (London) said that the paper merely reported that the solution in arachis oil was kept under ordinary conditions and there was no indication as to whether any precautions were taken to protect it from light. There was evidence of the deterioration of the solution and, as there was no recommendation in the B.P. about labelling the solution with an expiry date, it was a matter on which the author might express his views.

DR. G. E. FOSTER (Dartford) asked whether the method gave good results in the presence of cocoa butter, which introduced difficulties in the assay of vitamin D spectrophotometrically. Had the author any biological results to correlate with the chemical tests?

Mr. W. H. C. SHAW (Greenford) pointed out that the actual biological deterioration might be much greater than that shown by the chemical test. Was the author satisfied that the chromogenic material in the sample was still calciferol?

MR. A. R. ROGERS, in reply, said that he had carried out no chromatographic experiments, as chromatography would greatly lengthen the time taken for the assay. The conditions of storage for the samples were those described in the B.P. monograph. An extension of the work would be necessary before any expiry date could be stipulated, and biological figures would be required. He had not tried the method in the presence of cocoa butter, but it would probably behave similarly to other vegetable oils.